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Relation	

Fission yeast cells overproducing HSET/KIFC1 provides a useful tool for identification and evaluation of human kinesin-14 inhibitors

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Running head: Evaluation of kinesin-14 inhibitors

Keywords: cytotoxicity; **fission yeast; inhibitors; kinesin-14; off-target; overproduction**

ABSTRACT

Many human cancer cells contain more than two centrosomes, yet these cancer cells can form pseudo-bipolar spindles through the mechanism, called centrosome clustering, and survive, instead of committing lethal multipolar mitoses. Kinesin-14/HSET, a minus end-directed motor, plays a crucial role in centrosome clustering. Accordingly, HSET is deemed to be a promising chemotherapeutic target to selectively kill cancer cells. Recently, three HSET inhibitors (AZ82, CW069 and SR31527) have been reported, but their specificity and efficacy have not been evaluated rigorously. This downside partly stems from the lack of robust systems for the assessment of these drugs. Yeasts and filamentous fungi provide not only powerful models for basic and applied biology but also versatile tools for drug discovery and evaluation. Here we show that these three inhibitors on their own are cytotoxic to fission yeast, suggesting that they have off-targets in vivo except for kinesin-14. Nonetheless, intriguingly, AZ82 can neutralize otherwise toxic overproduced HSET; this includes a substantial reduction in the percentage of HSET-driven abnormal mitotic cells and partial suppression of its lethality. SR31527 also displays modest neutralizing activity, while we do not detect such activity in CW069. As an experimental proof-of-principle study, we have treated HSET-overproducing fission yeast cells with extracts prepared from various plant species and found activities that rescue HSET-driven lethality in those from *Chamaecyparis pisifera* and *Toxicodendron trichocarpum*. This methodology of protein overproduction in fission yeast, therefore, provides a convenient, functional assay system by which to screen for not only selective human kinesin-14 inhibitors but also those against other molecules of interest.

1. INTRODUCTION

Most animal cells have two centrosomes from which mitotic bipolar spindles assemble. This bipolarity is essential for equal segregation of genetic material, thereby ensuring genome stability. Like DNA duplication, a cell has a robust regulatory mechanism by which centrosome number is maintained strictly as one or two copies per cell, which orchestrates the chromosome cycle (Conduit et al., 2015; Fu et al., 2015). Interestingly, it is known that in many cancer cells, this synchrony between centrosome and chromosome cycles becomes uncoupled, by which such cells contain more than two centrosomes. Nonetheless, these cancer cells appear to divide normally by means of bipolar spindles without undergoing lethal multipolar mitoses (Quintyne et al., 2005). It has been shown that these cells could form pseudo-bipolar spindles by clustering the supernumerary centrosomes into two poles (Cosenza and Kramer, 2016; Gergely and Basto, 2008). Perturbations in centrosome clustering trigger multipolar spindle formation and mitotic catastrophe specifically in cancer cells with supernumerary centrosomes (Kwon et al., 2008; Quintyne et al., 2005).

Centrosome clustering is achieved by a side-by-side, rather than dispersed, positioning of individual centrosomes; this configuration results in the formation of two spindle poles that is facilitated through a microtubule-dependent inward force. Accordingly, a variety of cellular processes affecting microtubule-based tension and motility are involved in the clustering of supernumerary centrosomes (Godinho et al., 2009; Kramer et al., 2011; Leber et al., 2010; Rhys et al., 2018).

One of the crucial factors required for centrosome clustering is human HSET/KIFC1. This protein is a member of the kinesin-14 family of proteins that includes mouse Kifc2, *Xenopus* XCTK2, *Drosophila* Ncd, *C. elegans* KLP-15, *Arabidopsis* ATK5 and KCBP, *Aspergillus* KlpA, fission yeast Pkl1 and Klp2 and budding yeast Kar3 (Ambrose et al., 2005; Endow and Komma, 1996; Goshima and Vale, 2005; Hanlon et al., 1997; Meluh and Rose, 1990; O'Connell et al., 1993; Paluh et al., 2000; Robin et al., 2005; Saito et al., 1997; Troxell et al., 2001; Walczak et al., 1998; Yukawa et al., 2018). Kinesin-14 motor proteins have minus-end directionality and comprise three functional domains, an N-terminal tail domain, a central coiled-coil stalk domain and a C-terminal motor domain that possesses the ATPase activity (She and Yang, 2017). It is reported that HSET is abundantly expressed in several cancer cell lines including ovary, breast and lung cancer (Grinberg-Rashi et al., 2009; Pannu et al., 2015; Pawar et al., 2014). Intriguingly, knockdown of HSET in supernumerary centrosome-containing breast cancer cell lines prevents centrosome clustering and induces cell death by multipolarity in anaphase, while in normal cell lines that contain two centrosomes this treatment does not result in lethality (Kleylein-Sohn et al., 2012; Kwon et al., 2008). Therefore, specific targeting of HSET may provide a novel strategy by which to selectively kill cancer cells (Li et al., 2015; Xiao and Yang, 2016).

To date, three HSET inhibitors, designated AZ82, CW069 and SR31527 have been reported (Cosenza and Kramer, 2016; Watts et al., 2013; Wu et al., 2013; Xiao and Yang, 2016; Zhang et al., 2016) (Table 1). AZ82, which is the first HSET inhibitor to be identified, binds specifically to the HSET-microtubule binary complex, thereby inhibiting

the microtubule-stimulated ATPase activity of HSET (Park et al., 2017; Wu et al., 2013; Yang et al., 2014). Upon addition to cancer cells with supernumerary centrosomes, this small molecule inhibitor triggers multipolar spindle formation and mitotic catastrophe. The second inhibitor, CW069, was designed and synthesized according to in silico computational modeling for HSET binding (Watts et al., 2013). This compound binds to HSET in an allosteric manner and reduces its ATPase activity in vitro. While cells treated with monastrol, a kinesin-5 inhibitor, exhibit mitotic arrest with monopolar spindles, co-treatment with CW069 suppresses monopolarity induced by monastrol. CW069 also induces multipolar mitoses in cells containing supernumerary centrosomes (Watts et al., 2013). Finally, SR31527 was identified through a high-throughput screen based on an ATPase assay of HSET (Zhang et al., 2016). It inhibits HSET by binding directly to a novel allosteric site within the motor domain without involving microtubules. SR31527 reportedly prevents bipolar clustering of extra centrosomes in triple-negative breast cancer (TNBC) cells, and significantly reduces viability of TNBC cells. Despite the developments of these HSET inhibitors, their biological efficacy and off-target toxicity have not been evaluated properly, which is a crucial step towards clinical implementation of these drugs.

The fission yeast, *Schizosaccharomyces pombe*, has provided an excellent system not only as a model organism for basic biology but also as a useful tool for drug discovery and its assessment (Benko et al., 2016; Herrero et al., 2006; Lewis et al., 2017). Previously, we showed that HSET, when overexpressed in fission yeast, is toxic, and leads to mitotic arrest with monopolar spindles, reminiscent of overproduction of fission yeast kinesin-

14, Pkl1 or Klp2 (Yukawa et al., 2018). In this study, we have attempted to exploit this phenotype for the functional evaluation of the three known HSET inhibitors described earlier. We have found that all these reagents have HSET-independent cytotoxicity on fission yeast growth, indicating that they may not be specific to HSET. Intriguingly, however, AZ82 displays neutralizing activity against HSET-induced lethality. Furthermore, by applying this assay system to natural sources, we have identified HSET-suppressing activities in extracts prepared from plant species.

2. MATERIALS AND METHODS

2.1 Strains, media, and genetic methods

Fission yeast strains used in this study are listed in Table 2. Media, growth conditions, and manipulations were carried out as previously described (Bähler et al., 1998; Moreno et al., 1991; Sato et al., 2005). For most of the experiments, rich YE5S liquid media and agar plates were used. Wild-type strain (513; Table 2), drug-sensitive strains (YA8 and SAK931) were provided by P. Nurse (The Francis Crick Institute, London, England, UK), M. Yoshida (Chemical Genetics Laboratory, RIKEN, Saitama, Japan) and S. A. Kawashima (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan), respectively. For overexpression experiments using thiamine-repressible *nmt* series plasmids (Basi et al., 1993; Maundrell, 1990), cells were first grown overnight in Pombe Glutamate Medium (PMG, the medium in which the ammonium of EMM2 is replaced with 20 mM glutamic acid) with the required amino acid supplements in the presence of 15 μM thiamine. Thiamine was then washed out by filtration pump and cells

continued to be cultured in the same PMG media in the absence of thiamine for further 12-48 h as necessary. For spot tests, these cells were serially diluted 10-fold, from an initial concentration of 2 x 10^7 cells/ml, and spotted onto PMG plates with added supplements in the presence or absence of 15 μM thiamine. Plates were incubated at 27°C or 30°C.

2.2 Preparation and manipulation of nucleic acids

Enzymes were used as recommended by the suppliers (New England Biolabs Inc. Ipswich, MA and Takara Bio Inc., Shiga, Japan).

2.3 Chemical compounds

AZ82 was supplied from AstraZeneca (Boston, MA, U.S.A.). CW069 was a kind gift of S. V. Ley, the University of Cambridge. SR31527 was purchased from Vitas-M Laboratory (Apeldoorn, The Netherlands; Cat number: STK400735). All chemicals were dissolved in DMSO at 10 mM and stored at −20°C.

2.4 Treatment of HSET-overproducing fission yeast cells with kinesin 14 inhibitors

Cells were cultured in YE5S or PMG liquid media with the required amino acid supplements and thiamine until mid-log phase at 30°C. Thiamine was washed out and cells shifted to the same PMG liquid media without thiamine at a concentration of 1×10^5 cells/ml. The kinesin-14 inhibitors or DMSO (mock) were then added at various concentrations between 1 μM and 100 μM (0.01%–1% DMSO solution) and the cells were cultured at 30°C. OD measurements for yeast cultures were performed in a microplate reader (CHROMATE 4300; Awareness Technology Inc., Palm City, FL, U.S.A.), using an Iwaki Round Bottom 96-well microplate with lid and 200 μl per well

for all measurements. OD measurements were performed at 600 nm at 30°C. Yeast cell number was determined using an automated cell counter (F-820; Sysmex, Kobe, Japan). Images of culture were taken by FAS-IV gel imaging system (Nippon Genetics, Tokyo, Japan).

2.5 Preparation of plant extracts and assay for HSET-neutralizing activity

Extracts from plant species were prepared with standard procedures as previously described (Kimura et al., 2012) and dissolved in methanol (10 mg/ml). Minimal plates without thiamine were made by adding 50 ml of agar media containing fission yeast cells that overproduced HSET (4.6×10^4 cells/ml). Upon a series of 2-fold dilutions, 5 μl extracts were spotted onto these plates. Plates were then incubated at 28°C for 3 d.

2.6 Fluorescence microscopy

Fluorescence microscopy images were obtained using a DeltaVision microscope system (DeltaVision Elite; GE Healthcare, Chicago, IL, U.S.A.) comprising a wide-field inverted epifluorescence microscope (IX71; Olympus, Tokyo, Japan) and a Plan Apochromat 60×, NA 1.42, oil immersion objective (PLAPON 60×O; Olympus Tokyo, Japan). DeltaVision image acquisition software (softWoRx 6.5.2; GE Healthcare, Chicago, IL) equipped with a charge-coupled device camera (CoolSNAP HQ2; Photometrics, Tucson, AZ, U.S.A.) was used. Live cells were imaged in a glass-bottomed culture dish (MatTek Corporation, Ashland, MA, U.S.A.) coated with soybean lectin and incubated at 27°C. To keep cultures at the proper temperature, a temperature-controlled chamber (Air Therm SMT; World Precision Instruments Inc., Sarasota, FL, U.S.A.) was used. Images were taken as 14–16 sections along the z axis at 0.2 μm intervals; they were then deconvolved and merged into

a single projection. The sections of images acquired were compressed into a 2D projection using the DeltaVision maximum intensity algorithm. Deconvolution was applied before the 2D projection. Captured images were processed with Photoshop CS6 (version 13.0; Adobe, San Jose, CA, U.S.A.).

2.7 Statistical data analysis

We used the two-tailed $\chi2$ test to evaluate the significance of differences between frequencies of the cells with bipolar spindles in different strains. We used this key for asterisk placeholders to indicate p-values in the figures: e.g., ****, $P < 0.0001$. For growth curve experiments, the results are expressed as the mean ± standard error of the mean (SE).

3. RESULTS

3.1 The lethality of fission yeast cells overproducing HSET/kinesin-14 is rescued by co-overproduction of Cut7/kinesin-5

Bipolar spindle assembly requires proper force-balance generated by kinesin-5 and kinesin-14 (Sharp et al., 2000; She and Yang, 2017; Tanenbaum and Medema, 2010). In fission yeast, ectopic overproduction of kinesin-14 Pkl1 or inactivation of kinesin-5 Cut7 results in force imbalance leading to mitotic arrest with monopolar spindles (Hagan and Yanagida, 1990; Pidoux et al., 1996; Yukawa et al., 2018). Interestingly, mitotic arrest caused by Pkl1 overproduction is neutralized by co-overproduction of Cut7 and that cells overproducing both kinesins are capable of forming colonies (Rincon et al., 2017) (bottom two rows in Fig. 1A).

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We previously showed that overexpression of another kinesin-14, Klp2 or human HSET, is also lethal in fission yeast cells with a similar monopolar spindle phenotype (Yukawa et al., 2018). Therefore, we asked whether such Cut7-dependent rescue seen in Pkl1 overproducing cells is also observed in the case of overproduction of HSET or Klp2. As expected, Cut7 co-overproduction effectively suppressed the lethality of cells overproducing HSET or Klp2 (Fig. 1A and B). Observation of spindle morphology showed that while cells overproducing only Pkl1 or HSET displayed a high frequency of monopolar spindles (82%, n=151 or 85%, n=39 respectively), in those co-overproducing Pkl1 and Cut7 or HSET and Cut7, the frequency was substantially reduced to 12% (n=49) or 45% (n=38) respectively (Fig. 1C and D). Hence, HSET (and Klp2) is capable of generating inward pulling forces, thereby antagonizing outward pushing forces exerted by Cut7.

3.2 An assay system for evaluation of human kinesin-14 inhibitors using fission yeast

Yeast-based screening for biologically active small molecules has successfully been implemented for the identification of promising drugs against human cancer and other diseases (Mager and Winderickx, 2005). This strategy is also useful to develop reagents that exhibit a beneficial impact on normal cells, e.g. those promoting lifespan extension (Sarnoski et al., 2017). Fission yeast has been used for several screenings, such as purification of specific compounds produced by *Actinomycetes* (Lewis et al., 2017) and a functional assay for Indinavir, an inhibitor against Human Immunodeficiency Virus Type-1 (Benko et al., 2016; Benko et al., 2017; Yang et al., 2012). Having seen toxicity derived from overproduced HSET in fission yeast cells (see Fig. 1A), we exploited this

lethal phenotype for the biological evaluation of known HSET inhibitors described earlier (AZ82, CW069 and SR31527) (Cosenza and Kramer, 2016; Xiao and Yang, 2016; Watts et al., 2013; Wu et al., 2013; Zhang et al., 2016).

Since we can detect the inhibitory activity of compounds through a simple assay for cell growth properties, it is possible to assess the specificity and the efficacy of these inhibitors. If inhibitors were truly specific to HSET molecules, these compounds on their own would not make any adverse impact on fission yeast growth (top row in Fig. 2A, referred to as specific drugs). Upon overexpression of HSET, these inhibitors would now ameliorate viability loss resulting from HSET-mediated toxicity (bottom two rows in Fig. 2A). By contrast, if these small molecules could not inhibit HSET activity, viability would not be increased in their presence (second row in Fig. 2A).

On the other hand, if inhibitors on their own interfere with fission yeast growth, it implies that these compounds recognize molecules or inactivate some pathways that are essential for fission yeast cells independent of HSET; in other words, these inhibitors are not specific to HSET, but instead they are drugs that exhibit off-target effects (top row in Fig. 2B, referred to as multi-target drugs). Nonetheless, if HSET is effectively inhibited by these drugs, viability of fission yeast cells would be ameliorated to some extent compared to that without drug treatment (bottom row in Fig. 2B). Alternatively, if drugs' inhibitory activity is weaker, the degree of viability loss would not be additive when drug treatment and HSET overproduction are applied in combination compared to that under each condition (second row from the bottom in Fig. 2B).

3.3 All three known HSET inhibitors have non-specific cytotoxicity toward fission yeast cells

As aforementioned, if the HSET inhibitors were specific to human kinesin-14, addition of these molecules to wild type fission yeast should not incur any adverse effects on cell growth, as there are no targets (i.e. HSET) in these cells. Even if these reagents were to cross-inhibit fission yeast kinesin-14 molecules (i.e. Pkl1 and Klp2), they would not kill fission yeast cells, as deletion of either *pkl1* or *klp2*, or even double deletion is viable (Troxell et al., 2001).

To address the specificity of HSET inhibitors, we first treated wild type fission yeast cells individually with these drugs without introducing HSET. As shown in Fig. 3 (top row), AZ82 inhibited cell growth, while CW069 and SR31527 had no deleterious impact on growth curve, though SR31527 displayed a marginal inhibitory effect. It is known that wild type yeast cells are often inherently resistant to various exogenously added drugs. This multi-drug resistance can mainly be attributed to two reasons. One is that a thick cell wall prevents drugs entering the cells, and the other is that the presence of P-glycoprotein transporters actively pumps out incorporated drugs from the cells to the media (Nishi et al., 1992). Therefore, it is common in yeast studies to use drug-sensitive strains for this type of chemical biology (Aoi et al., 2014; Arita et al., 2011; Kawashima et al., 2012; Kawashima et al., 2013). Given this situation, we next exploited two genetically tractable strains that are specifically designed for a drug assessment (YA8 and SAK931, Table 2) (Kawashima et al., 2012; Kawashima et al., 2013; Takemoto et al., 2016). In these strains, genes involved in influx and efflux of exogenously added drugs

are multiply deleted; in YA8, genes encoding two major ATP-binding cassette (ABC) family transporters, Bfr1 and Pmd1, are deleted (*bfr1Δpmd1Δ*) (Arita et al., 2011), while SAK931 contains deletions of 5 additional genes (*7Δ*) (Takemoto et al., 2016). These 5 genes encode the major facilitator superfamily (MFS) transporters Caf5 and Mfs1, a P-type ATPase Dnf2, a C-22 sterol desaturase Erg5 and an AP-1-like transcription factor Pap1 (Aoi et al., 2014; Takemoto et al., 2016). While Caf5, Dnf2, Erg5 and Mfs1 inhibit influx and/or promote efflux of exogenously added drugs at the plasma membrane or the endoplasmic reticulum in collaboration with Bfr1 and Pmd1 (Kawashima et al., 2012; Kawashima et al., 2013; Takemoto et al., 2016), nuclear-localizing Pap1 regulates transcription of a wide range of genes involved in drug resistance (Toda et al., 1992; Toda et al., 1991; Toone et al., 1998).

As shown in Fig. 3 (bottom row), we found that all three drugs now displayed very strong growth-inhibitory effects on YA8 or SA931 at 100 μM. CW069 was not cytotoxic in YA8 cells, but was in SAK931; fission yeast cells are likely less permeable to CW069 than AZ82 or SR31527. These results indicated that all three HSET inhibitors targeted molecules besides HSET within fission yeast cells that are essential for cell viability. It is noted that no obvious alterations in cell morphology were observed as a result of treatment with these drugs (Lewis et al., 2017), of which currently, the molecular details of cytotoxicity remain to be dissected. In summary, we conclude that none of the three inhibitors examined is indisputably specific to HSET, but instead these small molecules interfere with unknown molecular pathways that are needed for cell viability of fission yeast.

3.4 AZ82 modestly rescues the lethality caused by HSET overproduction

We next sought to address whether these inhibitors are capable of rescuing the lethality of fission yeast cells resulting from forced expression of HSET. For this purpose, we assessed the number of cells in the presence or absence of inhibitors upon HSET overproduction. As the three kinesin-14 inhibitors displayed HSET-independent cytotoxicity (see Fig. 3), various concentrations of individual drugs were tested in YA8 and SAK931 cells to find optimal concentrations which would, on one hand, repress HSET-mediated lethality, and yet, on the other hand, display minimal cytotoxicity on their own. Intriguingly, we found that 10 µM AZ82 could rescue the lethality caused by HSET overproduction in YA8 cells (~3 fold increase of viability; 6% vs 17% in the absence and presence of AZ82 respectively, Fig. 4A and B). As the viability of cells treated with AZ82 alone dropped to 47%, the degree of rescue by AZ82 would be larger than 3 fold. We also examined microtubule structures in HSET-overproducing cells in the presence or absence of AZ82. Interestingly, consistent with growth recovery, HSET-overproducing cells treated with AZ82 exhibited the lower percentage of mitotic cells containing monopolar spindles (54% in the absence of AZ82 and 28% or 6% in the presence of 10 µM or 15 µM AZ82, respectively, Fig. 4C).

By contrast, neither CW069 or SR31527 exhibited noticeable recovery of cell viability at any concentrations examined (Fig. 4A and B). Nonetheless, it is noteworthy that no additive toxicity was observed between SR31527 and HSET overproduction, inviting the possibility that SR31527 also neutralized HSET-mediated lethality (see Fig. 2B), albeit in a somewhat modest manner. As for CW092, as far as we are concerned, we

failed to detect any HSET-neutralizing activity when assayed using the fission yeast system. Taking these results together, we consider that AZ82 and to a lesser extent SR31527 as well can block HSET function in fission yeast, although they possess off-target effects (Table 3).

3.5 Identification of activities in plant extracts that neutralize toxicity derived from HSET overproduction

As a first step towards proof-of-principal studies, we next sought to discover activities derived from natural products that can rescue HSET-mediated lethality (Fig. 5A). For this purpose, we used extracts that were prepared from various plants. As shown in Fig. 5B, we found such neutralizing activities in extracts prepared from *Chamaecyparis pisifera* (*Sawara cypress*, a species of false cypress) and *Toxicodendron trichocarpum* (*Japanese sumac*, an Asian tree species of genus *Toxicodendron*). Hence, the methodology developed in this study will be a versatile tool for the discovery of novel HSET inhibitors. It is, however, of note that although it was evident that the addition of extracts prepared from *Chamaecyparis pisifera* or *Toxicodendron trichocarpum* promoted growth of otherwise lethal HSET-overproducing cells, the growth inhibitory zone appeared in the middle of the spot (0.4 μg, Fig. 5B). This implied either that extracts contained two separate activities (HSET-neutralizing and cytotoxic towards fission yeast cells) or that these two activities were derived from the same compound as shown earlier for AZ82 (see Fig. 4B). Further characterization of these extracts, including biochemical fractionation and purification, is needed.

4. DISCUSSION

In this study, we have introduced a novel, simple assay system using fission yeast that enables us to monitor the specificity and efficacy of known inhibitors against human kinesin-14 proteins. Recently, much attention has been attracted to mitotic kinesins as novel antitumor targets and several kinesin inhibitors including those used in this study have been developed as potential cancer therapeutics (Al-Obaidi et al., 2016; Cosenza and Kramer, 2016; Huszar et al., 2009; Ma et al., 2014; Ohashi et al., 2015; Xiao and Yang, 2016). Among them, human kinesin-14 inhibitors are regarded to be promising, as many cancer cells display dysregulation of the centrosome cycle, resulting in the emergence of supernumerary centrosomes. Yet, these cells escape from lethal multipolarity, which is attributed to HSET-dependent centrosome clustering (Cosenza and Kramer, 2016; Gergely and Basto, 2008; Kwon et al., 2008). These drugs are expected to have minimal impact on non-transformed cells, as HSET is not essential for normal mitosis in human beings. Consistent with this notion, all three HSET inhibitors (AZ82, CW069 and SR31527) have been shown to inhibit motor activities of HSET in vitro and display growth-suppressing characteristics to some extent in human cancer cells that contain supernumerary centrosomes (Watts et al., 2013; Wu et al., 2013; Zhang et al., 2016). However, whether these drugs are specific to HSET has not rigorously been evaluated. One reason for this pitfall is that depletion or inactivation of HSET, a target of these drugs, in tumor cells results in low viability, which hampers accurate assessment of off-target effects in these cells.

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Given this complication, the fission yeast system introduced in this work is robust. As fission yeast cells are devoid of HSET, the existence of off-target effects are easily monitored and reliably assessed. Our results indicate that all three drugs have undesirable targets in fission yeast that are essential for cell viability. Accordingly, we ponder that in principle, none of these reagents is ideal as a specific HSET inhibitor. It is of note that consistent with this proposition, the existence of target molecules other than HSET was previously pointed out for all three drugs (Watts et al., 2013; Wu et al., 2013; Zhang et al., 2016).

Despite HSET-independent cytotoxic effects, we have found that AZ82 exhibits inhibitory activity toward otherwise lethal HSET overproduction in fission yeast and that SR31527 is likely to neutralize toxicity resulting from HSET overproduction, although their impact is less than that of AZ82. As we are able to monitor the specificity and the efficacy of HSET inhibitors by implementing this system, if new inhibitors were developed, our rapid and robust strategy would provide a powerful tool for their biological assessment in combination with a conventional human culture cell system.

In fission yeast, as in other organisms, simultaneous inactivation of kinesin-5 and kinesin-14 rescues lethality resulting from kinesin-5 inhibition alone (Civelekoglu-Scholey et al., 2010; Mountain et al., 1999; O'Connell et al., 1993; Pidoux et al., 1996; Rincon et al., 2017; Rodriguez et al., 2008; Saunders et al., 1997; Troxell et al., 2001; Wang et al., 2015; Yukawa et al., 2017; Yukawa et al., 2018). Thus, effective inhibitors against fission yeast kinesin-14s (Pkl1 and Klp2) could be identified using *cut7* temperature-sensitive mutants, as previously proposed in the *Aspergillus nidulans* system

(Wang et al., 2015). These inhibitors might also be effective to suppress HSET activities, as HSET and Pkl1/Klp2 are structurally conserved and HSET functionally replaces for Pkl1 or Klp2 when introduced into fission yeast (Yukawa et al., 2018).

Finally, we would like to point out that the methodology described in this study could be exploited as an assay system for inhibitors against any human proteins of interest. Provided that overproduction of those human proteins display some defective phenotypes including lethality, which is often the case (Benko et al., 2017; Matsuyama et al., 2006; Nkeze et al., 2015), small molecule libraries, natural products or extracts from any organism could be used to identify potential inhibitors (Fig. 5A). In fact, we have detected activities derived from *Chamaecyparis pisifera* (*Sawara cypress*) and *Toxicodendron trichocarpum* (*Japanese sumac*) that specifically rescued lethality caused by HSET overproduction (Fig. 5B). Detailed characterization of these molecules is in progress using both fission yeast and human cancer cells.

AUTHOR CONTRIBUTIONS

M.Y. S.A., K.K. and T.T. designed research. M.Y. T.Y. and N.K. performed experiments and analyzed the data. M.Y. and T.T. wrote the manuscript, and T.Y., Y.K., S.A., N.K. and K.K. made suggestions.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

REFERENCES

Al-Obaidi, N., et al., 2016. Identification of MAC1: a small molecule that rescues spindle bipolarity in monastrol-treated cells. ACS Chem Biol. 11**,** 1544-1551.

Aoi, Y., et al., 2014. Dissecting the first and the second meiotic divisions using a marker-less drug-hypersensitive fission yeast. Cell Cycle. 13, 1327 - 1334.

Ambrose, J. C., et al., 2005. A minus-end directed kinesin with +TIP activity Is involved in spindle morphogenesis. Mol. Biol. Cell. 16**,** 1584-1592.

Arita, Y., et al., 2011. Microarray-based target identification using drug hypersensitive fission yeast expressing ORFeome. Mol. Biosyst. 7**,** 1463-1472.

Bähler, J., et al., 1998. Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. Yeast. 14**,** 943-951.

Basi, G., et al., 1993. TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene. 123**,** 131-136.

Benko, Z., et al., 2016. HIV-1 protease in the fission yeast *Schizosaccharomyces pombe*. PLoS One. 11**,** e0151286.

Benko, Z., et al., 2017. A fission yeast cell-based system for multidrug resistant HIV-1 proteases. Cell Biosci. 7**,** 5.

Civelekoglu-Scholey, G., et al., 2010. Prometaphase spindle maintenance by an antagonistic motor-dependent force balance made robust by a disassembling lamin-B envelope. J Cell Biol. 188**,** 49-68.

Conduit, P. T., et al., 2015. Centrosome function and assembly in animal cells. Nat Rev
Mol Cell Biol. 16**,** 611-624.

Cosenza, M. R., Kramer, A., 2016. Centrosome amplification, chromosomal instability
and cancer: mechanistic, clinical and therapeutic issues. Chromosome Res. 24**,**
105-126.

Endow, S. A., Komma, D. J., 1996. Centrosome and spindle function of the *Drosophila*
Ncd microtubule motor visualized in live embryos using Ncd-GFP fusion
proteins. J Cell Sci. 109 (Pt 10)**,** 2429-2442.

Fu, J., et al., 2015. The centrosome and its duplication cycle. Cold Spring Harb Perspect
Biol. 7**,** a015800.

Gergely, F., Basto, R., 2008. Multiple centrosomes: together they stand, divided they
fall. Genes Dev. 22**,** 2291-2296.

Godinho, S. A., et al., 2009. Centrosomes and cancer: how cancer cells divide with too
many centrosomes. Cancer Metastasis Rev. 28**,** 85-98.

Goshima, G., Vale, R. D., 2005. Cell cycle-dependent dynamics and regulation of
mitotic kinesins in *Drosophila* S2 cells. Mol. Biol. Cell. 16**,** 3896-3907.

Grinberg-Rashi, H., et al., 2009. The expression of three genes in primary non-small
cell lung cancer is associated with metastatic spread to the brain. Clin Cancer
Res. 15**,** 1755-1761.

Hagan, I., Yanagida, M., 1990. Novel potential mitotic motor protein encoded by the
fission yeast *cut7*[+] gene. Nature. 347**,** 563-566.

Hanlon, D. W., et al., 1997. Characterization of KIFC2, a neuronal kinesin superfamily

    member in mouse. Neuron. 18**,** 439-451.

Herrero, A. B., et al., 2006. Cross-talk between nucleotide excision and homologous

    tecombination DNA repair pathways in the mechanism of action of antitumor

    Trabectedin. Cancer Res. 66, 8155-8162.

Huszar, D., et al., 2009. Kinesin motor proteins as targets for cancer therapy. Cancer

    Metastasis Rev. 28**,** 197-208.

Kawashima, S. A., et al., 2012. Analyzing fission yeast multidrug resistance

    mechanisms to develop a genetically tractable model system for chemical

    biology. Chem. Biol. 19, 893-901.

Kawashima, S. A., et al., 2013. A chemical biology strategy to analyze rheostat-like

    protein kinase-dependent regulation. Chem. Biol. 20, 262-271.

Kimura, K., et al., 2012. Kujigamberol, a new dinorlabdane diterpenoid isolated from

    85 million years old Kuji amber using a biotechnological assay. Fitoterapia. 83,

    907-912.

Kleylein-Sohn, J., et al., 2012. Acentrosomal spindle organization renders cancer cells

    dependent on the kinesin HSET. J Cell Sci. 125**,** 5391-5402.

Kramer, A., et al., 2011. Centrosome clustering and chromosomal (in)stability: a matter

    of life and death. Mol Oncol. 5**,** 324-335.

Kwon, M., et al., 2008. Mechanisms to suppress multipolar divisions in cancer cells

    with extra centrosomes. Genes Dev. 22**,** 2189-2203.

Leber, B., et al., 2010. Proteins required for centrosome clustering in cancer cells. Sci
Transl Med. 2**,** 33ra38.

Lewis, R. A., et al., 2017. Screening and purification of natural products from
actinomycetes that affect the cell shape of fission yeast. J Cell Sci. 130**,** 3173-
3185.

Li, Y., et al., 2015. KIFC1 is a novel potential therapeutic target for breast cancer.
Cancer Biol Ther. 16**,** 1316-1322.

Ma, H. T., et al., 2014. Synergism between inhibitors of Aurora A and KIF11
overcomes KIF15-dependent drug resistance. Mol Oncol. 8**,** 1404-1418.

Mager, W. H., Winderickx, J., 2005. Yeast as a model for medical and medicinal
research. Trends Pharmacol Sci. 26**,** 265-273.

Matsuyama, A., et al., 2006. ORFeome cloning and global analysis of protein
localization in the fission yeast *Schizosaccharomyces pombe*. Nat. Biotechnol.
24**,** 841-847.

Maundrell, K., 1990. *nmt1* of fission yeast. A highly transcribed gene completely
repressed by thiamine. J Biol Chem. 265**,** 10857-10864.

Meluh, P. B., Rose, M. D., 1990. KAR3, a kinesin-related gene required for yeast
nuclear fusion. Cell. 60**,** 1029-1041.

Moreno, S., et al., 1991. Molecular genetic analysis of fission yeast
Schizosaccharomyces pombe. Methods Enzymol. 194**,** 795-823.

Mountain, V., et al., 1999. The kinesin-related protein, HSET, opposes the activity of

    Eg5 and cross-links microtubules in the mammalian mitotic spindle. J Cell Biol.

    147**,** 351-366.

Nishi, K., et al., 1992. A leptomycin B resistance gene of *Schizosaccharomyces pombe*

    encodes a protein similar to the mammalian P-glycoproteins. Mol Microbiol. 6**,**

    761-769.

Nkeze, J., et al., 2015. Molecular characterization of HIV-1 genome in fission yeast.

    Cell Biosci. 5**,** 47.

O'Connell, M. J., et al., 1993. Suppression of the *bimC4* mitotic spindle defect by

    deletion of *klpA*, a gene encoding a KAR3-related kinesin-like protein in

    Aspergillus nidulans. J Cell Biol. 120**,** 153-162.

Ohashi, A., et al., 2015. A novel time-dependent CENP-E inhibitor with potent

    antitumor activity. PLoS One. 10**,** e0144675.

Paluh, J. L., et al., 2000. A mutation in γ-tubulin alters microtubule dynamics and

    organization and is synthetically lethal with the kinesin-like protein Pkl1p. Mol.

    Biol. Cell. 11**,** 1225-1239.

Pannu, V., et al., 2015. HSET overexpression fuels tumor progression via centrosome

    clustering-independent mechanisms in breast cancer patients. Oncotarget. 6**,**

    6076-6091.

Park, H. W., et al., 2017. Structural basis of small molecule ATPase inhibition of a

    human mitotic kinesin motor protein. Sci Rep. 7**,** 15121.

Pawar, S., et al., 2014. KIFCI, a novel putative prognostic biomarker for ovarian adenocarcinomas: delineating protein interaction networks and signaling circuitries. J Ovarian Res. 7**,** 53.

Pidoux, A. L., et al., 1996. Fission yeast pkl1 is a kinesis-related protein involved in mitotic spindle function. Mol. Biol. Cell. 7**,** 1639-1655.

Quintyne, N. J., et al., 2005. Spindle multipolarity is prevented by centrosomal clustering. Science. 307**,** 127-129.

Rhys, A. D., et al., 2018. Loss of E-cadherin provides tolerance to centrosome amplification in epithelial cancer cells. J Cell Biol. 217**,** 195-209.

Rincon, S. A., et al., 2017. Kinesin-5-independent mitotic spindle assembly requires the antiparallel microtubule crosslinker Ase1 in fission yeast. Nat Commun. 8**,** 15286.

Robin, G., et al., 2005. Essential kinesins: characterization of *Caenorhabditis elegans* KLP-15. Biochemistry. 44**,** 6526-6536.

Rodriguez, A. S., et al., 2008. Protein complexes at the microtubule organizing center regulate bipolar spindle assembly. Cell Cycle. 7**,** 1246-1253.

Saito, N., et al., 1997. KIFC2 is a novel neuron-specific C-terminal type kinesin superfamily motor for dendritic transport of multivesicular body-like organelles. Neuron. 18**,** 425-438.

Sarnoski, E. A., et al., 2017. A high-throughput screen for yeast replicative lifespan identifies lifespan-extending compounds. Cell Rep. 21**,** 2639-2646.

Sato, M., et al., 2005. New drug-resistant cassettes for gene disruption and epitope

tagging in *Schizosaccharomyces pombe*. Yeast. 22**,** 583-591.

Saunders, W., et al., 1997. Mitotic spindle function in *Saccharomyces cerevisiae*

requires a balance between different types of kinesin-related motors. Mol Biol

Cell. 8**,** 1025-1033.

Sharp, D. J., et al., 2000. Microtubule motors in mitosis. Nature [Lond.]. 407**,** 41-47.

She, Z. Y., Yang, W. X., 2017. Molecular mechanisms of kinesin-14 motors in spindle

assembly and chromosome segregation. J Cell Sci. 130**,** 2097-2110.

Takemoto, A., et al., 2016. Nuclear envelope expansion is critical for proper

chromosomal segregation during a closed mitosis. J Cell Sci. 129**,** 1250-1259.

Tanenbaum, M. E., Medema, R. H., 2010. Mechanisms of centrosome separation and

bipolar spindle assembly. Dev. Cell. 19**,** 797-806.

Toda, T., et al., 1992. Fission yeast pap1-dependent transcription is negatively regulated

by an essential nuclear protein, crm1. Mol Cell Biol. 12, 5474-5484.

Toda, T., et al., 1991. Fission yeast genes that confer resistance to staurosporine encode

an AP-1-like transcription factor and a protein kinase related to the mammalian

ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. Genes Dev. 5, 60-73.

Toone, W. M., et al., 1998. Regulation of the fission yeast transcription factor Pap1 by

oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and

the stress-activated MAP kinase, Sty1. Genes Dev. 12, 1453-1463.

Troxell, C. L., et al., 2001. *pkl1*[+] and *klp2*[+]: two kinesins of the Kar3 subfamily in

fission yeast perform different functions in both mitosis and meiosis. Mol. Biol.

Cell. 12**,** 3476-3488.

Walczak, C. E., et al., 1998. A model for the proposed roles of different microtubule-

based motor proteins in establishing spindle bipolarity. Curr. Biol. 8**,** 903-913.

Wang, B., et al., 2015. The *Aspergillus nidulans bimC4* mutation provides an excellent

tool for identification of kinesin-14 inhibitors. Fungal Genet Biol. 82**,** 51-55.

Watts, C. A., et al., 2013. Design, synthesis, and biological evaluation of an allosteric

inhibitor of HSET that targets cancer cells with supernumerary centrosomes.

Chem Biol. 20**,** 1399-1410.

Wu, J., et al., 2013. Discovery and mechanistic study of a small molecule inhibitor for

motor protein KIFC1. ACS Chem Biol. 8**,** 2201-2208.

Xiao, Y. X., Yang, W. X., 2016. KIFC1: a promising chemotherapy target for cancer

treatment? Oncotarget. 7**,** 48656-48670.

Yang, B., et al., 2014. Discovery of potent KIFC1 inhibitors using a method of

integrated high-throughput synthesis and screening. J Med Chem. 57**,** 9958-

9970.

Yang, H., et al., 2012. Effects of HIV-1 protease on cellular functions and their

potential applications in antiretroviral therapy. Cell Biosci. 2**,** 32.

Yukawa, M., et al., 2017. A microtubule polymerase cooperates with the Kinesin-6

motor and a microtubule crosslinker to promote bipolar spindle assembly in the

absence of Kinesin-5 and Kinesin-14 in fission yeast. Mol Biol Cell. 28**,** 3647-

3659.

Yukawa, M., et al., 2018. Two spatially distinct Kinesin-14 Pkl1 and Klp2 generate

collaborative inward forces against Kinesin-5 Cut7 in *S. pombe*. J Cell Sci. 131**,**

1-11.

Zhang, W., et al., 2016. Discovery of a novel inhibitor of kinesin-like protein KIFC1.

Biochem J. 473**,** 1027-1035.

Table 1: IC$_{50}$ of HSET/KIFC1 inhibitors

IC$_{50}$ (µM)	In vitro ATPase activity	In vivo cell viability	(Cell lines)
AZ82	0.3	N.D.	
CW069	75	86	(N1E-115)
SR31527	6.6	20 - 33	(MDA-MB-231, BT-549, MDA-MB-435S)

Microtubule-stimulated ATPase activity was measured using bacterially-produced HSET proteins. Viability was measured using cancer cell lines that contain excess centrosomes. Values (µM) are taken from the following references: AZ82 (Wu et al., 2013); SR31527 (Zhang et al., 2016); CW069 (Watts et al., 2013). N.D., not determined.

Table 2: Fission yeast strains used in this study

Strains	Genotypes	Figures used	Derivations
TY206	*h⁻ aur1R-Pnda3-mCherry-atb2 leu1 ura4*	1A, 1D	This study
TY223	*h⁺ kanR-Pnmt41-cut7 aur1R-Pnda3-mCherry-atb2 leu1 ura4 his2*	1A, 1D	This study
MY1212	*h⁻ lys1⁺-Pnmt41-GFP-HSET aur1R-Pnda3-mCherry-atb2 leu1 ura4 lys1*	1A, 1C-D	This study
TY241	*h⁻ kanR-Pnmt41-cut7 lys1⁺-Pnmt41-GFP-HSET aur1R-Pnda3-mCherry-atb2 leu1 ura4 lys1*	1A, 1C-D	This study
MY1230	*h⁻ lys1⁺-Pnmt1-GFP-pkl1 aur1R-Pnda3-mCherry-atb2 leu1 ura4 lys1*	1A, 1C-D	This study
TY245	*h⁻ kanR-Pnmt41-cut7 lys1⁺-Pnmt1-GFP-Pkl1 aur1R-Pnda3-mCherry-atb2 leu1 ura4 lys1*	1A, 1C-D	This study
TY208	*h⁻ leu1 ura4* [pREP41-GFP]	1B	This study
TY212	*h⁻ kanR-Pnmt41-cut7 leu1 ura4* [pREP41-GFP]	1B	This study
TY211	*h⁻ leu1 ura4* [pREP41-GFP-klp2]	1B	This study
TY215	*h⁻ kanR-Pnmt41-cut7 leu1 ura4* [pREP41-GFP-klp2]	1B	This study
513	*h⁻ leu1 ura4*	3	Our laboratory stock
YA8	*h⁺ bfr1::ura4⁺ pmd1::hisG leu1 ura4*	3, 4A	(Arita et al., 2011)
SAK931	*h⁻ caf5::bsdR pap1Δ pmd1Δ mfs1Δ bfr1Δ dnf2Δ erg5::ura4⁺ leu1 ura4 ade6-M210*	3	(Takemoto et al., 2016)
MY1204	*h⁻ lys1⁺-Pnmt1-HSET bfr1::ura4⁺ pmd1::hisG aur1R-Pnda3-mCherry-atb2 leu1 ura4 lys1*	4A	This study
TY250	*h⁻ caf5::bsdR pap1Δ pmd1Δ mfs1Δ bfr1Δ dnf2Δ erg5::ura4⁺ leu1 ura4 ade6-M210* [pREP41-GFP]	4A-B	This study
TY247	*h⁻ caf5::bsdR pap1Δ pmd1Δ mfs1Δ bfr1Δ dnf2Δ erg5::ura4⁺ leu1 ura4 ade6-M210* [pREP41-GFP-HSET]	4A-B	This study
TY26	*h⁺ bfr1::ura4⁺ pmd1::hisG leu1 ura4* [pREP41-GFP]	4B	This study
TY27	*h⁺ bfr1::ura4⁺ pmd1::hisG leu1 ura4* [pREP41-GFP-HSET]	4B-C, 5B	This study

Table 3: Effects of HSET/KIFC1 inhibitors in fission yeast

	Toxicity to *S. pombe*	Inhibition of HSET	Rescue of cell growth
AZ82	YES	YES	YES
CW069	YES	NO	NO
SR31527	YES	YES	NO

AZ82 is toxic to all three fission yeast strains tested (513: wild type; YA8: *bfr1Δpmd1Δ*; SAK931: *7Δ*). CW069 is toxic only to SAK931, while SR31527 is toxic to YA8 and SAK931.

LEGENDS FOR FIGURES

Fig. 1: Co-overproduction of kinesin-5 Cut7 neutralizes growth toxicity derived from kinesin-14 overproduction

(**A, B**) Spot test. Strains overexpressing *cut7* (*cut7^oe*) and/or a gene encoding kinesin-14 (**A**, fission yeast *pkl1^oe* or human *HSET^oe* or **B**, fission yeast *klp2^oe*) were serially (10-fold) diluted, spotted onto minimal plates in the presence or absence of thiamine (+Thi/repressed or −Thi/derepressed, respectively) and incubated at 30°C for 3 d. *cell conc.*, cell concentration. *klp2* was expressed ectopically on plasmids. *nmt1-pkl1* and *nmt41-HSET* were integrated at the *lys1* locus (Yukawa et al., 2018), while the *nmt41* promoter was integrated in front of the initiation codon of the *cut7* gene (Bähler et al., 1998). (**C**) Cellular localization of overexpressed Pkl1 and HSET and spindle morphology. Representative images are shown for each strain. Scale bars, 10 μm. (**D**) The percentage of bipolar (green) or monopolar spindles (magenta) upon overexpression of either *pkl1* or *HSET*. In each condition, more than 30 mitotic cells were counted (n>30). All p-values are derived from the two- tailed χ2 test (***P<0.001; ****P<0.0001).

Fig. 2: Strategy for evaluation of the specificity and efficacy of human kinesin-14 inhibitors using a fission yeast system

Inhibitors against human kinesin-14 are categorized into two classes. The first class is those that do not inhibit fission yeast cells on their own (**A,** specific drugs). The second class is those that possess growth-inhibiting activities on their own. It is likely that these drugs have some target molecules other than HSET that are essential for fission yeast cell

viability (**B**, multi-target drugs). In either type A or B, we could assess inhibitory activity toward HSET by monitoring growth properties of cells in which HSET is overproduced in the presence or absence of drug treatment.

Fig. 3: The three kinesin-14 inhibitors display off-target cytotoxicity in fission yeast

Growth suppression of fission yeast cells upon treatment with individual kinesin-14 inhibitors. Wild type (513, Table 2) (top row) and two types of drug-permeable fission yeast strains (YA8: *bfr1Δpmd1Δ* or SAK931: 7Δ, Table 2) were used (bottom row). Error bars (SE) are calculated from at least 2 independent experiments.

Fig. 4: AZ82 partially rescues HSET-induced lethality

(**A**) Growth characteristics of fission yeast cells upon HSET overproduction in the presence of individual inhibitors. YA8 (top) or SA931 cells (bottom) overexpressing the *HSET* gene were inoculated at 10^5 cells/ml in minimal medium in the absence of thiamine (derepressed condition, ON) in 96-hole microplates, and DMSO (mock) or drug (dissolved in DMSO) was added. The final concentration of DMSO was 1%. Cells were incubated for 2-3 d at 30°C. Note that small precipitates of cells are visible in HSET-overproducing YA8 cells containing 5 μM or 10 μM of AZ82. (**B**) Relative number of HSET-overproducing cells (*HSET^{oe}*) in the presence of individual drugs. Cell number in the absence of drugs and *HSET^{oe}* is set as 100%, and the relative value of individual conditions are calculated (n>3). All p-values are derived from the two-tailed $\chi 2$ test (**, $P < 0.01$, n.s., not significant). (**C**) The percentage of mitotic cells with monopolar (Mono)

or bipolar spindles (Bi). HSET-overproducing YA8 cells were treated with AZ82 (0, 10 or 15 μM) for 2 d at 30°C. On the left panel, representative images of mitotic cells containing monopolar (top row) or bipolar cells (bottom two rows) are shown. Mitotic spindles were visualized with GFP-HSET, which is colocalized with spindle microtubules (Yukawa et al., 2018). At least >200 cells were counted. All p-values are derived from the two-tailed $\chi2$ test (**, P < 0.01, ****, P < 0.0001). Scale bar, 10 μm.

Fig. 5: A general strategy for identification and evaluation of specific inhibitors against human proteins using a fission yeast system and the detection of HSET-inhibiting activities in extracts from plant species

(**A**) Provided that human genes overexpressed in fission yeast confer some phenotype, such as lethality, this overproducing strain can be used as an assay system in which to identify specific inhibitors against these proteins. Libraries consisting of small molecules or natural products, or extracts prepared from *Actinomycetes*, marine organisms or plants can be used as sources of inhibitor compounds. Molecules that show activity that rescues lethal overproduction of protein X without any adverse off-target effects toward fission yeast cells would be ideal. (**B**) Identification of HSET-neutralizing activity in plant extracts. Extracts from *Chamaecyparis pisifera* (*Sawara cypress*) and *Toxicodendron trichocarpum* (*Japanese sumac*) were prepared in methanol (10 mg/ml) (Kimura et al., 2012). 5 μl aliquots of extracts (each containing 0, 0.2 and 0.4 μg) were spotted onto agar plates. Plates were prepared from minimal media that contained HSET-overproducing fission yeast cells (4.6×10^4 cells/ml) and incubated at 28°C for 3 d.
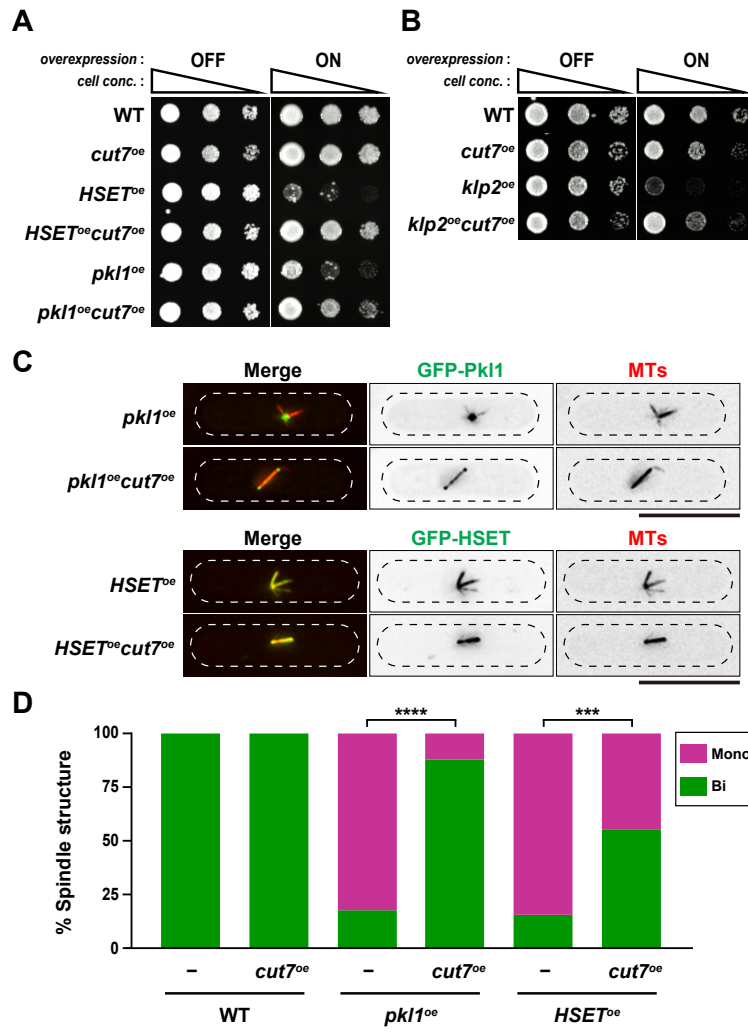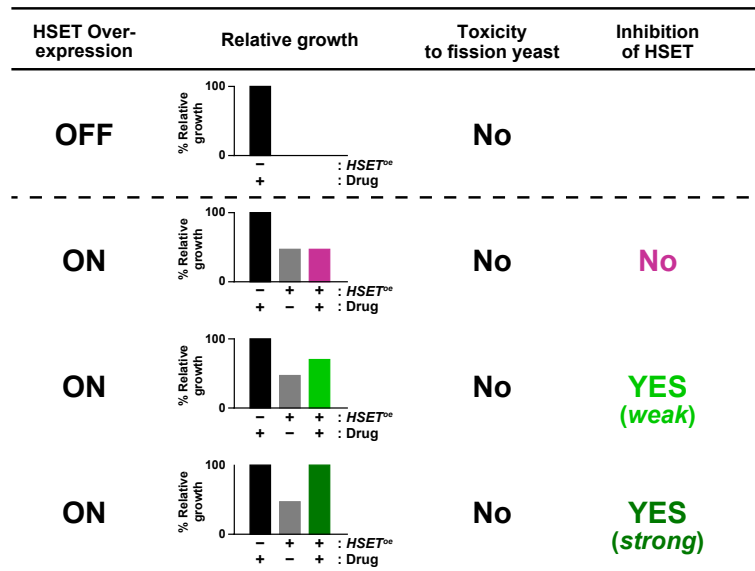
Figure 1. Yukawa *et al*.

A

overexpression : OFF | ON
cell conc. :

WT
cut7^{oe}
HSET^{oe}
HSET^{oe}*cut7*^{oe}
pkl1^{oe}
pkl1^{oe}*cut7*^{oe}

B

overexpression : OFF | ON
cell conc. :

WT
cut7^{oe}
klp2^{oe}
klp2^{oe}*cut7*^{oe}

C

| | Merge | GFP-Pkl1 | MTs |
pkl1^{oe}
pkl1^{oe}*cut7*^{oe}

| | Merge | GFP-HSET | MTs |
HSET^{oe}
HSET^{oe}*cut7*^{oe}

D

% Spindle structure

Mono
Bi

**** ***

WT: – , *cut7*^{oe}
pkl1^{oe}: – , *cut7*^{oe}
HSET^{oe}: – , *cut7*^{oe}

Figure 2. Yukawa *et al*.

A Specific drugs

HSET Over-expression	Relative growth	Toxicity to fission yeast	Inhibition of HSET
OFF		No	
ON		No	No
ON		No	YES (*weak*)
ON		No	YES (*strong*)

B Multi-target drugs

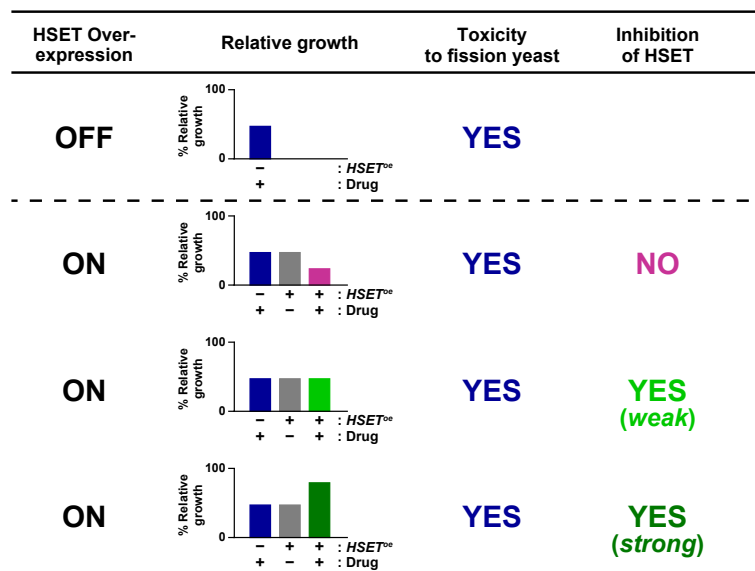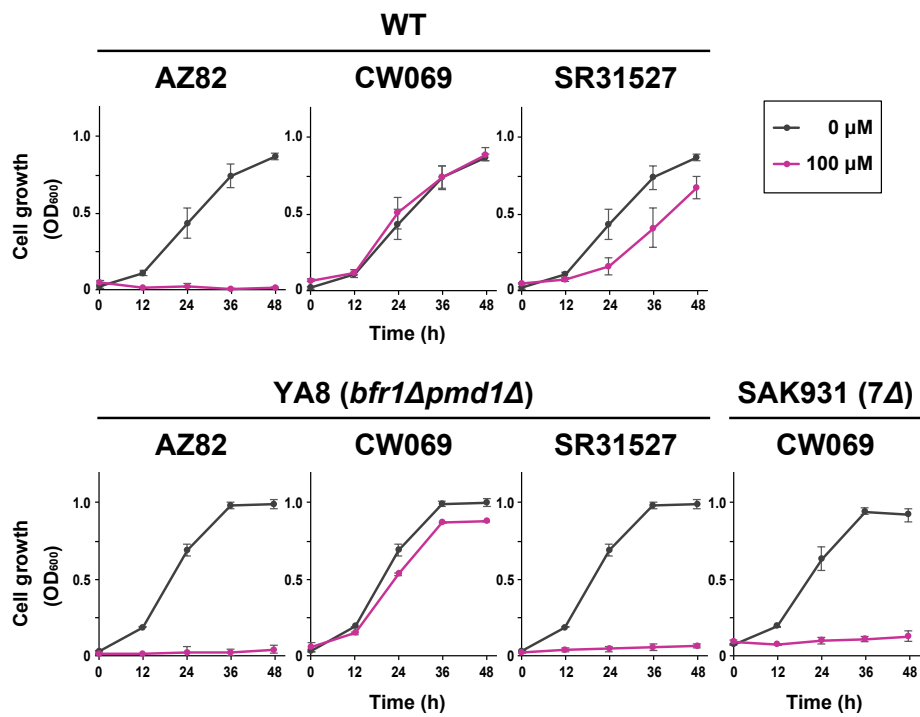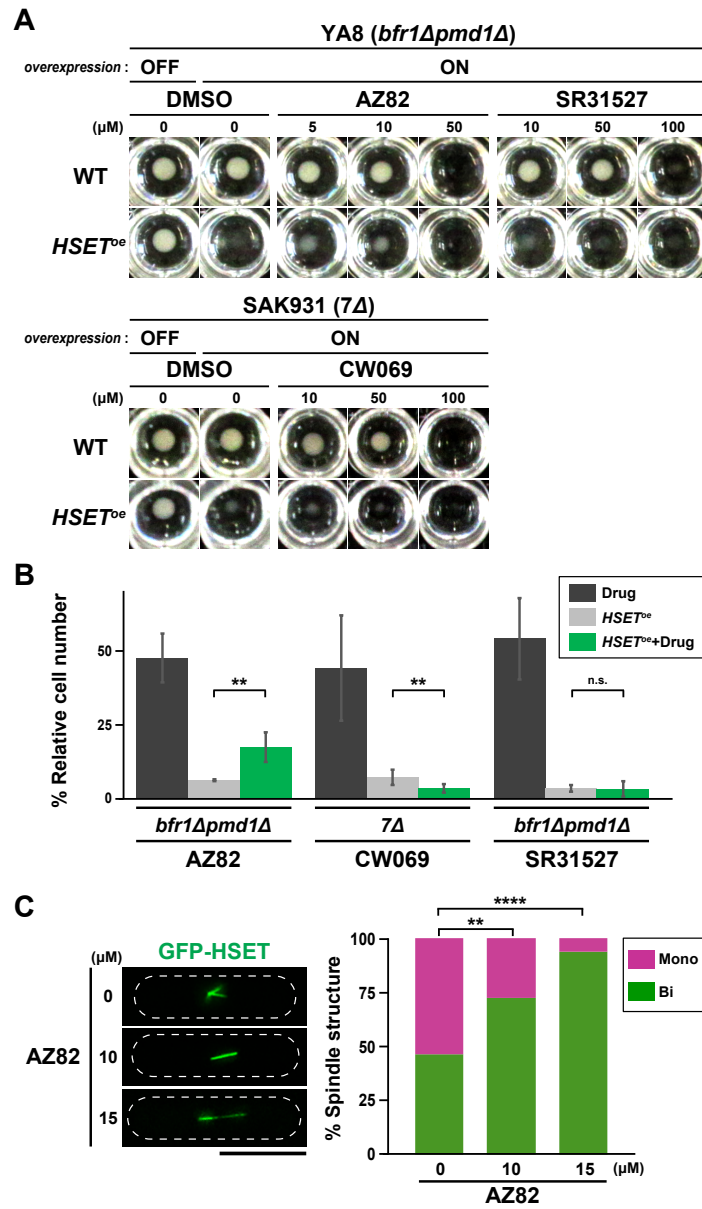| HSET Over-expression	Relative growth	Toxicity to fission yeast	Inhibition of HSET
OFF		YES	
ON		YES	NO
ON		YES	YES (*weak*)
ON		YES	YES (*strong*)

Figure 3. Yukawa *et al*.

Figure 4. Yukawa *et al*.

Figure 5. Yukawa *et al*.

A

Overproduced human proteins ▲

OFF → ON

Fission yeast

Lethal

Chemical library
or
Extract of natural sources

Viable

Inhibitor

B

Chamaecyparis pisifera

0   0.2   0.4   (µg)

HSET^OE

Toxicodendron trichocarpum

0   0.2   0.4   (µg)

HSET^OE