Small-Molecule Control of Kinesin-5 Proteins

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy In Biological Sciences

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> March 21, 2008 Blacksburg, Virginia

Keywords: albumin, ATPase, HsEg5, inhibitor, kinesin, microtubule(s), mitosis, monastrol

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Abstract

Mitosis, or cell division, is the mechanism by which cells divide and is an intricate process requiring the action and control of numerous proteins. Such proteins serve either as structural entities within the mitotic spindle, or perform the "work" within the apparatus. In particular, Kinesin-5 motor proteins, a subset within the kinesin motor protein superfamily, are primarily responsible for organization of microtubules (MTs) within the mitotic apparatus, and are consequently vital for efficient mitosis. These proteins utilize energy from ATP hydrolysis in order to "walk" along antiparallel MTs, positioning them into the bipolar mitotic spindle. Loss of Kinesin-5 activity results in formation of a monoastral spindle and subsequent cell cycle arrest.

Recently, a wide variety of small molecules have been identified that possess the ability to inhibit certain Kinesin-5 motors. Such compounds, including monastrol (the first Kinesin-5 inhibitor identified), have been employed to study Kinesin-5 activity. A thorough understanding of Kinesin-5 function, combined with the ability to specifically target these proteins with small molecules, may provide the capability to control cell division and may therefore have significant implications in anti-cancer therapies.

The following dissertation describes research that utilizes small molecules to probe the function (ATPase activity and MT interactions) of various Kinesin-5 proteins and provides information that will lead to a better understanding of exactly how such proteins function in vivo. Further, a greater knowledge of Kinesin-5 protein activity as well as specific interactions with small-molecule compounds, may lead to the development of more potent, less toxic anti-cancer drugs.

Grant Information

This work was supported by NSF (MCB-0130910 to R. Walker), NIH (GM066328 to E. Wojcik.), ASPIRES (S. Kim, R. Walker, and E. Wojcik), and the OSER / Carillion Biomedical Institute (E. Wojcik and S. Kim). S. Learman also obtained support from the Virginia Tech Graduate Research and Development Project and Sigma Xi.

Acknowledgements

I would like to take this opportunity to thank those people without whom this work could not have been completed. First and foremost, to my husband Deric, thank you for your neverending encouragement and wisdom. I would also like to thank my parents, Sue and Jim Hooper, I would not have made it this far without your love and support.

I would also like to thank each member of my advisory committee for his or her contributions to both my intellectual and personal development. To my advisor, Rich Walker, thank you for providing me with the opportunity to work in your lab. Thank you for your guidance, patience and for helping me to become an independent scientist. I feel confident pursuing a career with the knowledge and experience that I have obtained working with you. To Sunyoung Kim, thank you for knowing (and helping me see) what I am capable of, and for always pushing me towards it. To Jill Sible, thank you for including me in your lab gatherings, and for always making time to listen and offer advice (especially over a knitting break). I am also grateful to Edward Wojcik, for your valuable contributions to my research, and Daniela Cimini, for joining my committee at the last minute and still taking the time to help me develop as a scientist.

Josh Williams, thank you very much for allowing me to use (and for assistance with) the multi-well plate reader used for data collection in Chapters 2 and 3. Several undergraduates also contributed considerable effort towards completion of this project. Brie Barnes was a substantial help in making and running protein gels, among other things, and both Nathan Stevens and Kim Williams each collected data included in Chapters 2 and 3, respectively.

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List of Abbreviations

deoxyribonucleic acid: DNA microtubules: MTs adenosine-triphosphate: ATP malachite green: MG pyruvate kinase / lactate dehydrogenase: coupled amino acid: AA S-Trityl-L-cysteine: STLC Boc-S-Trityl-L-cysteine: BSTLC Fmoc-S-Trityl-L-cysteine: FSTLC wild type: WT bovine serum albumin: BSA human serum albumin: HSA

Chapter 1: Introduction

Literature Review

The Mitotic Apparatus and its Major Components: Microtubules and Microtubule-Dependent Motor Proteins

Cellular reproduction is characterized by the separation of replicated genetic material into two identical daughter cells. The mitotic apparatus, also known as the mitotic spindle, is responsible for physical movements of chromosomes within mitosis, such as alignment and segregation (Alberts et al., 2002; Lodish et al., 2000). The mitotic spindle is comprised of two major components: microtubules (MTs), a type of cytoskeletal fiber, and MT-dependent motor proteins that transport cellular cargo along the MTs (which serve as "railroads") (Alberts et al., 2002). Such motor proteins perform diverse functions within the mitotic apparatus. For example, some spindle motors are responsible for chromosomal movements, while others for the organization of MTs into a working bipolar spindle. MT-dependent motor proteins are also responsible for the transportation of other cellular cargo, such as vesicles, both within and independent of the mitotic apparatus (i.e. in interphase) (Alberts et al., 2002; Lodish et al., 2000; Mitchison and Kirschner, 1984).

Within the mitotic apparatus, two spindle poles migrate to separate sides of the cell, while spindle forming MTs assemble. Kinetochore-MTs then attach and align the chromosomes at the metaphase plate. Chromosome segregation takes place following sister chromatid separation as each sister moves poleward along its attached MT. After the chromosomes have moved a sufficient distance toward their respective spindle pole, cytokinesis occurs, characterized by cytoplasmic division and consequent separation of the newly formed daughter cells (Alberts et al., 2002; Lodish et al., 2000). Figure 1.1 illustrates a mitotic spindle in metaphase with the aligned chromosomes attached to kinetichores MTs that originate from spindle poles at either side of the apparatus.

The Kinesin Superfamily of Motor Proteins: Structure and Function

As previously mentioned, motor proteins serve as "laborers" within a typical eukaryotic cell. Functioning in both mitotic and non-mitotic events, MT-based motor proteins utilize energy from adenosine-triphosphate (ATP) hydrolysis in order to translocate along MTs. Motor protein reviews can be found in (Alberts et al., 2002; Mallik and Gross, 2004).

Based on sequence and structure, MT-based motor proteins are classified into two major families: dyneins and kinesins (Alberts et al., 2002). Because this work involves the study of kinesins, they will be discussed here in detail. The kinesin family is broken down into 14 subfamilies, each responsible for a different cellular function. Such kinesin motor protein activities include, but are not limited to: movement and transport of organelles, vesicles, protein complexes, MTs, and chromosomes (Alberts et al., 2002; Vale et al., 1985) (for a kinesin specific review, see (Miki et al., 2005). Criterion used to divide subfamilies is based on sequence similarity, primarily in the motor domain, which contains both the MT- and nucleotidebinding site (Alberts et al., 2002). Figure 1.2 shows the typical structure of a kinesin motor protein. In general, kinesins exist as dimers that possess two amino-terminal motor domains that perform the work of the protein (Alberts et al., 2002; Miki et al., 2005). Most kinesins also have an elongated α -helical stalk region whose presence facilitates dimer formation (Miki et al., 2005). This region is connected to the motor domain via a family-specific neck linker, which is thought to regulate motor activity and directionality (Higuchi and Endow, 2002; Miki et al., 2005; Rice et al., 1999). At the tail end, some kinesins associate with light chains that are responsible for the motor's interactions with cellular cargo (Alberts et al., 2002; Miki et al., 2005).

Although considerable research on the kinesin family's mechanism of action has been published, many details of the process are still poorly understood. It has been established, however, that kinesins act via nucleotide hydrolysis and consequent conformational changes that mimic a "walking" motion on MTs (Vale et al., 1996). The typical ATP hydrolysis cycle of kinesins is illustrated in Figure 1.3 and starts with an ADP-bound motor head binding a MT (labeled step 1 in Figure 1.3). MT binding stimulates ADP release, which is followed by ATP association (Hackney, 1996). Upon ATP binding, small movements within the protein regulate docking of the short neck linker domain parallel to the MT-bound motor head. This movement results in the positioning of the second motor head further down the MT where it can begin to associate with a new attachment site, step 2. Next, the energy released from ATP hydrolysis positions the second motor head to interact with the MT (further towards the plus end), step 3 (Hancock and Howard, 1999). As the first motor head, now with ADP bound, dissociates from the MT, the second motor head, also with ADP bound, tightly attaches to the MT, step 4 (Hancock and Howard, 1999).

The Kinesin-5 Motor Protein Subfamily: Structure and Function

There are 6 kinesin subfamilies that function within mitosis: Kinesin- 4, 5, 6, 7, 13, and 14 (Miki et al., 2005). The work described in subsequent chapters will primarily focus on the Kinesin-5 subfamily of kinesin motor proteins. The first Kinesin-5 protein to be discovered, bimC, was identified as a temperature-sensitive mutation that produced a "blocked in mitosis" phenotype in *A. nidulans* (Enos and Morris, 1990). Subsequently, other Kinesin-5 proteins have been identified in many eukaryotes and may be ubiquitous. Other organisms in which Kinesin-5 family proteins are present and have been studied include but are not limited to: *S. cerevisiae* (Hoyt et al., 1992), *S. compressa* (Peters and Kropf, 2006), *C. elegans* (Greene and Henikiff, 2005), *A. thaliana* (Greene and Henikiff, 2005), *D. melanogaster* (Heck et al., 1993), *X. laevis* (Le Guellec et al., 1991), and *H. sapiens* (Blangy et al., 1995).

With an N-terminal motor domain, short neck linker, an α -helical center stalk domain, and a C-terminal tail domain, Kinesin-5 family domain organization is consistent with that previously described for the kinesin superfamily (Blangy et al., 1995; DeBonis et al., 2003; Lawrence et al., 2004). However, instead of existing as dimers, Kinesin-5 proteins function as homotetramers, comprised of two dimers arranged "head to tail" (Kashina et al., 1996; Walczak and Mitchison, 1996). A schematic of how Kinesin-5 monomers form a dimer, and subsequently a functional homotetramer, is illustrated in Figure 1.4. Another factor that distinguishes Kinesin-5 proteins from the kinesin superfamily is the existence of an insertion loop, termed the L5 insertion loop, within helix $\alpha 2$ (Turner et al., 2001). Due to recent evidence that the Kinesin-5 L5 loop changes position relative to the motor's nucleotide state, it has been suggested that this loop is involved in motor activity (e.g. nucleotide binding / hydrolysis) (Brier et al., 2004; Maliga et al., 2002; Maliga and Mitchison, 2006; Mayer et al., 1999).

Functionally, Kinesin-5 proteins are indispensable for efficient cell division. Via MT plus end-directed motility, Kinesin-5 motor proteins are responsible for formation of the mitotic

spindle (Blangy et al., 1998; Enos and Morris, 1990; Greene and Henikiff, 2005; Sawin et al., 1992). With the energy released from ATP hydrolysis, each motor domain pair (located at opposite ends of the protein) binds antiparallel MTs originating from opposite spindle poles and pushes the MTs (and ultimately the spindle poles) apart in order to generate a bipolar mitotic spindle (Gilbert and Johnson, 1994; Kapitein et al., 2005; Kashina et al., 1997; Sharp et al., 2000b).

Kinesin-5 family function has been determined by inhibiting specific members, via temperature-inducible mutations in *A. nidulans* (bimC) (Enos and Morris, 1990), loss of function mutations in *D. melanogaster* (KLP61F) (Wilson et al., 1997), *X. laevis* (Eg5) (Sawin and Mitchison, 1995), and *H. sapiens* (HsEg5) (Blangy et al., 1998), RNA interference in HsEg5 (Stout et al., 2006), as well as small molecule inhibitors in Eg5 and HsEg5 (DeBonis et al., 2004; Kapoor et al., 2000; Mayer et al., 1999). In each case, inhibition of these Kinesin-5 family members results in formation of a monoastral spindle and mitotic arrest. This mitotic defect is termed monoastral because the resulting spindle contains both spindle poles centrally aggregated, with MTs protruding outwards in a sphere that is surrounded by a ring of attached chromosomes (Mayer et al., 1999). It has been hypothesized that this spindle structure is a result of the work of MT minus end-directed motor proteins (potentially cytoplasmic dyneins and members of the Kinesin-14 family), which would normally oppose the plus end-directed force of Kinesin-5 (Mayer et al., 1999; Sharp et al., 2000b). Figure 1.5 illustrates a monoastral phenotype resulting from the Kinesin-5 inhibition.

HsEg5: Structure and Mechanochemical Transduction

Due to their vital role in cell division, understanding all species' Kinesin-5 family members is of scientific relevance; however the following research will focus on vertebrate Kinesin-5s (Eg5), for application towards the Human Kinesin-5, HsEg5. Although basic Kinesin-5 structure and function were described above, there are a few additional HsEg5-specific features that may impact activity. The full length HsEg5 protein consists of a unique 20 amino acid N-terminal residue stretch (of unknown function), which is then followed by the conserved kinesin family motor domain, followed by a short neck linker region that connects the motor to an α -helix domain (consistent with the previously discussed structure) (DeBonis et al., 2003). Lastly, the C-terminal tail contains a cyclin-B/p34^{cdc2} phosphorylation site located at Thr-927 (Sawin and Mitchison, 1995). Phosphorylation at this site initiates localization and action of HsEg5 within the spindle complex (Blangy et al., 1997; Blangy et al., 1995; Sawin and Mitchison, 1995). Figure 1.6 shows a schematic representation of the HsEg5 motor protein domain organization.

The HsEg5 crystal structure illustrates Kinesin-5 specific structural entities previously described (e.g. the L5 loop) as well as those generally conserved in kinesin proteins, such as the nucleotide and MT binding sites (Turner et al., 2001). Other structural entities possessed by kinesin motors that are important for mechanotransduction are described as follows, with specific emphasis on HsEg5. Kinesin motor proteins possess two "switches" that function to relay subtle environmental changes (i.e. the presence or absence of gamma phosphate within the nucleotide binding pocket) to other parts of the motor. As illustrated by the crystal structure, and shown in Figure 1.7, HsEg5's switch I is a loop located at the end of helix α 3, and switch II (also called the "relay helix") is made up of helix $\alpha 4$ (Turner et al., 2001). In the presence of ATP, these two switches form contacts with ATP's gamma phosphate and with each other. Upon the conversion of ATP to ADP, and subsequent gamma phosphate release, the inter-switch contacts are eliminated. More specifically, in the ATP-bound state, switch II is in an "up" conformation, facilitating inter-switch and gamma phosphate communication. It has been suggested that the loss of the inter-switch communication that occurs in the presence of ADP results in a shift of switch II to a "down" position (Turner et al., 2001). Based on the aforementioned positional alterations during the process of ATP binding and hydrolysis, it is speculated that the accompanying structural changes within the motor domain of HsEg5 alter the motor's MT affinity, which is critical for mechanotransduction (Turner et al., 2001).

The last HsEg5 specific structural entity to be considered here resides at the C-terminal end of helix α 6. Here lies the neck linker, a short section of amino acids that connects the motor domain to the protein's helical stalk. As previously noted for kinesins in general, the neck linker plays a major role in motor force production as well as directionality (Higuchi and Endow, 2002; Miki et al., 2005; Rice et al., 1999). Interestingly, in the HsEg5 crystal structure, the neck linker takes on a conformation different from that previously observed for other kinesins. In the Kinesin-1 / ADP bound state, the neck linker is positioned parallel to the long axis of the motor domain while HsEg5's neck linker, as visualized in the crystal structure, is perpendicular to the motor domain. (Higuchi and Endow, 2002; Rice et al., 1999; Turner et al., 2001). This

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difference in HsEg5's neck linker positioning compared to Kinesin-1 may be a result of protein specific conformational changes within the nucleotidase cycle. In the presence of ADP, HsEg5's switch II is "down", thereby inducing steric interference that prevents association between the motor domain and the neck linker, forcing the neck linker to take a perpendicular position relative to the motor domain (Turner et al., 2001). However, when ATP binds, switch II moves upward to form contacts with the gamma phosphate, leaving room for the neck linker to move down and dock along the long axis of motor (Turner et al., 2001). The docking and undocking of the neck linker, and its dependence on nucleotide state, is hypothesized to result in alterations in MT affinity and HsEg5 movement along the MT (Turner et al., 2001).

Along with crystallography, kinetic experiments have been utilized to better understand the specific nucleotidase cycle and consequent mechanotransduction that occurs within the HsEg5 motor. ATPase analyses have shown that the motor's ATP hydrolysis rate is slower than that of ATP binding to the MT motor complex, suggesting that ATP binding is followed by a rate limiting conformational change that results in ATP hydrolysis (Cochran et al., 2004). A current model for Eg5's kinetic cycle suggests that this conformational change required for ATP hydrolysis is, in fact, the neck linker docking described above (Valentine and Gilbert, 2007). A brief description of HsEg5 ATPase cycle and subsequent MT transduction is as follows. As an ADP-bound HsEg5 motor head binds a MT, the ADP is rapidly released. ATP then associates with the MT bound motor head and provides the energy for positioning of the second motor head further towards the plus MT end. ATP hydrolysis occurs and the first motor head detaches from the MT as the second, more forward (towards the plus end), ADP-bound head binds the MT more tightly. It is this repetitive cycle that allows the HsEg5 motor protein to move "step wise" along the MT (Valentine and Gilbert, 2007).

HsEg5 Inhibition by Monastrol

As previously stated, inhibition of HsEg5 by several small molecules results in mitotic arrest. Monastrol, the first discovered small-molecule HsEg5 inhibitor, is a dihydropyrimidine derivative and specifically inhibits mitotic MT motility (via actions on HsEg5) inducing monoastral spindle formation (Mayer et al., 1999). The structure of monastrol is shown in Figure 1.8. In vertebrate cultured cells, monastrol does not affect a previously established bipolar spindle and does not delay cellular progression through the S and G phases of the cell

cycle (Kapoor et al., 2000). This indicates that monastrol only affects spindle formation and therefore only affects cells in the early stages of mitosis. Mayer et al. (1999) noted the molecule's specificity for monkey Eg5 with experiments revealing that monastrol does not inhibit conventional kinesin, some conventional kinesin homologs, or other kinesins/dyneins involved in vesicle transport (Mayer et al., 1999). Further, Eg5's in vitro ATPase activity decreases in the presence of monastrol both with and without MTs (MT-stimulated and basal, respectively) (Mayer et al., 1999). Monastrol does not affect chromosome attachment to MTs or MT assembly in mouse cells. In addition, the effects of monastrol may be reversed (spindle poles regain bipolarity, chromosomes re-align and mitosis continues to completion) with removal of the compound (Kapoor et al., 2000; Mayer et al., 1999).

Since its discovery, the mechanism of monastrol-induced Eg5 inhibition has been actively investigated. The monastrol / HsEg5 interaction is allosteric, and binding of the compound to the protein's motor domain induces conformational changes (discussed below) that prevent the motor's nucleotidase activity. Monastrol binds loosely to Eg5 when in the Eg5 : ATP complex, but tightly to the Eg5 : ADP complex, thereby inhibiting the conformational changes necessary for ADP release. Evidence also suggests that monastrol slows or even stops ADP (and potentially P_i) release both in the presence and the absence of MTs (Cochran et al., 2005; Cochran and Gilbert, 2005; Maliga et al., 2002). From these data, it has been hypothesized that monastrol actually binds Eg5 in an Eg5 : ADP complex (immediately after ATP is hydrolyzed), and facilitates an energetically-favorable state where the P_i immediately rebinds to form the monastrol-Eg5-ATP complex again without translocation along MTs (Cochran et al., 2005; Maliga et al., 2002).

The structural alterations that occur within the HsEg5 motor domain in the presence of monastrol result in the inability of the protein to hydrolyze ATP and consequently translocate along MTs. The crystal structures of HsEg5 : ADP and both with and without monastrol are illustrated in Figure 1.9. Monastrol binds 12Å from the motor's nucleotide binding site in a self-creating, induced-fit pocket between the α 2 and α 3 helices, enclosed by the L5-insertion loop (Cochran and Gilbert, 2005; DeBonis et al., 2003; Luo et al., 2004; Maliga et al., 2002; Wojcik et al., 2004; Yan et al., 2004). Structural alterations induced by monastrol include switch I (responsible for nucleotide binding, previously discussed) which, in the presence of monastrol, shifts ~6Å thereby altering the nucleotide binding site conformation (Yan et al., 2004). Further

structural changes seen when monastrol binds HsEg5 include reorganization of switch II and the C-terminal neck-linker (Yan et al., 2004). When bound by monastrol, switch II (usually involved in nucleotide binding, discussed above) angles outward causing the neck-linker to change conformation and "dock" along the motor domain, resulting in a loss of mobility and locking the protein into a nonfunctional position (Maliga et al., 2006; Yan et al., 2004). Also, revealed in Figure 1.9 is the apparent "closure" of HsEg5's L5- insertion loop in the presence of monastrol. It has been suggested that this movement of the L5 loop, "molding" around the compound, is the major conformational change that facilitates monastrol binding (Cochran and Gilbert, 2005; Yan et al., 2004).

Other monastrol-induced changes in HsEg5 protein secondary structure have been specifically illustrated through FT-IR experiments (Wojcik et al., 2004). These data indicate that in the presence of monastrol there is an increase in organized α -helices within the motor domain of the protein (Wojcik et al., 2004). This conclusion is consistent with interpretations of the HsEg5 : monastrol crystal structure, revealing that in the presence of monastrol the switch I part of the protein reorganizes into an α -helix (Yan et al., 2004). Taken together, these data indicate that monastrol allosterically binds HsEg5 outside the nucleotide-binding site and induces conformational changes that are communicated throughout the protein, consequently altering the motor's structure and function (Cochran et al., 2005; Kapoor et al., 2000; Luo et al., 2004; Wojcik et al., 2004).

Along with the known information on the effect of monastrol on HsEg5's nucleotide hydrolysis cycle, data regarding the consequences of monastrol on HsEg5's interactions with MTs are revealing as well. In MT motility assays, monastrol inhibits *Xenopus* Eg5-driven movement, but does not result in MT dissociation (Crevel et al., 2004). Monastrol also allows HsEg5 to maintain interactions with MTs although the stability of those interactions may be reduced (Cochran and Gilbert, 2005; Crevel et al., 2004). Together, these experiments demonstrate that along with preventing HsEg5's nucleotidase cycle, monastrol also weakens the affinity of HsEg5 for MTs, and even though HsEg5 can still bind, the interaction is so weak that the motor cannot generate the required force for efficient translocation (Cochran and Gilbert, 2005).

Site-Directed HsEg5 Mutagenesis: Interactions Between Mutant HsEg5 Proteins and Monastrol

Along with the large structural changes that occur within the motor domain of HsEg5 in the presence of monastrol, crystal analyses have illustrated specific amino acids (AA) within HsEg5 that might be responsible for monastrol inhibition and further examination of the specific interactions between HsEg5 and monastrol have been pursued with site-directed AA mutational experiments (Brier et al., 2006b; Maliga and Mitchison, 2006; Yan et al., 2004). Figure 1.10 shows the AA sequence of HsEg5 with such residues indicated. Specifically, R119 within the L5 loop has been suggested to move to accommodate space for monastrol as well as interact with the compound's phenol group (Brier et al., 2006b). Mutant proteins containing both R119E and R119A mutations lost monastrol sensitivity, indicating that this arginine residue is necessary for either monastrol binding or inhibition (or quite possibly both) (Brier et al., 2006b; Maliga and Mitchison, 2006). Two other AAs within the L5 loop as well as two AAs within helix $\alpha 2$ were also changed to alanine (D130A, P131A and I136A, V210A, respectively), each resulting in a loss of monastrol inhibition, again confirming their necessity for monastrol inhibition of HsEg5 (Brier et al., 2006b). Four AAs within helix α 3 were also chosen for mutational studies due to their potential interactions with monastrol. Y211 has been suggested to shift in the presence of monastrol in order to make room for the inhibitor (Yan et al., 2004). A Y211A construct was completely insensitive to monastrol, however a Y211M mutation amplified monastrol sensitivity. It is possible that the increased flexibility of the methionine side chain (as opposed to tyrosine or alanine) actually permits tighter binding of the drug (Brier et al., 2006b; Maliga and Mitchison, 2006). Another AA within helix α 3 has demonstrated importance in monastrol induced HsEg5 inhibition. L214 has been suggested to shift in the presence of monastrol to make room for the inhibitor, and mutation to alanine again abolishes the effect of monastrol on the protein (Brier et al., 2006b; Maliga and Mitchison, 2006). Alanine substitutions of both R221 and W127 resulted in the motor's loss of monastrol sensitivity, implying that these amino acids are also indispensable for monastrol-HsEg5 inhibition (Brier et al., 2006b). These mutational studies demonstrate that there are numerous AA required for monastrol binding and / or inhibition and also suggests that the HsEg5 : monastrol interaction is highly complex and involves multiple AA contacts (or conformational changes) to propagate inhibition.

Other Small-Molecule HsEg5 Targeting Compounds

Since the discovery of monastrol, a multitude of other small-molecule HsEg5 inhibitors have been identified and are presently being evaluated. Known IC₅₀s of certain Kinesin-5 targeting compounds, as well as categories of HsEg5 inhibitory compounds, are listed in Table 1.1. It is important to note that each HsEg5 inhibitor listed possesses a unique structure and that no common pharmacophore exists among them. The chemical diversity of such compounds is illustrated in the range of IC₅₀ values presented in Table 1.1, and suggests that each compound (or category of compounds) interacts with HsEg5 via a unique mechanism. The inhibitors utilized in subsequent chapters (other than monastrol) include S-trityl-L-cysteine, gossypol, flexeril, NSC 59349, NSC 169676, NSC 622124, Boc-S-trityl-L-cysteine, and Fmoc-S-trityl-Lcysteine. While these molecules have been shown to inhibit HsEg5 activity, not all demonstrate Kinesin-5 specificity. Further, not all members of the Kinesin 5 family are sensitive to these compounds, or even to monastrol (for example, the *D. melanogaster* Kinesin-5, KLP61F has demonstrated insensitivity to both monastrol (Maliga and Mitchison, 2006) and STLC (Chapter 3)).

First identified in a large scale screening experiment, S-trityl-L-cysteine (STLC) is more potent than monastrol in HsEg5 inhibition (Brier et al., 2004; DeBonis et al., 2004; Skoufias et al., 2006). STLC inhibits only mitotic cells and is not toxic to interphase cells, typical of Kinesin-5 specific inhibition. Characterization of the effect of STLC on cell cycle progression have revealed that addition of this compound to tumor cells does not affect cell cycle progression through the S or G2 phases; however the cell cycle halts at M phase with duplicated spindle poles and a monoastral spindle, again, characteristic of specific Kinesin-5 inhibition (DeBonis et al., 2004; Skoufias et al., 2006). Exchange experiments involving hydrogen and deuterium and mass spectrometry, along with site-directed mutagenesis, have shown that STLC's HsEg5 binding site is the same as that of monastrol, explaining how the two different compounds induce comparable results (Brier et al., 2004). Similar to monastrol, STLC binds Eg5 reversibly, as removal of the compound allows mitotically arrested cells to exit mitosis normally (Skoufias et al., 2006). More recent experiments have shown that STLC binds HsEg5 more tightly than monastrol, and because the IC₅₀ of STLC increases with increasing motor concentrations, it has been characterized as a "tight binding" inhibitor, as opposed to a "classic" inhibitor such as monastrol (IC₅₀ does not change with motor concentration) (Skoufias et al., 2006).

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While a crystal structure of HsEg5 and STLC has yet to be published, site directed mutagenesis of AAs thought to be involved in the HsEg5-monastrol interaction, have provided information about the motor's possible interactions with STLC. Figure 1.9 shows HsEg5's protein sequence and illustrates amino acids that have been mutated in order to evaluate specific interactions with STLC. Several mutations (D130A, V210A, and W127A) resulted in less potent STLC-HsEg5 inhibition however, the proteins still exhibited sensitivity to the drug (Brier et al., 2006b). Only one mutation tested, L214A, completely abolished STLC inhibition of HsEg5, implicating it as an essential AA for STLC specific HsEg5 inhibition (Brier et al., 2006b). Further, multiple site-specific AA mutations (R119A, P131A, Y211A, and R221A) resulted in a conversion of STLC binding and inhibition from tight to classical (Brier et al., 2006b). These data confirm that each HsEg5 targeting compound interacts and consequently inhibits the protein through different AA contacts and mechanism.

NSC 59349, NSC 169676, and NSC 622124, (from the National Cancer Institute) were also identified in the previously mentioned large-scale screen for Kinesin-5 inhibitors (DeBonis et al., 2004). NSC 59349 and 16976 are phenothiazine derivatives with low IC₅₀ values and high cytotoxic effects (DeBonis et al., 2004). The third compound NSC 622124, is not Kinesin-5 specific as it also targets the *D. melanogaster* Kinesin-14, Ncd (IC₅₀ 29 ± 9 μ M) (DeBonis et al., 2004). With a chemical formula of K₆Mo₁₈O₆₂P₂, NSC 622124 is comprised of 1 : 6 metal : oxygen clusters, making it a Wells-Dawson heteropolyoxometalate with an overall negative charge (Birand et al., 2002; Hopkins et al., 2000).

Gossypol, presently known for its use as a male anti-fertility drug (Dodou et al., 2005; Zatuchni and Osborn, 1981), has also been identified as an HsEg5 inhibitor (DeBonis et al., 2004). Addition of gossypol to mitotic cells results in inhibition of HsEg5 and subsequent monoastral spindle formation (DeBonis et al., 2004). In addition to HsEg5, the (-) enantiomer of gossypol demonstrates inhibitory activity on several different proteins: dehydrogenases, cathepsin L, protein kinase C, topoisomerase II, protein kinase A, and calcineruin (a serine / threonine protein phosphatase) (Adlakha et al., 1989; Baumgrass et al., 2001; Kimura et al., 1985; McDonald and Kadkhodayan, 1988; Meksongsee et al., 1970; Xiao et al., 1993).

Another small molecule that is currently being studied as an Eg5 inhibitor is flexeril. Known as a common muscle relaxant, flexeril also induces monoastral spindle formation (DeBonis et al., 2004). First established as an anti-depressant, flexeril is a tricyclic compound and has been suggested to target elements in the nervous system, specifically serotonin pathway somatic motor activity (Kobayashi et al., 1996; See and Ginzburg, 2008). Due to its impact on the nervous system, side effects are harsh and include severe neurological problems, blurred vision, as well as drowsiness / sedation and general body weakness (Toth and Urtis, 2004).

Because so much is known about the interactions between well-characterized inhibitors (e.g. monastrol and STLC) and HsEg5, researchers are also synthesizing derivatives of these drugs and evaluating them to better understand the effects of such compounds and in hopes of determining a common pharmacophore (Debonis et al., 2008; Klein et al., 2007; Ogo et al., 2007; Russowsky et al., 2006). Since STLC demonstrates more potent inhibition than monastrol, derivatives of STLC demonstrate potential for use as probes to study HsEg5 function as well in anti-mitotic drug development (Ogo et al., 2007). The work presented in Chapter 3 utilizes two derivatives of STLC: Boc-S-trityl-L-cysteine, and Fmoc-S-trityl-L-cysteine (BSTLC and FSTLC respectively). Both maintain the common STLC structure with either a Boc or Fmoc group attached to the cysteine amino group. Previous work on these compounds has demonstrated that modification of the STLC amino terminal group eliminates HsEg5 inhibition, both in vitro MT-stimulated ATPase assays, and in cell culture (Ogo et al., 2007), however our contradictory data (Chapter 3) suggests that these compounds may still be useful as tools to better understand HsEg5 function and inhibition.

Implications for Medicine

Cancer is a disease of uncontrolled cellular proliferation; therefore targeting specific elements involved in mitosis has proven moderately successful in existing cancer treatments (Sudakin and Yen, 2007). Many current anti-mitotic / anti-cancer therapies are aimed specifically at MTs and initiate mitotic arrest by interfering with their dynamics (Alberts et al., 2002; Brier et al., 2004; DeBonis et al., 2003; Gerdes and Katsanis, 2005; Miyamoto et al., 2003; Sudakin and Yen, 2007). Such drugs either stabilize MTs and prevent disassembly (Taxol), or prevent MT formation altogether (e.g. Nocodazole) (Alberts et al., 2002). However, while MTs are vital in mitotic cells, they perform important cellular activities in non-mitotic cells as well. This presents a problem because current MT targeting treatments do not discriminate, and abolish or impair MT activities in both dividing and non-dividing cells, cancerous or not. Because all cells utilize MTs for other functions, along with cell division, (i.e. neurons utilize

MTs for axonal transport) many cancer treatments produce undesirable side effects, including hair loss, death of "untargeted" cells (including red blood cells), as well as neurotoxicity (Gerdes and Katsanis, 2005; Rowinsky et al., 1993; Wood et al., 2001).

While there are several strategies presently being developed to decrease toxicity associated with MT-directed anti-cancer drugs (Wood et al., 2001), new alternatives that target other cellular elements, such as motor proteins, might provide less harsh anti-cancer treatments. Since Kinesin-5 proteins function predominately in mitosis, the identification of specific Kineisn-5 targeting compounds that innately discriminate between dividing and non-dividing cells, may represent a new generation of anti-cancer therapies. Currently, researchers are rapidly evaluating such Kinesin-5 targeting compounds in hopes of developing more specific anti-cancer treatments, with less severe side effects (Bergnes et al., 2005; Duhl and Renhowe, 2005).

A second possible medicinal application of these Kinesin-5 targeting compounds takes advantage of the fact that only specific species' versions of this protein are susceptible to these drugs (DeBonis et al., 2003; Maliga et al., 2006). In this manner, scientists might be able to prevent one organism's cell division without interfering with another's, potentially opening the door for development of drugs that could selectively target fungal and parasitic pathogens.

Objectives

The primary goal of the present study is to characterize the interactions between Kinesin-5 inhibitory compounds and their target proteins, in order to better understand protein function as well as small-molecule mode of inhibition for application in anti-cancer therapy. Specifically, my objectives are to:

- Identify small molecules' inhibitory binding sites on the HsEg5 molecule. Because HsEg5 inhibitory compounds induce motor inhibition similar to that of monastrol, it is likely that they bind the protein at or near the monastrol-binding site (Chapter 2).
- Determine susceptibility of the Drosophila melanogaster HsEg5 homolog, KLP61F, to other small-molecule inhibitors. KLP61F's monastrol insensitivity implies that the protein will not be susceptible to other HsEg5 targeting compounds (Chapter 2).
- 3) Evaluate the interactions between specific small molecules and Kinesin-5 proteins. Derivatives of the well-characterized Kineisn-5 inhibitor, STLC, should target HsEg5 via a similar mechanism (Chapter 3). Since NSC 622124 is not specific for Kinesin-5s, it inhibits HsEg5 differently than monastrol (Chapter 2).
- Characterize HsEg5 L5 loop mutant proteins and their interactions with smallmolecule inhibitors. Modifications within the monastrol-binding site will alter the compound's inhibitory effects (Chapter 4).
- Evaluate the interactions between monastrol and Bovine / Human Serum Albumins. Because BSA and HSA have shown concentration-dependent binding of various ligands, these proteins will also bind monastrol in a concentration-dependent manner (Chapter 5).



Figure 1.1: A Functional Mitotic Spindle. A mitotic (in metaphase) Madin-Darby canine kidney epithelial cell fixed and stained for MTs (green) and DNA (blue).



Figure 1.2: A Model of Kinesin Motor Protein Structure.

Shown are two kinesin monomers (respective domains are indicated) connected "head to head" via their central α -helical stalk domains.



Figure 1.3: A Schematic Model of Kinesin ATP Hydrolysis and Consequent MT Translocation.

Labels are as follows, D:ADP, T:ATP, D P_i : ADP and inorganic phosphate. Motor heads are numbered 1, and 2, (first and second) and cycle steps 1-4, as described in text. (a) and (b) denote alpha and beta tubulin, respectively.



Figure 1.4: Kinesin-5 Tetramer Formation.

Shown is the progression from Kinesin-5 monomer to tetramer formation. Protein domains are labeled.



Figure 1.5: A Monoastral Spindle Caused by Application of a Small Molecule Kinesin-5 Inhibitor.

Madin-Darby canine kidney epithelial cells were treated with 25 μ M S-trityl-L-cysteine for 20 hours then fixed and stained for MTs (green) and DNA (blue).



Figure 1.6: Schematic Diagram of the HsEg5 Motor Protein.

Shown is a linear diagram of the HsEg5 motor protein with specific amino acids and domains designated.



Figure 1.7: The HsEg5 Motor Domain with MgADP Bound.

Illustrated are structural entities that are unique to Kinesin-5 protiens (the L5 loop) or involved in ATPase activity (Switches I and II) (Adapted from Turner et al., 2001).



Figure 1.8: The Structure of Monastrol. The chemical structure of the Kinesin-5 inhibitor, monastrol, is illustrated.



Figure 1.9: The HsEg5 Motor Domain with MgADP, With and Without Monastrol.

Illustrated are structural entities that are drastically altered in the presence of monastrol, as described in text. (A) The motor domain of HsEg5 in the absence of monastrol is presented again for ease of comparison between the two states. (B) The motor domain on HsEg5 with bound monastrol. (Adapted from Turner et al., 2001, and Yan et al., 2004).

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Figure 1.10: HsEg5 Motor Domain Amino Acid Sequence.

Shown is the protein sequence of HsEg5. Amino acid numbers are in parentheses. Amino acid mutations discussed in Chapter 1 are denoted with either a (*) or a (^) for monastrol or STLC mutational analyses, respectively (Brier et al., 2006b; Maliga and Mitchison, 2006). The Kinesin-5 specific L5 loop, and α -helices 2 and 3 are also indicated (Wojcik et al., 2004).

Inhibitor Name	Reported IC ₅₀
Adociasulfates ^(1, 2)	3.5 µM (- MTs)
Biphenyl compounds ⁽³⁾	2 nM (+ MTs)
Chlorpromazine ⁽⁴⁾	$5 \ \mu M (+ MTs)$
Dihydropyrazole based compound ⁽⁵⁻⁸⁾	0.2 nM (+ MTs)
Dihydropyrazolobenzoazine based compounds ⁽⁹⁾	1.6 nM (+ MTs)
Dihydropyrrole based compounds (10-12)	2 nM (+ MTs)
Flexeril* ⁽¹³⁾	36 µM (+ MTs)
Gossypol* ⁽¹³⁾	10.8 µM (+ MTs)
Indole based compounds ^(2,14, 15)	90 nM (MT Motility)
Monastrol* and derivatives (16-20)	200 nM (+ MTs)
Polyoxometalate: NSC 622124* ⁽¹³⁾	12 µM (- MTs)
Phenothiazine based compounds*: NSC 59349, NSC 169676 ⁽¹³⁾	6 µM (- MTs)
Pyrrolotriazine based compounds ⁽²¹⁾	50 nM (Cytotoxicity)
Quinazolinone based compounds ^(2, 3, 22-24)	1.7 nM (+ MTs)
S-trityl-L-cysteine and derivatives ^{* (13, 25, 26)}	$0.15 \ \mu M \ (+ MTs)$
Tetrahydro-beta-carboline based compounds ^(15, 27)	650 nM (+ MTs)
Tetrahydroisoquinoline based compounds ⁽²⁸⁾	234 nM (Cell Proliferation)
Thiazole based compounds ⁽³⁾	6 nM (- MTs)
Thiophene based compounds ⁽²⁹⁾	480 nM (+ MTs)

Table 1.1: IC₅₀ Values of Certain HsEg5 Inhibitors.

Shown is a list of known HsEg5 inhibitors and inhibitor families, as well as reported IC₅₀ values. Values are presented as either basal (- MTs) or MT-stimulated (+MTs). IC₅₀ values were obtained via an in vitro assay unless otherwise indicated, and are the most potent reported to date. Asterix denote compounds used in discussed research. References are as follows: ¹(Bergnes et al., 2005) ²(Brier et al., 2006a) ³(Parrish et al., 2007) ⁴(Lee et al., 2007) ⁵(Cox et al., 2005) ⁶(Cox et al., 2006) ⁷(Coleman et al., 2007) ⁸(Roecker et al., 2007) ⁹(Garbaccio et al., 2007) ¹⁰(Fraley et al., 2006) ¹¹(Cox et al., 2007) ¹²(Garbaccio et al., 2006) ¹³(DeBonis et al., 2004) ¹⁴(Nakazawa et al., 2003) ¹⁵(Hotha et al., 2003) ¹⁶(Mayer et al., 1999) ¹⁷(Maliga et al., 2002) ¹⁸(Gartner et al., 2005) ²⁰(Klein et al., 2007) ²¹(Kim et al., 2006) ²²(Sakowicz et al., 2004) ²³(Zhang et al., 2005) ²⁴(Lad et al., 2008) ²⁵(Debonis et al., 2008) ²⁶(Ogo et al., 2007) ²⁷(Sunder-Plassmann et al., 2005) ²⁸(Tarby et al., 2006) ²⁹(Pinkerton et al., 2007)

Chapter 2: NSC 622124 Inhibits Human Eg5 and Other Kinesins Via Binding to the Conserved Microtubule-Binding Site

This work has been submitted to the Journal of Medicinal Chemistry with the following list of authors: Sarah S. Learman, Nathanial S. Stevens, Sunyoung Kim, Ed J. Wojcik, and Richard A. Walker.

Abstract

Unlike human Kinesin-5 (HsEg5) inhibitors that target the monastrol-binding site, the HsEg5 inhibitor NSC 622124 did not compete with ¹⁴C-monastrol in HsEg5 binding assays. NSC 622124 inhibited the basal- and microtubule-stimulated ATPase activities of monastrolinsensitive KLP61F, was found to compete with microtubules in HsEg5 ATPase assays, and also to disrupt binding of HsEg5 and Kinesin-1 to microtubules. Taken together, our data indicate NSC 622124 inhibits kinesins by targeting the conserved microtubule-binding site.

Introduction

Kinesin-5 motor proteins act to separate the spindle poles during formation of the bipolar mitotic spindle (reviewed in (Sharp et al., 2000a)). Certain Kinesin-5 family members, e.g., the human Eg5 protein (HsEg5), represent targets of an expanding collection of chemically diverse, small-molecule inhibitors (DeBonis et al., 2004; Mayer et al., 1999). The mechanism of HsEg5 inhibition, as well as the search for more potent inhibitors, is of particular interest since HsEg5 inactivation leads to cell cycle arrest, and thus inhibitors of this motor have potential as anti-cancer drugs (Bergnes et al., 2005; Miyamoto et al., 2003; Sakowicz et al., 2004).

Monastrol, the first recognized HsEg5 inhibitor, allosterically inhibits the motor's basal and microtubule (MT)-stimulated ATPase activities, and therefore mechanochemical transduction (Cochran and Gilbert, 2005; Mayer et al., 1999). The monastrol binding site is 12 Å from the nucleotide-binding site and is formed by elements of helix α 2, insertion loop L5, and helix α 3 (Yan et al., 2004). Recent characterization of other HsEg5 inhibitors suggests the L5 loop and structurally adjacent regions represent a "hot spot" that modulates allosteric inhibition by many different compounds (Brier et al., 2004; Brier et al., 2006c).

The vast majority of HsEg5 inhibitors, including monastrol, are highly specific for Kinesin-5 proteins and have little or no effect on members of the other thirteen kinesin families. However, one recently identified inhibitor, the polyoxometalate NSC 622124 ($K_6Mo_{18}O_{62}P_2$) shown in Figure 2.1, has been reported to inhibit Ncd (DeBonis et al., 2004), a member of the Kinesin-14 family. Since Ncd does not contain a well-defined monastrol-binding pocket (Wojcik et al., 2004), NSC 622124 may instead target a conserved site present in both HsEg5 and Ncd. The present study examines the interactions between NSC 622124 and kinesin proteins in order to better understand this compound's mechanism of Kinesin-5 inhibition as well as identify its potential as a probe to study Kinesin-5 function.

Materials and Methods

Reagent Information

Racemic ¹⁴C-monastrol (specific activity: 50 mCi/mmol) was synthesized from ethyl acetoacetate, 3-hydroxybenzaldehyde and ¹⁴C -thiourea (American Radiolabeled Chemicals, Inc.) (Kappe, 2000). HPLC analysis and UV-vis spectroscopy were employed to isolate and to confirm the identity of the compound, respectively. NSC 59349, NSC 169676, and NSC 622124 were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. S-trityl-L-cysteine (STLC) and flexeril were obtained from Sigma-Aldrich. Inhibitors were prepared in DMSO as 50 mM solutions, with the exceptions of monastrol (100 mM in DMSO), ¹⁴C-monastrol (10 mM in DMSO), and flexeril (50 mM in water).

Protein Expression and Purification

The plasmid used for expression of HsEg5 was described previously (Wojcik et al., 2004). A cDNA, encoding residues 1-367 of *D. melanogaster* KLP61F, was amplified from clone LD15641 (Berkeley Drosophila Genome Project) using Pfu polymerase (Stratagene), a forward primer containing an NdeI site, and a reverse primer containing an XhoI site. The

product was digested with NdeI and XhoI and inserted into pET-21a (Novagen). Sequencing of both insert strands confirmed no mutations occurred during amplification. Plasmids were transformed into BL21 Codon-plus (DE3)-RIL cells (Stratagene) for protein expression.

Overnight cultures of transformed cells were diluted 1:100 into LB media supplemented with 100 μ g/ml ampicillin and grown at 37°C for 2.5 hours. Protein expression was induced with 0.2 mM IPTG, and after 4 hours at room temperature, cells were pelleted, washed once with 25 mM PIPES pH 6.9, 0.25 mM MgSO₄, 0.5 mM EGTA, and frozen at -80°C until use. Frozen cells were thawed in 50 mM HEPES (pH 7.5), 75 mM NaCl, 1 mM PMSF, 0.1 mM MgATP, 40 μ g/mL DNase, 0.3 mg/ml lysozyme, 10 mM MgCl₂, and 1 mM DTT, and passed through a French Press three times to ensure adequate lysis. Cell lysates were then centrifuged at 20,500g for 30 minutes at 4°C, and the resulting supernatant was passed over a 5 ml SP-Sepharose column. After washing with 50 mM HEPES (pH 7.5), 0.2 mM MgATP, 1 mM DTT and 250 mM NaCl. The eluate was immediately mixed with an equal volume of 50 mM HEPES (pH 7.5), supplemented with glycerol (to 10%), frozen on dry ice, and stored at -80°C. Protein concentrations were measured by Bradford Assay (Biorad) with BSA as the standard.

Full length *D. melanogaster* Kinesin-1 was expressed and bacterial cells were lysed and centrifuged as described for HsEg5 and KLP61F. The supernatant was then centrifuged at 100,000 x g for 15 minutes at 4°C (Yang et al., 1990), and the resulting high speed supernatant was used directly in MT motility experiments.

Binding and Competition Experiments

Columns were prepared with fine grade G25 Sephadex and Micro Bio-Spin Chromatography columns (Biorad). Sephadex was prepared per manufacturer's instructions, exchanged into HEM buffer (20 mM HEPES, pH 7.2, 1 mM EDTA, and 1 mM MgCl₂), and added to each column to generate a packed resin bed of 0.7 ml. Just prior to use, columns were centrifuged (1500g, 4 minutes) to remove excess liquid.

Binding reactions (130 μ l final volume) containing 1 mg/ml (~24 μ M) motor protein and ¹⁴C-monastrol (0.9 mM) were prepared in HEM buffer, incubated at room temperature for 10 minutes, then 50 μ l was applied to each of two spin columns. Columns were immediately centrifuged (1500g, 4 minutes), and samples of the initial reaction as well as each column's
"flow-through" were analyzed by Bradford assay and liquid scintillation counting to quantify protein and ¹⁴C-monastrol, respectively.

For competition experiments, motor protein was incubated with 0.5 mM inhibitor for 20 minutes at room temperature prior to addition of 0.9 mM ¹⁴C-monastrol, and then subjected to size exclusion spin chromatography after another 10-minute incubation at room temperature. Statistical analyses (t-tests) were performed using Prism 4 software (GraphPad).

ATPase Assays

All assays were conducted at room temperature in 50 mM Tris-acetate, pH 7.4, 2 mM MgCl₂. Control reactions were supplemented with DMSO to match the concentration of DMSO carried over with inhibitors. Basal and microtubule (MT) -stimulated ATPase rates presented in Figure 2.3 were measured with a coupled pyruvate kinase / lactate dehydrogenase assay (Deavours et al., 1998; Moore et al., 1996) and normalized to 100% of the control rate (no inhibitor). Basal ATPase reactions contained 5 μ M motor, while MT-stimulated ATPase reactions contained 200 nM motor, 20 μ M paclitaxel and GTP-depleted, paclitaxel-stabilized MTs (2.3 μ M bovine or bison tubulin). Basal inhibitor concentrations were either 200 μ M (monastrol) or 100 μ M (all others). In order to maintain the motor protein : compound ratio in the basal assays, the inhibitor concentration was set to 4 μ M in MT-stimulated reactions. Data outliers were identified (Tukey, 1977) and omitted from calculations.

Data presented in Figure 2.4 was collected via the malachite green phosphate assay. Briefly, 50 μ l reactions, containing 100 nM motor protein, 20 μ M paclitaxel, GTP-depleted paclitaxel-stabilized MTs and a range of NSC 622124 concentrations (0, 0.002, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 25, 50, and 100 μ M) prepared in 50 mM Tris-acetate, pH 7.4, 2 mM MgCl₂, were initiated by the addition of MgATP. Specifically, reactions presented in Figure 2.4a received 0.5 μ M bovine or bison tubulin over a range of MgATP concentrations (0.005, 0.01, 0.032, 0.045, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.8, 1, and 2 mM). Reactions presented in Figure 2.4b received 2 mM MgATP, over a range of tubulin concentrations (0.0625, 0.125, 0.25, 0.4, 0.5, 1, 2, 4, and 8 μ M). Aliquots (5, 10 or 15 μ l) removed at 2, 4 or 5 minutes were added immediately to dilute malachite green reagent (BioAssay Systems) in 96-well plates. Time points at "zero" minutes were obtained by addition of MgATP after dilution of sample aliquots with malachite green reagent. After 15-30 minutes at room temperature, the A650 of samples were measured with a SpectraFluor Plus microplate reader (Tecan), and the rate of P_i production was determined.

To determine the IC₅₀ for NSC 622124 inhibition of the MT-stimulated ATPase activity of HsEg5 (Figure 2.7) ATPase rates in the presence of MTs were measured, via the malachite green assay, as a function of NSC 622124 concentration. Data outliers were identified (Tukey, 1977) and omitted from calculations. IC₅₀ value was calculated from means for each drug concentration as described (Maliga et al., 2002). Curve fits were performed using Prism 4 (GraphPad) and included data through 100 μ M NSC 622124 (while only data through 1 μ M is plotted).

Co-sedimentation Assays

MT co-sedimentation assays were prepared in 50 mM PIPES, pH 6.9, 1 mM EGTA, and 0.5 mM MgCl₂ and contained 20 μ M paclitaxel, 5 μ M tubulin (as paclitaxel-stabilized MTs), 2.5 μ M HsEg5, 1 mM MgAMPPNP, and 25 μ M NSC 622124 (or an equivalent volume of DMSO as a control). Reactions were incubated at room temperature for 15 minutes and centrifuged at 110,000 x g spun in a Beckman TLA 100.3 rotor at 25°C for 15 minutes. Supernatants and pellets were analyzed by SDS-PAGE.

MT-Motility Assays

Full length *D. melanogaster* Kinesin-1 was purified (as described above) and directly applied to slide-coverslip chambers constructed with double-sided tape. After a wash step with 50 mM PIPES, pH 6.9, 1 mM EGTA, 0.5 mM MgCl₂, 0.1 μ M paclitaxel-stabilized MTs (bovine tubulin), and 1 mM MgATP / MgAMPPNP, the same buffer with 5 μ M NSC 622124 was perfused into the chamber. Samples were observed at room temperature by video-enhanced differential interference contrast microscopy (Karabay and Walker, 1999).

Results and Discussion

To address the possibility that NSC 622124 binds HsEg5 at a site distinct from monastrol, we synthesized ¹⁴C-monastrol and utilized size exclusion spin chromatography to isolate motor with bound ¹⁴C-monastrol in the absence or presence of selected HsEg5 inhibitors, including NSC 622124. Comprised of both S- and R- enantiomers, the ¹⁴C-monastrol was similar to commercially available racemic monastrol in ability to inhibit HsEg5 ATPase activity (data not shown). Consistent with monastrol's moderate binding affinity (in the μ M range) (Maliga et al., 2002), and specificity (Maliga et al., 2006), each molecule of HsEg5 that passed through the column retained 0.34 ± 0.02 mol ¹⁴C-monastrol. In comparison, the monastrolinsensitive *D. melanogaster* Kinesin-5, KLP61F (Maliga et al., 2006), did not exhibit measurable binding to ¹⁴C-monastrol (Figure 2.2), indicating that KLP61F's monastrol insensitivity stems from the inability of the protein to bind the compound. Pre-incubation of HsEg5 with four inhibitors reported to target the monastrol-binding site (Brier et al., 2006c) either completely (for STLC, NSC 169676 and NSC 59349) or significantly (for flexeril, unpaired t-test, p = 0.027) reduced the binding of ¹⁴C-monastrol to HsEg5. However, NSC 622124 did not significantly reduce bound ¹⁴C-monastrol (unpaired t-test).

Since NSC 622124 did not appear to target the monastrol-binding site but was active against Ncd (DeBonis et al., 2004), we investigated whether this compound affected either the basal or MT-stimulated ATPase activities of monastrol-insensitive (Maliga and Mitchison, 2006) KLP61F (Figure 2.3). As expected from both previous work (Maliga et al., 2006), and the inability of KLP61F to bind ¹⁴C-monastrol (Figure 2.2), inhibitors that target the monastrol binding-site had no effect on KLP61F ATPase activity either with or without MTs (Figure 2.3). In contrast, NSC 622124 significantly inhibited both basal and MT-stimulated ATPase activities of KLP61F.

The ability of NSC 622124 to inhibit both monastrol-sensitive and monastrol-insensitive kinesins suggests the compound targets a site conserved across kinesins, such as the ATP- or MT-binding site. To determine if NSC 622124 competes with MgATP for binding to HsEg5, the MT-stimulated ATPase activity of HsEg5 was measured via the coupled ATPase assay at several MgATP concentrations at various NSC 622124 concentrations. All data were fit to the

Michaelis-Menten equation and the resulting curves along with the calculated V_{max} and K_m values are presented in Figure 2.4a. The decreasing trends observed for both V_{max} and K_m as NSC 622124 concentrations increased (also presented in Figure 2.4a), suggests that the interaction between MgATP and NSC 622124 is not competitive.

The MT-binding site is another conserved site between HsEg5 and KLP61F that may serve as a binding site for NSC 622124. To determine if NSC 622124 competes with MTs for binding to HsEg5, coupled MT-stimulated ATPase assays were performed in which either MTs or NSC 622124 were varied. Data were analyzed similarly to the ATP-competitive data and resulting calculated V_{max} values remained constant while the K_m values (Figure 2.4b) showed an increasing trend with NSC 622124 concentration, indicative of a competitive interaction between NSC 622124 and MTs.

Our results demonstrating that NSC 622124 competes with MTs but not with ATP for association with HsEg5 predicts that the inhibitor should interfere with the ability of HsEg5, and perhaps kinesins from outside the Kinesin-5 family, to bind MTs. To test this possibility, HsEg5 MT co-sedimentation assays with and without NSC 622124 were performed. MT co-sedimentation results (Figure 2.5) showed that NSC 622124 completely disrupted HsEg5 binding to MTs, even in the presence of MgAMPPNP, consistent with our biochemical data.

The ability of NSC 622124 to interfere with HsEg5 MT-binding implies that the compound will similarly interfere with other kinesin proteins' MT interactions, as the MT-binding site is conserved across the kinesin superfamily. The effect of NSC 622124 on the *D. melanogaster* Kinesin-1 MT motility in the presence of either 1 mM MgATP (Figure 2.6, left panel) or MgAMPPNP (Figure 2.6, right panel) was observed by video-enhanced differential interference contrast microscopy. Images were collected one minute before (Figure 2.6, top row) and 5 min after chamber perfusion with NSC 622124 (Figure 2.6, bottom row). For experiments with MgATP, the majority of MTs released from the coverslip during the time course of NSC 622124 perfusion (< 25 sec) and the few MTs that remained attached showed no directed movement, and instead exhibited thermal movements consistent with single-point attachment. Experiments performed in the presence of MgAMPPNP provided similar results in terms of reduction in the number of attached microtubules and increased evidence of single-point attachment. In contrast to these results, replacement of the chamber volume with buffer containing paclitaxel

and identical nucleotide had no effect on the number of MTs attached to the surface (and for MgATP, no effect on the rate of gliding $(0.47 \pm 0.03 \ \mu \text{m/sec}, n = 10, \text{data not shown})$).

Taken together, the simplest explanation for our results is that NSC 622124 binds at or adjacent to the conserved kinesin MT-binding site and consequently alters the affinity of the motor for MTs. At least two other compounds, adociasulfate-2 (AS-2) (Sakowicz et al., 1998) and rose bengal lactone (RBL) (Hopkins et al., 2000), have also been reported to bind at/near the MT-binding site and to inhibit the activity of more than one kinesin. Both compounds inhibit the MT-stimulated ATPase activity of Kinesin-1 and at least one other kinesin motor, and both compounds compete with MTs but not ATP for binding to the motor. Further, AS-2 and RBL inhibit the interaction between Kinesin-1 and MTs in motility assays and in MT cosedimentation assays, similar to our NSC 622124 data (Hopkins et al., 2000; Sakowicz et al., 1998).

The mechanism by which AS-2, RBL, and NSC 622124 inhibit both MT-stimulated ATPase activity and interaction with MTs most likely involves direct competition between each compound and MTs. However, these compounds exhibit dramatically different efficacies on MT-stimulated ATPase activity. With an IC₅₀ value of 69 ± 15 nM for HsEg5 (Figure 2.7), NSC 622124 is among the most effective inhibitors of HsEg5 MT-stimulated ATPase activity reported to date, regardless of binding site on the motor (Bergnes et al., 2005; DeBonis et al., 2004; Kim et al., 2006; Tarby et al., 2006). In comparison, AS-2 is ~100 fold less effective against HsEg5, and AS-2 and RBL are similarly less effective against Kinesin-1 (Brier et al., 2006a; Hopkins et al., 2000; Sakowicz et al., 1998). In fact, the ability of NSC 622124 to inhibit the basal ATPase activity of HsEg5 allowed the inhibitor to "survive" a screen designed to eliminate compounds that affected MT assembly or motor binding to MTs (DeBonis et al., 2004).

How might NSC 622124 interact with the MT-binding site of kinesin motors? AS-2 has been suggested to act as a MT "mimic" in which the compound's sulfate groups function analogously to the negatively charged C-terminus of tubulin and consequently associate with basic residues in the motor's MT-binding site (Sakowicz et al., 1998). In support of this model, AS-2 has been shown to enhance Kinesin-1 basal ATPase activity (Sakowicz et al., 1998). Given its small size (\sim 12 x 15 Å) and negatively charged surface, NSC 622124 could easily fit into the MT-binding site and could, as suggested for AS-2, interact with basic residues in the MT-binding site (Woehlke et al., 1997). However, as noted above, rather than acting as an

enhancer of basal ATPase activity, NSC 622124 instead acts to inhibit this activity. In comparison, RBL exhibits a more complicated effect and either modestly enhances (at 10 μ M) or completely inhibits (at 40 μ M) Kinesin-1 basal ATPase activity (Hopkins et al., 2000). Taken together, although the simplest explanation for our results is that NSC 622124 associates with the MT-binding site, NSC 622124 does not appear to act as a MT mimic and it remains formally possible that this compound interacts with an unidentified site conserved across kinesins, which allows for spatially distant control over the MT-binding site. Resolution of the exact NSC 622124 binding site will likely depend on co-crystallization of the compound and HsEg5 or other kinesins.

Overall, our data reinforce the concept that small molecules can control kinesins through sites other than the L5 loop found specifically in Kinesin-5 motors. Although a pan-kinesin inhibitor that targets a shared, conserved site may not initially appear promising for therapeutic uses, recent work has identified a novel class of HsEg5, ATP-competitive inhibitors that interact either directly with the nucleotide binding site (Parrish et al., 2007; Rickert et al., 2008) or via a separate allosteric (Luo et al., 2007) site. The ability of these compounds to target a conserved binding site shared by all kinesins yet still retain specificity to a select few suggests that it may be possible to generate NSC 622124 derivatives that show specificity for certain kinesins and thereby selectively interfere with cell processes that depend on those motors.

Conclusion

We have characterized the interactions between three kinesin proteins and the Kinesin-5 inhibitor, NSC 622124. Rather than interacting with the monastrol-binding pocket (i.e., the typical Kinesin-5 inhibitor binding site), NSC 622124 targets the MT-binding site of HsEg5 and two monastrol-insensitive kinesin proteins. Further, this compounds inhibits rather than enhances motor basal ATPase activity and thus acts as a negative regulator via a site traditionally viewed as a binding site for a positive regulators (i.e., MTs). Our work emphasizes the concept that MT motors may be controlled at multiple sites by both positive and negative regulators.



Figure 2.1: NSC 622124 is the Potassium Salt of the Polyoxometalate $(Mo_{18}O_{62}P_2)$ Shown. Oxygen atoms are illustrated in red, molybdenum atoms in green, and phosphorous in yellow.



Figure 2.2: NSC 622124 Does Not Interfere with ¹⁴**C-Monastrol Binding to HsEg5.** Reactions containing motor protein (HsEg5 or KLP61F) and ¹⁴C-monastrol (± indicated competitor) were subjected to size exclusion spin chromatography and the amount of protein and bound ¹⁴C-monastrol, determined.



Figure 2.3: NSC 622124 Inhibits KLP61F Basal and MT-Stimulated ATPase Activities. Normalized KLP61F steady-state basal (open bars) and MT-stimulated (solid bars) ATPase rates were determined for the indicated inhibitors.



Symbol	NSC 622124 (nM)	V _{max} (nM)	$K_m(nM)$
	0	8.7 ± 0.3	80 ± 15
*	2	8.9 ± 0.5	86 ± 22
0	20	9.2 ± 0.5	86 ± 21
•	50	7.7 ± 0.5	60 ± 20
•··-··	100	6.7 ± 0.5	55 ± 23
•	200	3.5 ± 0.3	15 ± 9

Symbol	NSC 622124 (nM)	V _{max} (nM)	K _m (nM)
•	0	8.6 ± 0.7	291 ± 97
▲	20	8.2 ± 0.6	431 ± 105
•	100	9.7 ± 0.7	875 ± 192

Figure 2.4: NSC 622124 Competes with MTs but not ATP for Association With HsEg5. HsEg5 MT-stimulated ATPase assays were performed in the presence of NSC 622124 in which either ATP (a) or MTs (tubulin) (b) were varied. Data were fit to the Michaelis-Menten equation and the resulting curves are plotted along with the mean rates at each ATP / tubulin concentration Calculated V_{max} and K_m values from competitive ATPase assays are also presented.



Figure 2.5: NSC 622124 Prevents HsEg5 From Binding MTs.

Supernatant and pellet fractions from a MT co-sedimentation assay containing either DMSO or NSC 622124 were analyzed by SDS-PAGE. (S) and (P) denote supernatant and pellet samples from the assay centrifugation step. (T) and (E) denote tubulin and HsEg5, respectively.



Figure 2.6: NSC 622124 Disrupts Kinesin-1 MT Attachment and Motility.

Snapshots from a Kinesin-1 MT motility assay before (-1 min) and after NSC 622124 perfusion (5 min) are shown. Arrows point to MTs. Each panel is $\sim 27 \ \mu m$ wide.



Figure 2.7: NSC 622124 HsEg5 MT-Stimulated IC_{50} Determination. MT-stimulated HsEg5 ATPase assays were performed with a range of NSC 622124 concentrations and the IC_{50} calculated.

Chapter 3: Evaluation of the Interactions Between Boc- and Fmoc- S-trityl-Lcysteine Derivatives and Kinesin Proteins

Abstract

Early in mitosis, Kinesin-5 motor proteins drive spindle pole separation in order to form the bipolar mitotic spindle. The human Kinesin-5 motor protein (HsEg5) is of particular scientific interest due to its fundamental role in mitosis and recent recognition as an anti-cancer target. Study of HsEg5 has been facilitated by the identification of numerous small molecules that specifically inhibit the motor, prevent spindle formation, and lead to mitotic arrest. Established HsEg5 inhibitors, as well as chemically-modified derivatives, are also currently being evaluated for their anti-mitotic, and consequently anti-cancer activities. S-trityl-Lcysteine, STLC, (a well characterized HsEg5 inhibitor) has demonstrated significant potency in its HsEg5 inhibition and previous work has shown that chemical modifications of this compound may improve its efficacy. In this study the effects of two STLC derivatives (Boc- and Fmoc-STLC) on the ATPase activities of various kinesin proteins were analyzed. Our results suggest that experimental and cellular conditions may influence the mechanism by which small molecules interact with and subsequently affect the activity of Kinesin-5 proteins.

Introduction

Kinesin-5 motor proteins are primarily responsible for separating the spindle poles during mitotic spindle formation. Using energy from ATP hydrolysis for microtubule (MT) plus enddirected movement, these proteins arrange antiparallel MTs into a functional bipolar spindle (Blangy et al., 1998; Enos and Morris, 1990; Greene and Henikiff, 2005; Sawin et al., 1992). When mutated (Blangy et al., 1998; Enos and Morris, 1990; Sawin and Mitchison, 1995) or inhibited (siRNA, small molecule compounds, antibodies) (Blangy et al., 1995; Mayer et al., 1999; Weil et al., 2002), these proteins can no longer properly assemble the mitotic spindle and as the cell cannot efficiently progress through mitosis, it arrests (Enos and Morris, 1990; Mayer et al., 1999). Since inhibition of Kinesin-5 proteins, namely the human version (HsEg5), halts cell division, the ability to control these proteins (and consequently control cell division) with small molecules is currently under active investigation, as these compounds may be useful in the development of novel anti-cancer therapies.

Monastrol, the first small molecule HsEg5 inhibitor identified, was so named because treatment of cells resulted in a non-functional, monoastral spindle (Mayer et al., 1999). This monoastral spindle has both centrosomes centrally located with MTs extending outwards, and attached chromosomes surrounding in a sphere (Mayer et al., 1999). Further characterization of the monastrol-HsEg5 interaction has determined that the compound allosterically inhibits HsEg5 ATPase activity through binding an induced-fit pocket formed by elements of helix α 2, insertion loop L5, and helix α 3 (DeBonis et al., 2003; Maliga et al., 2002; Mayer et al., 1999; Wojcik et al., 2004; Yan et al., 2004). Upon binding, monastrol induces conformational changes within the HsEg5 motor domain that prevent the protein from efficiently progressing through its nucleotidase cycle, therefore resulting in lack of motor function (Cochran et al., 2005; Cochran and Gilbert, 2005; Crevel et al., 2004; Kapoor et al., 2000; Krzysiak et al., 2006; Maliga et al., 2002; Maliga et al., 2006).

Since the discovery of monastrol, numerous small molecule Kinesin-5 inhibitors have been identified. Many of these compounds are of particular interest due to their ability to prevent mitosis at lower concentrations than monastrol. S-trityl-L-cysteine (STLC) (DeBonis et al., 2004) for example, has demonstrated IC₅₀s of HsEg5's basal and MT-stimulated ATPase activity at 1 μ M and 140 nM (Brier et al., 2004; DeBonis et al., 2004), respectively, whereas monastrol's reported basal and MT-stimulated ATPase IC₅₀s are 9 μ M (Brier et al., 2004; DeBonis et al., 2003) and 34 μ M (Maliga et al., 2002), respectively. Like monastrol, STLC induces monoastral spindle formation and consequently cell cycle arrest (Skoufias et al., 2006). STLC reversibly associates with HsEg5 at the same site as monastrol (Brier et al., 2004; Brier et al., 2006c; Skoufias et al., 2006), but, while monastrol is an established classical binding inhibitor (IC₅₀ value changes with motor concentration), STLC has been identified as a tight binding inhibitor (IC₅₀ remains constant, regardless of motor concentration), a possible explanation for the difference between the two drugs' potency (Skoufias et al., 2006). Initial characterization of STLC (primarily due to its high efficacy) suggests that it may represent a new class of compounds for both the study of Kinesin-5 mechanotransduction and development of therapeutics to control cell reproduction.

The present study utilizes two well-known STLC derivatives previously used in peptide synthesis: Boc-S-trityl-L-cysteine and Fmoc-S-trityl-L-cysteine (BSTLC and FSTLC respectively). The effects of these compounds on the steady state ATPase activities of Kinesin-5 proteins including HsEg5, the monastrol insensitive *D. melanogaster* homologue KLP61F (Maliga and Mitchison, 2006), as well as a KLP61F-like mutant form of HsEg5, E116V+E118N, were evaluated. Further, the effects of these STLC derivatives on a Kinesin-14 motor protein, Ncd, were assessed.

Our data shows that the basal ATPase activity of Kinesin-5 proteins is surprisingly enhanced by both STLC derivatives, with BSTLC being the most effective. However, in the presence of MTs, each compound exhibited inhibitory activity. Our data confirms that Kinesin-5 proteins possess multiple levels of regulation, and that in vivo control of these proteins may require the coordination of both inhibitory and stimulatory allosteric regulators.

Materials and Methods

Reagents

S-trityl-L-cysteine and monastrol were obtained from Sigma-Aldrich. Boc- S-trityl-L-cysteine and Fmoc- S-trityl-L-cysteine were purchased from NovaBioChem. Compounds were prepared in DMSO as 50 mM solutions with the exception of monastrol, which was prepared as 100 mM.

Protein Expression and Purification

The HsEg5 motor domain, composed of HsEg5 residues 1-370 and a C-terminal 6-His tag, was expressed as previously described (Wojcik et al., 2004). A cDNA encoding residues 1-367 of *Drosophila melanogaster* KLP61 was amplified from clone LD15641 (Berkeley *Drosophila* Genome Project) by PCR using Pfu polymerase (Stratagene), a forward primer containing an NdeI site, and a reverse primer containing an XhoI site. The product was digested with NdeI and XhoI and inserted into pET-21a (Novagen). Sequencing confirmed that no

mutations occurred during amplification. Site directed mutagenesis of the HsEg5 motor domain to create the E116V+E118N double mutant protein was performed by S. Kim. Briefly, two mutagenic oligonucleotide primers with the desired mutations were designed and synthesized (forward 5' CTTTTACAATGGTGGGTAACAGGTCACCTAAT 3' and reverse 5' ATTAGGTGACCTGTTACCCACCATTGTAAAAG3'). Mutant HsEg5 was generated and amplified via PCR. To confirm only the desired mutations were acquired, resulting DNA was sequenced. A plasmid encoding the Ncd motor domain (MC6), amino acids 333-700 was a gift from S. Endow (Chandra et al., 1993).

All plasmids were transformed into BL21 Codon-plus (DE3)-RIL cells (Stratagene) for protein expression. Overnight cultures of transformed cells were diluted 1:100 into LB media supplemented with 100 μ g/ml ampicillin and grown at 37°C for 2.5 hours. Protein expression was induced with 0.2 mM IPTG, and after 4 hours at room temperature, cells were pelleted, washed once with 25 mM PIPES pH 6.9, 0.25 mM MgSO₄, 0.5 mM EGTA, and frozen at -80°C until use. Frozen cells were thawed in 50 mM HEPES (pH 7.5), 75 mM NaCl, 1 mM PMSF, 0.1 mM MgATP, 40 μ g/mL DNase, 0.3 mg/ml lysozyme, 10 mM MgCl₂, and 1 mM DTT, and passed through a French Press three times. Cell lysates were then centrifuged at 20,500g for 30 minutes at 4°C, and the resulting supernatant was passed over a 5 ml SP-Sepharose resin. After a wash with 50 mM HEPES (pH 7.5), 0.1 mM MgATP, and 1 mM DTT, the protein was eluted with 50 mM HEPES (pH 7.5), 0.2 mM MgATP, 1 mM DTT and 250 mM NaCl. The protein-containing eluate was immediately mixed with an equal volume of 50 mM HEPES (pH 7.5), supplemented with glycerol (to 10%), frozen on dry ice, and stored at -80°C until use. A Bradford Assay (Biorad), with BSA as the standard, was used to determine protein concentrations.

Coupled ATPase Assay:

Steady-state basal ATPase activities presented in Figures 3.2, 3.3 and Table 3.1 were measured with the pyruvate kinase / lactate dehydrogenase coupled enzyme assay (Deavours et al., 1998; Moore et al., 1996). Coupled assays were carried out at room temperature in 50 mM Tris-acetate, pH 7.4, 2 mM MgCl₂. Reactions contained 5 μ M motor and STLC, BSTLC, or FSTLC to the indicated concentration. Control reactions were supplemented with DMSO to match the concentration of DMSO carried over with inhibitors. IC₅₀ values were calculated from

means for each drug concentration as described (Maliga et al., 2002). Curve fits and statistical analyses were done using Prism 4 (GraphPad). Data points represent the mean \pm SEM. In order to maintain a constant scale for Figures 3.2 and 3.3, presented values were normalized against the control rate (100%). Coupled MT-stimulated assays at 100 μ M drug were performed to confirm trends seen with the malachite green (MG) assay (Table 3.1). MT-stimulated assays were identical to basal reactions except for motor concentration (200 nM) and the addition of 20 μ M paclitaxel and 1.14 to 2.23 μ M GTP-depleted, paclitaxel-stabilized MTs (bovine or bison tubulin). Non-normalized basal and MT-stimulated coupled ATPase rates (control and at 100 μ M drug) are presented in Table 3.1.

Malachite Green ATPase Assay:

Steady-state MT-stimulated ATPase rates presented in Figure 3.4 and Table 3.1 were measured using the malachite green phosphate (MG) assay (BioAssay Systems). Briefly, 50 µl reactions contained 100 nM motor, 20 μ M paclitaxel, 1.25 μ M bovine tubulin (as GTP-depleted, paclitaxel-stabilized MTs), and the indicated drug concentration. Control reactions were supplemented with DMSO to match the concentration of DMSO carried over with inhibitors. Each reaction was prepared in 50 mM Tris-acetate, pH 7.4, 2 mM MgCl₂, and initiated by the addition of 1 μ M MgATP. Aliquots (10 μ l) were removed at 2 and 4 minutes and immediately added to dilute malachite green reagent in 96-well plates. Time-zero points were obtained by addition of MgATP after dilution of sample aliquots with malachite green reagent. After 15-20 minutes at room temperature, the A₆₅₀ of samples and P_i standards were measured with a SpectraFluor Plus microplate reader (Tecan), and plots of P_i versus time were used to determine the rate of P_i production. IC₅₀ values were calculated from means for each drug concentration as described (Maliga et al., 2002). Curve fits and statistical analyses were done using Prism 4 (GraphPad). Data points represent the mean \pm SEM. In order to maintain a constant scale for figure 3.4, presented values were normalized against the control rate (100%). MG basal assays at 100 μ M drug were also performed to confirm trends seen with the coupled basal experiments (Table 3.1). Assays were set up identical to MT reactions except with 2, 4, and 6 minute time points, 5 μ M motor protein, and the omission of paclitaxel and MTs. Non-normalized basal and MT-stimulated MG ATPase rates (control and at 100 μ M drug) are presented in Table 3.1.

Results and Discussion

Recently researchers have been synthesizing and testing chemically altered versions (or derivatives) of various Kinesin-5 inhibitors for use as probes to study motor function or as potential anti-cancer drugs (Debonis et al., 2008; Gartner et al., 2005; Ogo et al., 2007; Russowsky et al., 2006). In particular, structural modifications of S-trityl-L-cysteine (STLC), a specific and potent Kinesin-5 inhibitor (structure illustrated in Figure 3.1), alter its efficacy (Debonis et al., 2008; DeBonis et al., 2004; Ogo et al., 2007). For example, S-trityl-L-cysteine-glycine showed lower effectiveness when screened for Kinesin-5 inhibition, while another STLC derivative with an –OMe group attached to the central ring, demonstrated over 10-fold more potent Kinesin-5 inhibition than its parent compound (Ogo et al., 2007). We obtained two derivatives of STLC: Boc-STLC (BSTLC), and Fmoc-STLC (FSTLC), which are commonly used for incorporation of cysteine residues during peptide synthesis. Each contains the same base S-trityl-L-cysteine structure with either a Boc or Fmoc group attached to the cysteine amino group (Figure 3.1). The current work describes the use of these compounds to study kinesin protein activity in order to appreciate how Kinesin-5s may be controlled in vivo.

Basal ATPase rates for HsEg5 and the *D. melanogaster* Kinesin-5, KLP61F, in the presence of STLC, BSTLC and FSTLC were determined. Data presented in Figure 3.2 were measured with the coupled ATPase assay and were normalized to 100% control rates for ease of comparison. Raw data for ATP hydrolysis $\pm 100 \ \mu$ M drug are presented in Table 3.1. With a calculated IC₅₀ of 2.2 μ M, STLC inhibited HsEg5 basal ATPase activity comparable to published data (IC₅₀ of 1 μ M) (DeBonis et al., 2004). Surprisingly, over a concentration range from zero to 100 μ M, BSTLC enhanced the basal ATPase activity of HsEg5 (Figure 3.2a) by approximately 2-fold. This is the first record to date of a small-molecule compound stimulating the ATPase activity as drastically as BSTLC; however, we did observe a slight but significant enhancement in rate at 100 μ M (unpaired t-test, P < 0.0001) (Figure 3.2a and Table 3.1). In order to corroborate trends seen with the coupled assay, experiments were performed with HsEg5 and all three compounds at 100 μ M via the malachite green (MG) ATPase assay. The results presented in Table 3.1, confirm that the compound's apparent enhancements are not an artifact of the coupled assay.

The same sets of experiments were performed with *D. melanogaster*'s Kinesin-5 homolog, KLP61F (Figure 3.2b). Consistent with KLP61F insensitivity to HsEg5 monastrol binding-site targeting compounds (Maliga and Mitchison, 2006), STLC had no effect on the basal activity of this protein. However, BSTLC and FSTLC increased KLP61F's basal ATP hydrolysis rate (with BSTLC's enhancement being more dramatic) although the enhancement seen with each compound was not as extensive as that seen with HsEg5 (Figure 3.2 and Table 3.1). Again, trends were confirmed at 100 μ M with the MG assay (Table 3.1).

Because similar results were obtained with both BSTLC and FSTLC for two different Kinesin-5 proteins, one sensitive to STLC and one insensitive, we tested the efficacy of these compounds on the motor domain of a non-Kinesin-5 protein, Kinesin-14, Non-claret Disjunction (Ncd). Figure 3.3 shows the normalized coupled basal ATPase activity of HsEg5, KLP61F and Ncd with either 0 or 100 μ M STLC, BSTLC, or FSTLC. HsEg5 and KLP61F data are repeated from Figure 3.2 and are presented again for comparison with Ncd. Also, presented in Table 3.1 are the non-normalized values for Ncd and each compound. At 100 μ M, STLC did not significantly inhibit Ncd, and no significant enhancement with either BSTLC or FSTLC was observed (unpaired t-tests), indicating that these compounds specifically target Kinesin-5s.

We next evaluated the effects of STLC, BSTLC and FSTLC on Kinesin-5 motor proteins in MT-stimulated ATPase assays. Due to the high cost of tubulin (for MTs), lower reaction volume and fewer reagents needed (with comparable assay sensitivity), the MG assay was principally employed to evaluate kinesin MT-stimulated ATPase activities (Figure 3.4 and Table 3.1). Results obtained in KLP61F MT-stimulated MG experiments demonstrated large amounts of scatter and higher error than any of our other data sets. This result may be explained by the temperamental nature of the MG assay (generally higher error is obtained with this assay, compared to the coupled assay), in combination with the naturally slow ATPase activity of KLP61F. Because KLP61F hydrolyzes ATP at such a slow rate (15-fold slower than HsEg5, Table 3.1), small changes in activity are more amplified and therefore produce more error.

In spite of the aforementioned potential assay difficulties, we were able to collect interpretable data under each condition. Both HsEg5 and KLP61F responded to STLC in the presence of MTs as they did in the absence; HsEg5 demonstrating sensitivity (calculated IC₅₀ = 0.5μ M) and KLP61F exhibiting insensitivity (Figure 3.4). However, each protein was affected by BSTLC and FSTLC quite differently in MT-stimulated assays compared to basal

experiments. With a calculated IC₅₀ of 2.7 μ M, HsEg5 was potently inhibited by FSTLC in the presence of MTs. This result is inconsistent with a previous report of the FSTLC HsEg5 MT-stimulated IC₅₀ (55 μ M (Ogo et al., 2007)). MT-stimulated experiments also illustrated an altered interaction between BSTLC and HsEg5, as the compound exhibited no effect on activity (enhancement or inhibition) in the presence of MTs. These data are consistent with previous reports that BSTLC does not inhibit HsEg5 MT-stimulated ATPase activity (Debonis et al., 2008; Ogo et al., 2007). The effects of these compounds on the MT-stimulated ATPase rate of KLP61F are also relatively different from those observed in the absence of MTs; both BSTLC and FSTLC's KLP61F enhancement is lost and the rates shift back near that of the control. Non-normalized ATPase data points from each assay at 100 μ M are presented in Table 3.1.

The enhancement of both Kinesin-5 protein's basal ATPase rates in the presence of BSTLC and FSTLC was consistent in both the coupled and the MG assay indicating a genuine drug / protein effect. Also consistent between the proteins was the decrease in rate from maximum enhancement (at 100 μ M) back to that of the control rate, at higher concentrations of compound (Figures 3.2 and 3.4). It is important to note here that reactions containing BSTLC or FSTLC in concentrations higher than 100 μ M formed an opaque precipitate (FSTLC being more severe than BSTLC) that made reactions in both the coupled and the MG assays cloudy, potentially indicative of the compound losing solubility and precipitating out of solution. A decrease of available drug in solution is a possible explanation for the loss of basal Kinesin-5 enhancement observed at concentrations over 100 μ M. The insolubility of these compounds at high concentrations (the extent of which remains unknown) implies that we may only be able to accurately monitor the compounds interactions with kinesin proteins at concentrations lower than 100 μ M.

Our results show that the effects of these derivatives are much more pronounced on HsEg5 than KLP61F, supporting conclusions that HsEg5 is more susceptible to small molecule control than its *D. melanogaster* homolog (Brier et al., 2006c; Maliga et al., 2006). The inability of KLP61F to bind radiolabeled monastrol (Chapter 2) suggests that key residue differences in the monastrol-binding pocket determine a given Kinesin-5 motor's sensitivity to monastrol (and presumably other inhibitors that target the same site). Thus, correlation of sequence and monastrol sensitivity for Kinesin-5 motors may be useful in identification of residues essential for recognizing compounds that target the monastrol-binding site (Brier et al., 2006b; Maliga and

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Mitchison, 2006). A recent FT-IR study determined that monastrol alters one or more carboxylic acids within the HsEg5 motor domain in the presence of ATP (Wojcik et al., 2004). Figure 3.5 shows a sequence alignment of the motor domains of HsEg5, KLP61F, and Ncd, and illustrates two HsEg5 specific glutamic acid residues near the N-terminus of the L5 loop (monastrol binding site) E116 and E118. Because these residues are not conserved in KLP61F (valine and asparagine are present at the equivalent position), they may be required for small molecule sensitivity ((Wojcik et al., 2004) Chapter 4). In order to determine the importance of these residues in HsEg5 small molecule inhibition, we generated a mutant version of the HsEg5 motor domain, E116V+E118N, and tested the ability of this KLP61F-like HsEg5 mutant to hydrolyze ATP in the presence of BSTLC.

Basal and MT-stimulated ATPase activities of the E116V+E118N double mutant protein in the presence of 100 μ M BSTC were measured using the coupled and MG assays, respectively. Due to FSTLC's questionable solubility and because its apparent basal enhancement was much less dramatic than BSTLC, experiments with FSTLC were not conducted with the E116V+E118N protein. Presented in Table 3.1, and similar to results seen with KLP61F, BSTLC mildly but significantly (unpaired t-test p = 0.0011) enhanced the basal ATPase rate of E116V+E118N, and was not significantly effective in the presence of MTs (unpaired t-test), similar to results obtained with the other Kinesin-5 proteins. Our E116V+E118N ATPase data also confirm reports that point mutations within the L5 loop of HsEg5 can alter the motor's ability to hydrolyze ATP (Brier et al., 2006b; Maliga and Mitchison, 2006). While there are several other amino acid differences between the two proteins, both within the L5 loop area and outside of it, we show that this KLP61F-like, HsEg5 double mutant responds to BSTLC and FSTLC more similarly to KLP61F than its parent protein HsEg5, implying that amino acids 116 and 118 of KLP61 affect the proteins interactions with HsEg5 L5 loop-targeting compounds.

Despite the fact that each Kinesin-5 tested was enhanced by BSTLC (and mildly with FSTLC) in the absence of MTs, the Kinesin-14, Ncd, typically not sensitive to Kinesin-5 targeting compounds, was unaffected (DeBonis et al., 2004; Maliga and Mitchison, 2006). However, KLP61F, which has also demonstrated insensitivity to L5 targeting drugs ((Maliga and Mitchison, 2006) Figures 3.2b and 3.4b), is affected by both compounds tested. The sequence alignment in Figure 3.5 shows the amino acid variations between the two Kinesin-5s (HsEg5 and KLP61F) and the Kinesin-14 (Ncd). To allow for Kinesin-5 specificity, the BSTLC

binding site is more than likely a protein section that is either missing or very different in Ncd, compared to HsEg5 and KLP61F. As previously mentioned Kinesin-5 proteins possess a L5 insertion loop that serves as the HsEg5 / STLC binding site (Brier et al., 2004). While monastrol / STLC sensitive as well as insensitive Kinesin-5 proteins possess this insertion loop, amino acid differences between species' are believed to confer sensitivity to such small molecules (Maliga and Mitchison, 2006). Interestingly, where HsEg5 and KLP61 contain 21 amino acids in their L5 loops, Ncd only contains 11 (Figure 3.5). Therefore it remains possible that the STLC derivatives tested target both Kinesin-5s via the L5 loop (regardless of the amino acid differences that prevent KLP61F- monastrol / STLC inhibition) and because this segment is absent in Ncd, the motor is insensitive. Binding competition assays with BSTLC and FSTLC and ¹⁴C-monastrol (as described in Chapter 2) to determine if these compounds bind the L5 site, were not interpretable given the high concentrations of drugs necessary for these experiments and the insolubility of the drugs at these concentrations. Another area that varies between our Kinesin-5s and Kinesin-14 constructs is the neck linker (Figure 3.5). This portion of amino acids is highly conserved between individual kinesin subfamilies and is responsible for motor directionality (Case et al., 1997; Sablin et al., 1996). The Ncd construct's neck linker is much shorter those of HsEg5 or KLP61F, and it is also plausible that BSTLC targets this area. Experiments testing BSTLC's effects on different kinesin subfamily proteins, each with different neck linker should be performed in order to determine if the compound associates here.

Our data shows that Kinesin-5 proteins, specifically HsEg5, are both mildly enhanced by the FSTLC. While the rate simulation seen with FSTLC is significant, the enhancement seen in the presence of BSTLC is much more dramatic. The enhancement ability of a compound structurally similar to one of the most potent established HsEg5 inhibitors is unprecedented. While we report it to be the first Kinesin-5 stimulatory compound, BSTLC is not the first small molecule to enhance the basal ATPase rate of a kinesin motor protein. AS-2, a natural marine product has demonstrated Kinesin-1 basal enhancement by interacting with the MT-binding site of the motor (Brier et al., 2006a; Reddie et al., 2006; Sakowicz et al., 1998). Again, because BSTLC / FSTLC had no effect on Kinesin-14 Ncd, it is unlikely that these compounds target the kinesin MT binding site, like AS-2.

While our BSTLC and FSTLC binding site studies were inconclusive, it remains likely, due to overwhelming structural similarities that BSTLC binds HsEg5 at the same site as STLC,

the L5 loop. Further, our results signifying altered BSTLC / HsEg5 interactions with L5 loop mutations, argue that the compound does, in fact, associate at or near that site. Further, Ncd does not possess a loop comparable to the Kinesin-5 L5 loop (Figure 3.5), again suggesting that the Kinesin-5 insertion loop confers sensitivity to BSTLC. If this is true, why would this compound affect the STLC-insensitive protein, KLP61F, or more specifically, if STLC does not interact with KLP61F's L5 loop, why would BSTLC? Perhaps the structural differences between the two compounds are enough to allow for an interaction between KLP61F and BSTLC, while STLC is ineffective. The ability of BSTLC to enhance Kinesin-5 motor protein ATP hydrolysis through interactions with a site previously identified solely for inhibitors, implies the existence of a more complicated mode of kinesin motor protein regulation (specifically for HsEg5) than previously thought (Maliga et al., 2002; Mayer et al., 1999; Yan et al., 2004).

To add another level of complexity to the system is addition of one of the protein's natural allosteric regulators, MTs. Upon association with MTs, kinesin proteins undergo conformational changes that result in ATP hydrolysis. Consistent with the altered experimental conditions, our MT-stimulated ATPase data with both HsEg5 and KLP61F implicates a different interaction between the compounds and kinesin motor proteins compared to the basal conditions. Under conditions in which MTs are present, HsEg5 is severely inhibited by FSTLC, implying that the presence of MTs alters the system and consequently allows the motor to either bind the inhibitor or undergo the conformational changes necessary for inhibition. Similarly, in the presence of MTs the enhancement seen with BSTLC in basal assays is lost. Perhaps as a result of a binding site competition or an alternate conformational pathway following interactions between the motor and MTs, the stimulatory effects of BSTLC are eliminated.

Interestingly, a recent publication by Ogo et al. determined that modifications at STLC's amino group (where the Boc- and Fmoc- groups are located, Figure 3.1) result in a loss of MT-stimulated ATPase inhibition, compared to STLC (Ogo et al., 2007). They determined the HsEg5 MT-stimulated IC₅₀ of BSTLC to be > 63 μ M, which is consistent with our data that BSTLC is not inhibitory in MT-stimulated assays (Figure 4a). They also report the MT-stimulated IC₅₀ of FSTLC to be 55 μ M, which is over 15-fold less potent than our data (IC₅₀ = 2.7 μ M). The difference between our IC₅₀ calculation and that reported (Ogo et al., 2007) may be due to the use of different experimental conditions or measurement assays. Specifically, our data was measured with the coupled assay, which measures a decrease in NADH absorbance as a

function of ADP production, and time (from ATP hydrolysis), while Ogo et al. performed the Kinase-glo luminescent assay (Promega), which measures the amount of ATP remaining in solution following a given reaction. Further, it is possible that the protein construct used by Ogo et al. (details not indicated in literature) is different than our construct, and subsequently hydrolyzes ATP at an altered rate (with or without small molecule).

The ability of monastrol and other HsEg5 inhibitors to arrest mitosis, and thus cell proliferation, and the ensuing potential they show as anti-cancer agents, has led to a recent surge of research and study focused on HsEg5 and precisely how such small molecules can specifically control it. As the search for small-molecule compounds that specifically target HsEg5 continues, derivatives of already identified, well characterized HsEg5 inhibitors lead the way towards more potent, specific cell cycle control (Cox et al., 2005; Debonis et al., 2008; Gartner et al., 2005; Ogo et al., 2007; Sunder-Plassmann et al., 2005). Our characterization of two STLC derivatives has shown that KLP61F (and potentially other monastrol / STLC-insensitive Kinesin-5s) can be targeted with small molecules that also target HsEg5, by possible interactions with the Kinesin-5 specific L5 loop. We also show that the presence of MTs, a native kinesin allosteric regulator, changes the interactions of such small molecules and Kinesin-5 proteins. This is not the first account of a compound that exhibits different effects on a kinesin motor in the presence / absence of MTs. As previously discussed, through kinesin MT- binding site interactions AS-2 mimics MTs in Kinesin-1 basal assays, resulting in an enhanced ATPase rate; however in MTstimulated experiments, the compound was found to inhibit the motor (Sakowicz et al., 1998). The ability of MTs to change the effect of a compound on protein activity will certainly have an impact on its effectiveness as an anti-cancer treatment. Precisely how MTs change such compound / HsEg5 interactions (i.e. directly or through allosterics) will need to be determined before such compounds may be applied in vivo.



STLC





Figure 3.1: Chemical Structures of STLC, BSTLC, and FSTLC.



Figure 3.2: BSTLC and FSTLC Enhance the Basal ATPase Activity of the Kinesin-5 Motor Proteins: HsEg5 and KLP61.

ATP hydrolysis was measured with the pyruvate kinase / lactate dehydrogenase coupled assay. Values were normalized against the uninhibited rate of the appropriate motor. The plots show the average basal ATPase values \pm SEM for HsEg5 in 3.2a (n = 2 to 35) and KLP61F in 3.2b (n = 3 to 75), respectively, in the presence of STLC (black boxes), BSTLC (open triangles), or FSTLC (grey circles).

	Basal		MT-Stimulated	
	Coupled Assay	MG Assay	Coupled Assay	MG Assay
	ADP/Motor/Sec	P/Motor/Sec	ADP/Motor/Sec	P/Motor/Sec
HsEg5 Control	0.15 ± 0.002	0.073 ± 0.003	7.3 ± .42	9.1 ± 0.15
HsEg5 + STLC	$0.02 \pm 0.001*$	$0.003 \pm 0.001*$	$0.15 \pm 0.02*$	$0.5 \pm 0.1*$
HsEg5 + BSTLC	$0.3 \pm 0.004*$	$0.15 \pm 0.009*$	7.8 ± 1.2	$8.4 \pm 0.28^*$
HsEg5 + FSTLC	$0.17 \pm 0.007*$	0.06 ± 0.001 *	$2.2 \pm 1.3^*$	$0.25 \pm 0.15^*$
KLP61F Control	0.05 ± 0.001	0.024 ± 0.00	0.56 ± 0.07	0.6 ± 0.12
KLP61F + STLC	0.05 ± 0.003	0.029 ± 0.003	0.57 ± 0.10	0.7 ± 0.06
KLP61F + BSTLC	$0.08 \pm 0.005*$	$0.044 \pm 0.002*$	ND	0.5 ± 0.12
KLP61F + FSTLC	$0.07 \pm 0.004*$	$0.033 \pm 0.001*$	ND	0.4 ± 0.15
MC6 Control	0.12 ± 0.005	ND	ND	ND
MC6 + STLC	0.12 ± 0.001	ND	ND	ND
MC6 + BSTLC	0.11 ± 0.006	ND	ND	ND
MC6 + FSTLC	0.13 ± 0.002	ND	ND	ND
		- 		
VN Control	0.18 ± 0.003	0.13 ± 0.02	ND	7.4 ± 0.63
VN + STLC	0.21 ± 0.014	ND	ND	ND
VN + BSTLC	0.22 ± 0.001	ND	ND	5.8 ± 0.16

Table 3.1: Effect of STLC, BSTLC and FSTLC on the ATPase Rates of Selected Kinesin Proteins.

Shown are the mean \pm SEM ATPase values for the indicated kinesin protein in the presence of 100 μ M of the indicated compound as measured by either the pyruvate kinase / lactate dehydrogenase coupled assay (Figure 3.2, 3.3) or the malachite green assay (Figure 3.4). N values not already presented are as follows: HsEg5 basal MG (n = 3), KLP61F basal MG (n = 3), HsEg5 MT-stimulated coupled (n = 3 to 16), KLP61F MT-stimulated coupled (n = 5 to 22), E116V+E118N (VN) basal coupled (n = 8 to 46) and E116V+E118N (VN) MT-stimulated MG (n = 4 to 8). ND: Not Determined. * Mean is statistically different from control (Unpaired t-test).



Figure 3.3: STLC Derivatives Do Not Affect the Basal ATPase Activity of the Kinesin-14 Motor Protein, Ncd.

ATP hydrolysis of the Ncd motor domain was measured at 100 μ M drug concentration with the pyruvate kinase / lactate dehydrogenase coupled assay (n = 2 to 6). Also shown are the HsEg5 (n = 10 to 35) and KLP61 (n = 16 to 75) ATPase activities at 100 μ M drug (also presented in Figure 3.2). Control values are presented as black columns, STLC in white, BSTLC in light gray, and FSTLC in dark gray. Values were normalized against the uninhibited rate of the appropriate motor.



Figure 3.4: MTs Alter the Effects of STLC Derivatives on Kinesin-5 Proteins. ATP hydrolysis was measured with the malachite green phosphate assay. Values were normalized against the uninhibited rate of the appropriate motor. Figures a and b show MTstimulated ATPase values for HsEg5 (n = 3) and KLP61F (n = 3), respectively, in the presence of varied STLC (black boxes), BSTLC (open triangles), or FSTLC (grey circles).

Human_HsEg5 Drosophila_KLP61 Drosophila_Ncd_MC6	-MASQPNSSAKKKEEKGKNIQVVVRCRPFNLAERKASAHSIVECDPVRKEVSVRTGGLAD MDISGGNTSRQPQKKSNQNIQVYVRVRPLNSRERCIRSAEVVDVVGPREVVTRHTLD
prosobulia_wcg_wcg	RELENIVHULKGNIKVICKIKPPLESEENKMCCIWIIHUESIVELQSIUAQARS
Human HsEq5	KSSRKTYTFDMVFGASTKOIDVYRSVVCPILDEVIMGYNCTIFAYGOTGTGKTFTMEGER
Drosophila KLP61	SKLTKKFTFDRSFGPESKOCDVYSVVVSPLIEEVLNGYNCTVFAYGOTGTGKTHTMVGNE
Drosophila Ncd MC6	KMGQQIFSFDQVFHPLSSQSDIF-EMVSPLIQSALDGYNICIFAYGQTGSGKTYTMDGVP
	ATP L5
Uuman Hellah	
Drosophila KLP61	TARLKSSWEDDSDIGIIPRALSHLFDELRMMEVEYTMRISYLELVNEELCDLLST
Drosophila Ncd MC6	ESVGVIPRTVDLLFDSIRGYRNLGWEYEIKATFLEIYNEVLYDLLS
	L5 MT
Human HsEg5	DVSERLQMFDDPRNKRGVIIKGLEEITVHNKDEVYQILEKGAAKRTTAATLMNAYSSRSH
Drosophila_KLP61	DDTTKIRIFDDSTKKGSVIIQGLEEIPVHSKDDVYKLLEKGKERRKTATTLMNAQSSRSH
Drosophila_Ncd_MC6	NEQKDMEIRMAKNNKNDIYVSNITEETVLDPNHLRHLMHTAKMNRATASTAG <u>NERSSRSH</u>
	ATP
Human HsEq5	SVFSVTIHMKETTIDGEELVKIGKLNLVDLAGSENIGRSG-AVDKRAREAGNINOSLLTL
Drosophila KLP61	TVFSIVVHIRENGIEGEDMLKIGKLNLVDLAGSENVSKAGNEKGIRVRETVNINQSLLTL
Drosophila_Ncd_MC6	AVTKLELIGRHAEKQEISVGSINLVDLAGSESPKTSTRMTETKNINRSLSEL
	ATP
Human_HsEg5	GRVITALVERTPHVPYRESKLTRILQDSLGGRTRTSIIATISPASLNLEETLSTLEYAHR
Drosophila_KLP61	GRVITALVDRAPHVPYRESKLTRLLQESLGGRTKTSIIATISPGHKDIEETLSTLEYAHR
Drosophila_Ncd_MC6	TNVILALLQKQD <u>HIPYR</u> NSKLTHLLMPSLGGNSKTLMFINVSPFQDCFQESVKSLRFAAS
	мт
Human_HsEg5	AKNILNKPEVNQKLT
Drosophila_KLP61	AKNIQNKPEVNQKLT
Drosophila_Ncd_MC6	VN
	Neck Linker

Figure 3.5: Sequence Alignment of Kinesin-5 proteins and Ncd.

Shown is a ClustalW sequence alignment of Kinesin-5 proteins, HsEg5 and KLP61F, and the Kinesin-14 Ncd. Amino acids responsible for motor directionality (neck linker), nucleotide binding and hydrolysis (ATP), or MT interactions (MT) are indicated. The Kinesin-5 specific L5 loop (L5) with amino acids mutated to create the E116V+E118N mutant, (Table 3.1) HsEg5 E116 and E118 (6, 8, above the sequence, respectively), are also indicated (Grant et al., 2007; Sablin et al., 1996; Wojcik et al., 2004).

Chapter 4: Characterizing the Roles of E116 / E118 in HsEg5 ATP Hydrolysis and Small-Molecule Sensitivity

Abstract

Kinesin-5 proteins are primarily responsible for organization of the bipolar mitotic spindle. The human version, HsEg5, has demonstrated sensitivity to numerous small-molecules that specifically inhibit protein activity and subsequently control cell division. The most common binding site for such small molecules is an induced-fit pocket enclosed by the Kinesin-5-specific, L5 insertion loop. Crystallographic and FT-IR analyses identified several amino acids within the HsEg5 L5 loop potentially required for small-molecule inhibition. The present study utilizes site-directed mutagenesis to evaluate the importance of two glutamic acids in the L5 loop, both in the presence and absence of inhibitors. Our results suggest the existence of one or more electrostatic interaction(s) between specific amino acid side chains. These interactions may also be involved in motor domain movements necessary both for ATP hydrolysis and monastrol-induced inhibition.

Introduction

Kinesin-5 proteins are responsible for microtubule (MT) movements early in mitosis that result in organization of the bipolar mitotic spindle (Kashina et al., 1997; Sawin et al., 1992). Because of their requirement in mitosis, understanding specifically how Kinesin-5s function in vivo (chiefly the human version, HsEg5), as well as the ability to control motor activity by small-molecule compounds, is of particular interest (Bergnes et al., 2005).

The interactions between HsEg5 and monastrol, the first identified Kinesin-5 inhibitor (Mayer et al., 1999), have been well characterized. Monastrol allosterically inhibits HsEg5 ATPase activity by binding to an induced-fit site enclosed by the L5 insertion loop (Maliga et al., 2002; Yan et al., 2004). Monastrol binding causes the L5 loop of the protein to fold over, like a hinge, and enclose the monastrol-binding pocket (Cochran et al., 2005; Yan et al., 2004).

Similar loops have been identified in various kinesins, as well as actin and myosin (in the absence of small molecule) and have been linked to larger structural alterations within the catalytic core of the protein (Brier et al., 2004; Clarke et al., 1986; Cochran and Gilbert, 2005; Fetrow et al., 1997; Gerstein and Echols, 2004; Lesk and Chothia, 1984; Maliga et al., 2006; Sweeney et al., 1998; Yang and Miles, 1992). Such conformational changes may result in more global protein movements and functions, such as MT binding or nucleotide hydrolysis. By a similar mechanism, HsEg5's L5 loop may be involved in regulation of protein activity in the absence of inhibitor, and when bound by an L5 targeting compound, such movements may be altered (Maliga et al., 2006).

Crystallographic data of the HsEg5 motor domain both with and without monastrol present have identified specific amino acids that line the HsEg5 / monastrol binding site. Due to their potential involvement in motor / drug interactions, and toward a better understanding of structural elements that confer HsEg5 small-molecule sensitivity, amino acids that line the monastrol binding pocket are attractive candidates for site-directed mutagenesis (Brier et al., 2006b; Maliga and Mitchison, 2006). Some specific mutations and their effects on monastrol-and STLC- mediated inhibition are discussed in Chapter 1.

Since the HsEg5 / monastrol structure was published, other methods have been utilized to illustrate structural alterations that occur within the motor domain in the presence of small-molecule compounds. In 2004, Wojcik et al. used FT-IR spectroscopy to visualize secondary structure changes within the HsEg5 motor domain and noted that in the presence of monastrol and ATP, alterations in one or more carboxylic acid(s) occur and may permit monastrol inhibition (Wojcik et al., 2004). Analysis of sequence alignments comparing the HsEg5 L5 loop to monastrol-sensitive (*X. laevis* Eg5 (Mayer et al., 1999)) and insensitive (*D. melanogaster* KLP61F (Maliga and Mitchison, 2006)) Kinesin-5 proteins shows conservation of two glutamic acid residues among monastrol-sensitive proteins that could be responsible for the visualized spectral shifts: E116 and / or E118 (Figure 4.1). While neither of these residues have been directly implicated in contacting monastrol (Brier et al., 2006b; Maliga and Mitchison, 2006; Yan et al., 2004), they may confer L5 loop's hinge flexibility required for monastrol binding, or for ATPase activity in the absence of inhibitor (S. Kim, manuscript in preparation). To test the functional significance of these residues for HsEg5 ATP hydrolysis, as well as determine if these residues are required for HsEg5 inhibition by small molecules, we created and evaluated nine

HsEg5 mutants, in which one or both of these glutamic acid residues were substituted as follows: E116D, E116V, E116L, E118D, E118N, E118L, E116D+E118D, E116V+E118N, and E116L+E118L.

Materials and Methods

Reagents:

S-trityl-L-cysteine (STLC) and monastrol were obtained from Sigma-Aldrich. Both compounds were prepared in DMAO, STLC at 50 mM and monastrol at 100 mM.

Protein Cloning, and Site-Directed Mutagenesis:

The wild type (WT) HsEg5 motor domain, composed of HsEg5 residues 1-370 and a Cterminal 6-His tag, was cloned as previously described (Wojcik et al., 2004). Site-directed mutagenesis of the HsEg5 motor domain to create the described mutant proteins was performed by S. Kim. Briefly, two mutagenic oligonucleotide primers with the desired mutations were designed and synthesized. Mutant HsEg5 was generated and amplified via PCR. To confirm only the desired mutations were acquired, the resulting DNA was sequenced. Following are the primers used to generate each mutant protein:

E116V

forward 5' ACAATGGTGGGTGAAAGGTCACCTAA 3' reverse 5' TTTCACCCACCATTGTAAAAGTTTTTCC 3'

E116L

forward 5' TACAATGCTGGGTGAAAGGTCACCTAAT 3' reverse 5' TTTCACCCAGCATTGTAAAAGTTTTTCCAG 3'

E116D

forward 5' AATGGACGGTGAAAGGTCACCTAA 3' reverse 5' TTCACCGTCCATTGTAAAAGTTTTTCC 3'

E118N

forward 5' GAAGGTAACAGGTCACCTAATGAAGAG 3' reverse 5' GTGACCTGTTACCTTCCATTGTAAAAGTTT 3'

E118L

forward 5' GAAGGTCTGAGGTCACCTAATGAAGAG 3' reverse 5' GTGACCTCAGACCTTCCATTGTAAAAGTTT 3'

E118D

forward 5' AGGTGACAGGTCACCTAATGAAGA 3' reverse 5' TGACCTGTCACCTTCCATTGTAAAAG 3'

E116L+E118L

forward 5' TTTACAATGCTGGGTCTGAGGTCACCTAATGAAG 3' reverse 5' CTTCATTAGGTGACCTCAGACCCAGCATTGTAAA 3'

E116V+E118V

forward 5' CTTTTACAATGGTGGGTAACAGGTCACCTAAT 3' reverse 5' ATTAGGTGACCTGTTACCCACCATTGTAAAAG3'

E116D+E118D

forward 5' CTTTTACAATGGACGGTGACAGGTCACCTAAT 3' reverse 5' ATTAGGTGACCTGTCACCGTCCATTGTAAAAG 3'

Protein Expression, and Purification:

All plasmids were transformed into BL21 Codon-plus (DE3)-RIL cells (Stratagene) for protein expression. Overnight cultures of transformed cells were diluted 1:100 into LB media supplemented with 100 µg/ml ampicillin and grown at 37°C for 2.5 hours. Protein expression was induced with 0.2 mM IPTG, and after 4 hours at room temperature, cells were pelleted, washed once with 25 mM PIPES pH 6.9, 0.25 mM MgSO₄, 0.5 mM EGTA, and frozen at -80°C until use. Frozen cells were thawed in 50 mM HEPES (pH 7.5), 75 mM NaCl, 1 mM PMSF, 0.1 mM MgATP, 40 µg/mL DNAse, 0.3 mg/ml lysozyme, 10 mM MgCl₂, and 1 mM DTT, and passed through a French Press three times to ensure adequate lysis. Cell lysates were then centrifuged at 20,500g for 30 minutes at 4°C, and the resulting supernatant was passed over a 5 ml SP-Sepharose resin. After a wash with 50 mM HEPES (pH 7.5), 0.1 mM MgATP, and 1 mM DTT, the protein was eluted with 50 mM HEPES (pH 7.5), 0.2 mM MgATP, 1 mM DTT and 250 mM NaCl. For desalted fractions, the protein-containing eluate was immediately passed over a PD-10 desalting column and eluted with 50 mM HEPES (pH 7.5), 0.1 mM MgATP, and 1 mM DTT. Eluates were immediately mixed with an equal volume of 50 mM HEPES (pH 7.5), supplemented with glycerol (to 10%), frozen on dry ice, and stored at -80°C until use. A Bradford Assay (Biorad), with BSA as the standard, was used to determine protein concentrations.

ATPase Assays:

Steady-state basal ATPase activities were measured with the pyruvate kinase / lactate dehydrogenase coupled enzyme assay (Deavours et al., 1998; Moore et al., 1996). Assays were carried out at room temperature in 50 mM Tris-acetate, pH 7.4, 2 mM MgCl₂. Reactions contained 5 μ M motor and monastrol or STLC at indicated concentrations. Control reactions were supplemented with DMSO to match the concentration of DMSO carried over with inhibitors. NaCl concentration in all assays was < 15 mM unless otherwise indicated. IC₅₀ values were calculated from means for each drug concentration as described (Maliga et al., 2002). Curve fits were done using Prism 4 (GraphPad).

SDS-PAGE

A 10% SDS-PAGE gel was prepared and 1 μ g of each protein (indicated) was loaded in each lane. Gel was run at 200 V for approximately 30 minutes and stained with Biosafe Coomassie (Bio-Rad).

Results and Discussion

Previous work has suggested that HsEg5 residues E116 and / or E118, may be involved in HsEg5 / monastrol sensitivity ((Wojcik et al., 2004), S. Kim manuscript in preparation). To determine the importance of these residues in motor activity and inhibitor sensitivity, we created nine HsEg5 mutants with substitutions at the E116 and / or the E118 residue(s) (Figure 4.1). Six of the nine amino acid mutations were based solely on amino acid chemistry. Since the wild type protein contains two glutamic acid residues, each amino acid (or both) were switched to an aspartic acid, maintaining similar chemistry (hydrophilic, negative charge) but with a shorter side chain. In order to change amino acid more substantially, residues were also mutated to a nonpolar leucine. The last three mutations were derived from the sequence of monastrol insensitive Kinesin-5, KLP61F (Maliga and Mitchison, 2006). This *D. melanogaster* protein possesses a valine and an asparagine at the respective positions (Figure 4.1), so a set of HsEg5 mutations were constructed containing either or both of these substitutions. Evaluation of the KLP61F-like mutants may help to clarify differences between KLP61F and HsEg5 that result in
such different ATPase activities, as well as which specific amino acids confer HsEg5 sensitivity to small molecules.

Mutant HsEg5 Basal ATPase Activity

To determine if E116 and / or E118 are required for basal protein activity, we performed steady state basal ATPase assays with each mutant. Results indicate that modification of the E116 / E118 residue(s) affects ATPase activity; compared to the WT protein, each of the E116 mutations displayed a slower ATP hydrolysis rate, while each of the E118 mutants hydrolyzed ATP faster (Table 4.1). This phenomenon is best visualized in the leucine single mutant proteins. The removal of negative charge (glutamic acid) and introduction of hydrophobicity (leucine) altered HsEg5 ATPase activity the most severely; with E116L demonstrating the slowest ATP hydrolysis rate of all mutants tested, and the E118L mutant demonstrating the fastest. Because each mutation of the E116 residue resulted in impaired ATPase ability, our results suggest that this residue is required to maintain efficient ATPase activity. On the other hand, a glutamic acid residue at the 118 position may act as a negative regulator of motor activity, as modification of this residue results in an increase in ATPase activity.

The mutant proteins with the most conservative change are E116D, E118D, and E116D+E118D. Aspartic acid maintains the same charge as glutamic acid, however the loss of a methyl group makes the side chain shorter, and may consequently eliminate contacts or interactions with small molecules or other distant elements within the protein. This chemical change, regardless of how small, was visualized in the altered ability of these mutants to hydrolyze ATP: with the E116D mutant's decrease in ATPase rate, and E118D's increase (Table 4.1).

Interestingly, the E116D+E118D protein hydrolyzes ATP comparably to the E116D mutant; this trend is also observed for E116L+E118L, as its basal ATPase rate is more similar to its E116L counterpart. The tendency for these double mutant proteins to behave more similarly to their respective 116 mutants reinforces the conclusion that the loss of activity due to mutations at the 116 position prevents the protein from efficiently hydrolyzing ATP regardless of amino acid composition at position 118. E116V+E118N contrarily, hydrolyzes ATP at a similar rate to its E118N counterpart. As mentioned earlier, the E116V, E118N, and E116V+E118N set of mutations were based on the monastrol-insensitive *D. melanogaster* Kinesin-5 homologue,

KLP61F, (Maliga and Mitchison, 2006), which possesses a valine and an asparagine at positions equivalent to HsEg5's 116 and 118, respectively. Of the 116 mutations tested, the E116V basal ATPase activity is the closest to that of WT, however its basal ATPase rate is still considerably lower. While the value side chain is hydrophobic like leucine, it is smaller and therefore may introduce less steric interference and / or less hydrophobicity (Black and Mould, 1991) than the E116L mutant and therefore allows more ATPase activity. Consistent with the other 118 mutants, E118N displays faster ATPase activity than WT. The introduction of an asparagine at the 118 location maintains the residue's hydrophilic nature, though without the negative charge and may be responsible for the observation that E118N hydrolyzes ATP similarly to the WT protein and our E118D mutant, but not as efficiently as the hydrophobic E118L mutant. Again, E116V+E118N is the only double mutant tested that does not behave in the same way as its E116V counterpart. This protein hydrolyzes ATP faster than WT, while the other double mutants are slower, and more similar to their 116 versions. The ability of E116V to maintain moderate ATPase activity, compared to the other E116 mutant proteins, may permit the protein to be enhanced in the presence of the E118N mutation. Moreover, the other E116 mutant's slow rates could not be overcome by their 118 counterparts.

Mutant HsEg5 Basal ATPase Activity: in the presence of monastrol and STLC

In order to determine if E116 and / or E118 confer specificity for small-molecule HsEg5 inhibition, we examined each mutant's ability to hydrolyze ATP in the presence of different monastrol concentrations. Presented in Table 4.1 are the calculated IC_{50} values for monastrol and each protein. As visualized by the increase in mutant IC_{50} relative to WT, alteration of E116 and / or E118 resulted in a loss of monastrol potency. Further, each double mutant demonstrated a loss in monastrol sensitivity comparable to their E116 single mutant counterpart. A mild enhancement was surprisingly observed for E116L and E116L+E118L in the presence of monastrol (EC₅₀ values of approximately 0.3 μ M each, Table 4.1).

Table 4.1 also shows results for each mutant in the presence of STLC. Interestingly, while E116D was the only E116 single mutant to retain STLC sensitivity, each E118 single mutant was potently inhibited (IC₅₀s of approximately 2 μ M each, Table 4.1). Further, E116V, E116L and their corresponding double mutants were not only uninhibited by STLC, they demonstrated considerable rate enhancement (EC₅₀s are presented in Table 4.1). Since only

proteins possessing an acidic residue at position 116 were inhibited by STLC, this suggests that the presence of a negatively charged residue at the E116 position is required for inhibition by the compound. Previous reports have demonstrated that fewer amino acids are required for STLC-HsEg5 inhibition monastrol (Brier et al., 2006b; Maliga and Mitchison, 2006); this finding is confirmed by our results. Drug size is a possible explanation for the observed differences in inhibition between the two compounds. Monastrol is much smaller than STLC (for structures of these compounds see Figures 1.7 and 3.1) and as a result may fit better in the binding pocket and permit more contacts (either direct or allosteric), thereby making more residues required (to varying degrees) for monastrol-induced sensitivity. On the other hand, STLC may dock into the protein, and make few but essential contacts in order to prevent HsEg5 ATPase activity; we demonstrate that in order to inhibit HsEg5, STLC requires a hydrophilic, or negatively charged residue at position 116. Our results confirm previous reports that modification of residues that are critical for monastrol inhibition may not alter STLC interactions, and although STLC and monastrol share a binding site, the two compounds affect HsEg5 by different mechanisms (Brier et al., 2004; DeBonis et al., 2004; Skoufias et al., 2006).

As previously mentioned, in the presence of monastrol (and potentially other small molecules), HsEg5's L5 loop "closes" like a hinge, over the binding pocket. This conformational change results in more global structural alterations within the catalytic core of the protein that subsequently prevent ATPase activity (Maliga et al., 2006). Motor proteins such as kinesin-1, myosin and actin also contain loop structures, distinct from the L5 loop of Kinesin-5 proteins (i.e. the P loop responsible for nucleotide recognition) known to regulate motor activities such as MT binding or nucleotide hydrolysis (in the absence of small-molecule) (Amos and Hirose, 2007; Nitta et al., 2004; Sweeney et al., 1998; Umeki et al., 2006). In addition to small-molecule binding interactions, the L5 loop has also been implicated in regulation of HsEg5 ATPase activity. For example, in the absence of small-molecule, L5 loop movements have been directly linked to movements of switches I and II, involved in nucleotide binding and hydrolysis (Cochran and Gilbert, 2005; Maliga et al., 2006). Further, replacement of HsEg5's L5 loop with an equivalent segment from rat Kinesin-1 resulted in a 2-fold decrease in ATPase activity, confirming the presence of HsEg5's L5 loop is important for maintaining its ATPase activity (Brier et al., 2004). Our mutational analysis suggests that E116 is required for efficient HsEg5 ATP hydrolysis, as each modification of this residue tested resulted in a decrease in motor

activity. E116 is located at the N-terminus of the loop and may act to confer the loop's hingelike flexibility, without which the protein's ability to hydrolyze ATP is impaired.

Mutant HsEg5 Basal ATPase Activity: the effects of ionic strength

Our results demonstrated that glutamic acid residues in the HsEg5 L5 loop are important for basal ATPase activity. What role could these residues be playing in this protein? Through interactions such as salt bridges, charged residues along with intracellular salt concentrations, are critical in protein regulation and consequently cell activity (Alberts et al., 2002; Hackney and Stock, 2000). Salt bridges in proteins serve as non-covalent bonds between charged amino acids (within close proximity to each other) and contribute to protein structure and function (Bosshard et al., 2004). Such a salt bridge could, as a result of stable amino acid interactions (and consequently protein secondary structure), increase motor domain stability and control movements required for ATPase activity (Perutz, 1978). The side chain shifts induced by ligand binding (small molecule or nucleotide), may, through changes in salt bridge stability, be responsible for the larger scale protein domain movements that result in either ATP hydrolysis, or motor inhibition (in the small-molecule-bound state). Thus, the effect of ionic strength on the in vitro ATPase activity of motor proteins is directly applicable towards appreciating in vivo effects. If there is a salt bridge within the WT HsEg5 motor domain then it will be susceptible to altered ionic conditions, and the protein's ATPase activity should change accordingly. Further, if E116 or E118 (or both) are participating in such an electrostatic interaction, mutations at these residues will demonstrate altered ATPase activities in response to ionic strength.

Towards understanding the in vivo effects of salt on HsEg5 ATPase activity, and to determine if E116 / E118 might participate in a salt bridge, we performed a series of basal coupled ATPase assays with each of our mutant proteins in the presence of 0, 25, 75, and 150 mM NaCl. To determine the effects of ionic strength on the ability of monastrol to inhibit each mutant protein, we repeated the same series of experiments in the presence of 200 μ M monastrol. Data for each protein are shown in Figure 4.2. Results presented in Figure 4.2 have been normalized to emphasize the effects of NaCl on each individual protein.

With increasing NaCl concentrations, each protein demonstrated an enhancement in ATPase rate, to varying degrees. All E116 single and double mutants were enhanced approximately 2-fold, while the E118 single mutants were each enhanced to a lesser extent,

indicating that alteration of the glutamic acid at the 116 position allows for increased sensitivity to salt concentrations. E116L, the slowest mutant protein (Table 4.1), was the most sensitive to NaCl-induced rate enhancement (Figure 4.2). This implies that while the replacement of glutamic acid with leucine at the 116 position may reduce motor activity under certain conditions (in the absence of MTs and at low salt), this mutant protein is still able to function. Furthermore, HsEg5 maintains its ability to hydrolyze ATP despite each mutation tested here, however the rate of ATP hydrolysis is dependent on experimental conditions.

We next evaluated the ability of each mutant to be stimulated by NaCl in the presence of 200 μ M monastrol (Figure 4.2). Data obtained for each mutant protein and monastrol at varied NaCl concentrations is consistent with the monastrol IC₅₀ data (Table 4.1). Specifically, the presence of NaCl did not dramatically alter monastrol sensitivity: E116L and E116L+E118L remaining insensitive and E116V, E116V+E118N remaining less sensitive than the other proteins tested. Further, E116D, E118N, E118L, E118D, E116D+E118D, and WT all maintained equivalent monastrol sensitivity at 0 versus high 150 mM salt. This is inconsistent with previous reports that HsEg5 is more sensitive to monastrol under high salt conditions, (Luo et al., 2004). A possible explanation for this discrepancy is that our experiments used a high concentrations were tested, it may have been possible to visualize an increase in sensitivity at higher salt.

Possibly the most striking feature of Figure 4.2 is the apparent loss in NaCl sensitivity (loss of rate enhancement) in the presence of monastrol exhibited by the E118 single mutants, E118N, E118L, and E118D (Figure 4.2). Each of these proteins is completely inhibited by monastrol and no increase in rate is observed at high ionic strength in monastrol experiments. On the other hand, E116V, E116D, and E116D+E118D, each exhibit sensitivity to monastrol (as their rates in the presence of monastrol are slower than in the absence), however these proteins are still sensitive to NaCl and undergo rate enhancement with increasing NaCl concentrations, while E116L, E116V+E118N, and E116L+E118L rates are enhanced by NaCl regardless of monastrol.

The observation that NaCl affects each of the mutant proteins to different extents suggests ionic strength is genuinely affecting the proteins (as opposed to another component of the assay) and supports the existence of a salt bridge within the HsEg5 motor domain. How

might increased ionic strength affect an electrostatic protein interaction, such as a salt bridge? Moreover, how might salt bridge alterations affect motor protein ATPase activity? In the presence of high salt, interactions between ions involved in salt bridges will be weakened. Loss of the salt bridge due to altered electrostatic interactions will result in the loss of structural stability conferred by the presence of a salt bridge, and permit the conformational changes necessary for protein activity (e.g. nucleotide hydrolysis as visualized in our experiments) (Perutz, 1978). Moreover, motor proteins such as myosin and some kinesin subfamily members contain arginine-glutamic acid salt bridges that are critical in ATP hydrolysis (Auerbach and Johnson, 2005; Farrell et al., 2002; Klumpp et al., 2003; Minehardt et al., 2001; Muller et al., 1999; Okimoto et al., 2001; Onishi et al., 2004; Rice et al., 1999; Song et al., 2001; Yun et al., 2001). Extending these findings, we propose the existence of a salt bridge within the HsEg5 motor domain and because such salt bridge interactions regulate similar activities in other kinesin proteins, HsEg5's salt bridge also functions as a means to regulate motor activity. Further, we advocate that this salt bridge is located at the N-terminus of the HsEg5 L5 loop and through manipulation of small-scale side chain interactions, regulates loop movements that consequently control catalytic activity (e.g. ATP hydrolysis via switches I and II).

Results from monastrol experiments at high ionic strength suggest that the presence of the compound alters the dynamics of the prospective salt bridge within the HsEg5 motor domain. Data implicates one of the two amino acids mutated in this study to be part of the proposed HsEg5 salt bridge: E116. The only proteins that were not sensitive to high ionic strength in the presence of monastrol were E118N, E118L, and E118D. This suggests that the ability of monastrol to inhibit HsEg5 proteins that possess a glutamic acid at the 116 position is not sensitive to NaCl. IC₅₀ data suggests that E116 is required for basal ATPase activity in the absence of small molecule, as each mutation at this site resulted in a decrease in control rate (Table 4.1). Furthermore, experiments in high ionic strength in the absence of monastrol, showed that collectively, E116 mutant proteins are more sensitive to NaCl enhancement, suggesting that under high ionic strength, the available ions in the system may compensate for the absent negative charge, and salt bridge donor, in the E116 mutants, and allow for ATP hydrolysis.

By definition, amino acid residues that represent candidates for a simple salt bridge interaction are positioned within 4Å between oppositely charged side chain atoms (Barlow and

Thornton, 1983; Bosshard et al., 2004). Examination of the HsEg5 protein crystal structure revealed one candidate residue that fits the criteria to be a salt bridge counterpart for E116: R221 (S. Kim, manuscript in preparation). However, no candidate salt bridge partners for E118 were identified following the aforementioned criteria (S. Kim, manuscript in preparation). Therefore, it is our conclusion that HsEg5 utilizes a salt bridge as a means to regulate ATP hydrolysis (by regulating the L5 loop and other global protein movements) in vivo, and that one part of this salt bridge resides at position E116. Further, because monastrol selectively inhibits proteins with a glutamic acid residue at the 116 position, we suggest that monastrol stabilizes the E116 salt bridge, resulting in a loss of ATPase activity.

Mutant HsEg5 Gel Migration Patterns

During the course of protein purification, we recognized that various mutants did not migrate at the same molecular weight, as visualized by SDS-PAGE, as the WT protein. Sequencing of mutant DNA (see methods) confirmed the composition of each construct, so the altered migration pattern of each mutant must be a direct result of corresponding amino acid composition.

Figure 4.3 is a picture of the HsEg5 mutant proteins resolved by SDS-PAGE. Interestingly, the proteins that retain a negative charge at position 116, 118, or both, migrate higher than those proteins that lack a negative charge at those positions. The observation that the presence of a negative charge at either or both of these positions, causes the protein to exhibit a more retarded migration is very similar to the observation that phosphorylation of a protein (and consequent addition of a negative charge) also causes retardation in relative mobility (J. Sible personal communication). It is unclear whether the shift in mobility is simply due to the charge difference, or to a localized structural change as a result of the negative charge.

Overall, our results suggest that E116 may form a salt bridge within the HsEg5 motor domain, which stabilizes the L5 loop in order to regulate ATP hydrolysis. Further, we propose that monastrol reinforces the stability of this salt bridge and prevents ATPase activity. These conclusions provide a basis for understanding how ligand-induced (nucleotide of small-molecule) allostery modulates HsEg5 activity.

* * Human_HsEg5 MEGERSPNEEYTWEEDPLAGI Xenopus_Eg5 MEGERSSDEEFTWEQDPLAGI Drosophila_KLP61F MVGNESPNEEYTWEEDPLAGI

Figure 4.1: Sequences of the HsEg5, Eg5, and KLP61F L5 Loops.

Shown are the amino acid sequences of the monastrol-sensitive Human HsEg5 (Mayer et al., 1999), *Xenopus* Eg5 (Mayer et al., 1999), and the monastrol-insensitive *Drosophila* KLP61F (Maliga and Mitchison, 2006) L5 insertion loops. Specific amino acids mutated in this study (E116 and E118) are denoted with an asterix.

	Control	Monastrol	Monastrol	Monastrol	STLC	STLC	STLC
Protein	Rate	$200 \mu M$	$IC_{50} \mu M$	$\mathrm{EC}_{50}\mu\mathrm{M}$	$100 \ \mu M$	IC50 µM	$EC_{50} \mu M$
WT	0.138 ± 0.002	0.025 ± 0.001	3.8 ± 0.8	ND	0.023 ± 0.001	2.3 ± 1.0	ND
E116D+E118D	0.059 ± 0.002	0.040 ± 0.002	27.4 ± 19.9	ND	0.033 ± 0.002	2.4 ± 1.6	ND
E116D	0.064 ± 0.002	0.037 ± 0.001	34.3 ± 10.7	ND	0.024 ± 0.001	1.8 ± 0.3	ND
E118D	0.164 ± 0.003	0.050 ± 0.002	14.9 ± 2.6	ND	0.032 ± 0.001	2.1 ± .3	ND
E116L+E118L	0.034 ± 0.001	0.040 ± 0.001	ND	0.28 ± 0.2	0.041 ± 0.001	ND	23.8 ± 22.2
E116L	0.023 ± 0.001	0.027 ± 0.001	ND	0.25 ± 0.5	0.030 ± 0.001	ND	64.0 ± 121.6
E118L	0.224 ± 0.004	0.033 ± 0.005	13.2 ± 2.2	ND	0.022 ± 0.001	2.3 ± 0.3	ND
E116V+E118N	0.176 ± 0.003	0.136 ± 0.003	116.9 ± 139.6	ND	0.210 ± 0.014	ND	2.2 ± 1.5
E116V	0.095 ± 0.002	0.083 ± 0.003	87.5 ± 76.3	ND	0.128 ± 0.002	ND	31.8 ± 58.2
E118N	0.187 ± 0.013	0.025 ± 0.001	15.8 ± 2.6	ND	0.015 ± 0.001	2.0 ± 0.8	ND

Table 4.1: Basal ATPase Rates for HsEg5 Mutants ± Monastrol or STLC.

Basal ATPase rates (ADP / motor / second), as determined by the coupled assay, are presented. Also presented are the calculated IC_{50} values for monastrol or STLC and each mutant protein. In the event that a protein was enhanced by the compound, an EC_{50} was calculated. Means \pm SEM are presented with n values for each protein as follows: (WT = 2 to 84), (E116V = 4 to 17), (E116L = 4 to 17), (E116D = 4 to 23), (E118N = 4 to 17), (E118L = 4 to 17), (E118D = 2 to 31), (E116V+E118N = 3 to 49), (E116L+E118L = 3 to 28), and (E116D+E118D = 2 to 32). ND: Not Determined



Figure 4.2: NaCl Stimulates HsEg5 ATPase Activity and Alters Protein Sensitivity to Monastrol.

Normalized ATPase rates are shown for each mutant protein at 0 - 150 mM NaCl, in the presence (black triangles with black dashed line) or the absence (black triangles with black solid line) of 200 μ M monastrol. WT rates are presented in each mutant plot for comparison (gray squares and lines). Data points represent the mean ± SEM with n values for each protein as follows: (WT = 3 to 23), (E116V = 4 to 10), (E116L = 4 to 10), (E116D = 4), (E118N = 4 to 10), (E118L = 4 to 10), (E118D = 4), (E116V+E118N = 8 to 21), (E116L+E118L = 5 to 10), and (E116D+E118D = 7 to 12).



Figure 4.3: Negative Charge Effects HsEg5 SDS-PAGE Migration Patterns.

Each mutant protein $(1 \mu g)$ was analyzed by 10% SDS-PAGE. Standard molecular weights and the specific proteins loaded in each lane are indicated.

Chapter 5: Radiolabeled Monastrol Binds Human and Bovine Serum Albumin

Abstract

Monastrol, an inhibitor of the human Kinesin-5 protein, HsEg5, arrests cells in mitosis and consequently blocks cell reproduction. The ability to reversibly target and control an essential component of the mitotic spindle has lead to identification and characterization of additional HsEg5 inhibitors for use in anti-cancer therapies. In assays using size exclusion spin columns to evaluate the binding of ¹⁴C-monastrol to HsEg5, we serendipitously discovered that ¹⁴C-monastrol binds to various bovine serum albumin (BSA) and human serum albumin (HSA) preparations in a concentration-dependent manner. If monastrol association disrupts the activity of HSA, human application of this anti-cancer compound could have unanticipated negative side effects; or if HSA binds monastrol without releasing it, then monastrol's efficacy may be lost. Conversely, if HSA continues to function with monastrol bound, it may be possible to utilize HSA as a natural transport system to deliver monastrol directly to tumors.

Introduction

Cancer is a disease of unregulated cell division. Many current anti-cancer treatments target microtubules (MTs), which perform the structural and mechanical work of the mitotic spindle (Alberts et al., 2002; Brier et al., 2004; DeBonis et al., 2003; Gerdes and Katsanis, 2005; Miyamoto et al., 2003; Sudakin and Yen, 2007). While these therapies are effective in their ability to disrupt cell division via altered MT dynamics, they do not discriminate between dividing and non-dividing cells, which also rely on MTs for other cellular functions, such as vesicle transport and organelle positioning. As a result, these compounds impact non-dividing cells as well as both healthy and cancerous dividing cells. Therefore with the use of MT-targeting drugs in anti-cancer treatments comes an increased likelihood of severe side effects,

such as untargeted cell death and neurotoxicity (Gerdes and Katsanis, 2005; Rowinsky et al., 1993; Wood et al., 2001).

The ability to control mitosis more exclusively has been made possible with the recent discovery of small molecules that specifically target the human Kinesin-5 motor protein, HsEg5 (Bergnes et al., 2005; Duhl and Renhowe, 2005; Mayer et al., 1999). Direct inhibition of this protein results in mitotic arrest due to monoastral spindle formation and therefore these compounds have little or no effect on non-dividing cells (Mayer et al., 1999). The first identified inhibitor, monastrol (named so after the phenotype resulting from cellular application), (Mayer et al., 1999) is a dihydropyrimidine derivative and has proven to specifically and reversibly target HsEg5 within mitotic cells. Thus monastrol, monastrol derivatives, or other compounds that specifically inhibit HsEg5 have potential to be effective anti-cancer agent (Bergnes et al., 2005; DeBonis et al., 2004; Duhl and Renhowe, 2005; Marcus et al., 2005; Muller et al., 2007; Sakowicz et al., 2004; Sudakin and Yen, 2007; Wood et al., 2001).

While evaluating the monastrol HsEg5 interaction (Chapter 2), we discovered that monastrol binds to bovine serum albumin (BSA) as well as various preparations of human serum albumin (HSA). At a concentration of 0.6 mM, serum albumin is the most abundant protein in human blood plasma and is responsible for solute transport and distribution (via the blood stream) as well as maintenance of blood pH and osmotic pressure (Curry et al., 1998; Sugio et al., 1999). Since HSA is one of the few proteins other than HsEg5 that binds monastrol (Peters et al., 2006), and due to recent interest in the use of monastrol for anti-cancer treatments, the interaction of monastrol and HSA has important implications for medical use. For example, the association between monastrol and HSA might interfere with serum albumin function and cause unintended negative side effects. On the other hand, if the binding of monastrol does not disrupt HSA's function, therein lies the potential to utilize HSA as a natural transport system in order to deliver anti-cancer drugs exclusively to the cancerous organ.

Materials and Methods

Protein Preparation and Reagent Information:

HsEg5 and KLP61F cloning, expression, and purification methods are described in Chapter 2. Racemic ¹⁴C-monastrol (specific activity: 50 mCi/mmol) was synthesized from ethyl acetoacetate, 3-hydroxybenzaldehyde and ¹⁴C-thiourea (American Radiolabeled Chemicals, Inc.) (Kappe, 2000). HPLC analysis and UV-vis spectroscopy were employed to isolate and to confirm the identity of the compound, respectively. Monastrol, as well as bovine and human serum albumins, were purchased from Sigma-Aldrich. Catalogue numbers and details on each serum albumin type are as follows: BSA A7906; HSA, A1653 (96-99%, remainder mostly globulins); HSA, A3782 (~99%, fatty acid free, essentially globulin free); HSA, A8763 (~99%). For convenience, the HSA types are referred to as (1), (2), and (3), respectively throughout the following work.

¹⁴C-monastrol Binding Assays:

HsEg5 and KLP61F binding assays were performed using G25 Sephadex columns as described in Chapter 2. BSA and HSA binding assays were performed with Pierce protein desalting columns equilibrated in HEM buffer (20 mM HEPES, pH 7.2, 1 mM EDTA, and 1 mM MgCl₂. Reactions were set up for each experimental condition, containing 1 mM ¹⁴C-monastrol, indicated protein concentration, and HEM buffer to 100 μ L. After a 5 minute incubation at room temperature, 80 μ L of each reaction was passed through the size exclusion resin via centrifugation at 1500 x g for 4 minutes. The initial reaction and resulting flow-through were analyzed by Bradford protein concentration assay (Bio-Rad) and liquid scintillation counting.

ATPase Assays

Steady-state basal ATPase activities were measured with the pyruvate kinase / lactate dehydrogenase coupled enzyme assay (Deavours et al., 1998; Moore et al., 1996). Assays were carried out at room temperature in 50 mM Tris-acetate, pH 7.4, 2 mM MgCl₂. Reactions contained 5 μ M HsEg5 and monastrol / bovine serum albumin at the indicated concentrations. Control reactions were supplemented with DMSO to match the concentration of DMSO carried

over with monastrol. IC_{50} values were calculated from means for each drug concentration as described (Maliga et al., 2002). Curve fits were done using Prism 4 (GraphPad).

Results and Discussion

As reported in Chapter 2, ¹⁴C-monastrol binding to Kinesin-5 proteins was evaluated by size exclusion chromatography. We attempted to utilize bovine serum albumin (BSA) as a control for these binding assays but surprisingly discovered a significant binding interaction between BSA and ¹⁴C-monastrol (Figure 5.1). In order to further evaluate the interaction between BSA and ¹⁴C-monastrol binding assays with the radiolabeled compound and a range of BSA concentrations were performed. Protein versus ¹⁴C-monastrol, recovered in the flow-through from each reaction, is plotted in Figure 5.2. Our data shows that the radioactivity recovered in each sample directly corresponds with protein concentration indicating that ¹⁴C-monastrol binds BSA in a concentration-dependent manner. Linear regression analysis of our data resulted in a best-fit line with a slope of 0.7, which represents the average binding ratio for ¹⁴C-monastrol:BSA over the range of BSA concentrations in our experiments.

To determine if the presence of BSA altered the amount of monastrol available to inhibit HsEg5, we also performed a series of pyruvate kinase / lactate dehydrogenase coupled enzyme ATPase assays with HsEg5 and monastrol in the presence of BSA. If BSA binds monastrol, the compound will be unable to inhibit HsEg5 as potently in assays with BSA present. With an IC₅₀ of 11 ± 3 μ M, our observed monastrol - HsEg5 inhibition is consistent with previous reports (9 μ M (DeBonis et al., 2003)). Interestingly, in the presence of 50 μ M BSA, monastrol's HsEg5 IC₅₀ increases to 19 ± 8 μ M, suggesting that the BSA present in the assay is binding some of the available monastrol, and as a result more of the compound is required to inhibit HsEg5. It is important to note that while the IC₅₀ only increases from 11 μ M to 19 μ M in the presence of BSA, only 50 μ M BSA was used in experiments, and our BSA binding data (Figure 5.2) utilized BSA concentrations up to 200 μ M (188 μ M BSA was measured in the flow-through of this particular experiment, data not shown) without achieving saturation. More ATPase assays with higher concentrations of BSA would need to be performed in order to visualize a more dramatic effect on the monastrol-inhibited HsEg5 ATPase activity in the presence of BSA.

We next assessed the ability of ¹⁴C-monastrol to bind three different preparations of human serum albumin (HSA). Figure 5.3 shows recovered HSA (nmol) plotted against recovered ¹⁴C-monastrol (nmol). Linear regression of each data set resulted in the following average binding ratios: HSA (1) 0.7, HSA (2) 0.6, and HSA (3) 0.1, suggesting that each HSA preparation binds ¹⁴C-monastrol with a unique affinity. In sum, our data verify that ¹⁴C-monastrol binds each of the HSA preparations tested (to different degrees), in a concentration-dependent manner.

As previously discussed, HSA performs many functions within the bloodstream, including solute transport. HSA utilizes allosteric interactions in order to bind, carry, and even deliver biological and synthetic compounds throughout the human body (Sudlow et al., 1975). The various binding capabilities of HSA have been visualized by crystallography, molecular modeling, biointeraction chromatography, liquid chromatography, displacement studies, NMR, UV, and FT-IR spectroscopy (among others) (Chen and Hage, 2004; Millot et al., 2001; Sarver et al., 2005; Simard et al., 2006; Zsila et al., 2005; Zunszain et al., 2003). HSA possesses no less than 15 allosteric binding sites (two of which have been identified as important drug-binding sites) each with a unique ligand affinity (Bhattacharya et al., 2000b; Curry et al., 1998; Sudlow et al., 1975). Further, the extent of allostery, or structural changes within HSA induced by ligand binding undoubtedly varies depending on individual ligand and particular binding site (Bhattacharya et al., 2000b; Curry et al., 1998; Sudlow et al., 1975). HSA utilizes its ability to bind various ligands at different affinities in order to deliver such ligands to target locations. Delivery of compounds carried by HSA occurs when a second HSA binding solute (such as a fatty acid) binds the complex and through either allosteric interactions or direct competition, permits release of the first compound.

For simplicity, one example binding site / drug interaction will be described here. Reports have shown that warfarin, an anti-coagulant, binds HSA at one of the major drug binding sites, Sudlow's site I, and that drug binding is sensitive to long chain fatty acid concentration (Birkett et al., 1977; Curry et al., 1998; Petitpas et al., 2001; Vorum and Honore, 1996). The presence of up to 4 mol of long chain fatty acid per mol HSA can, through association with HSA and induced structural alterations within Sudlow's site I, increase HSA's warfarin affinity up to 3-fold (Birkett et al., 1977; Curry et al., 1998; Petitpas et al., 2001; Vorum and Honore, 1996). Contrarily, at higher fatty acid concentrations, the amount of warfarin bound to HSA decreases, due to direct competition for binding between the drug and available fatty acid to site I (Birkett et al., 1977; Vorum and Honore, 1996). Thus, drug affinity for binding Sudlow's site I, is directly dependent on the concentration of fatty acid, or other binding solute, via a combination of both allosteric and direct binding site competition. In sum, HSA solute (or drug) delivery is a highly complicated process with multiple levels of regulation. Specifically, an unexpected increase in endogenous ligand (e.g. fatty acid or heme) may stimulate premature drug release and subsequent toxicity (Fasano et al., 2005). This notwithstanding, HSA has been exploited in order to deliver synthetic compounds, such as anti-coagulants, anesthetics, ibuprofen, and HIV medications, to specific organs and may be applicable for monastrol delivery in cancer therapies (An et al., 2004; Bhattacharya et al., 2000a; Bhattacharya et al., 2005; Sudlow et al., 1975; Sugio et al., 1999; Zsila et al., 2005; Zunszain et al., 2003).

Our results confirm that HSA's ligation state designates ligand affinity. The ability of HSA (1) and HSA (2) to bind radiolabeled compound with comparable affinity (0.7 and 0.6, respectively) confirms that the compound targets these HSA preparations with high affinity. However, with an average binding ratio of 0.1, the third HSA preparation demonstrated a significantly lower affinity for monastrol than the other two. Interestingly, this preparation was the only preparation tested not free of fatty acids (see methods), suggesting that fatty acids present in the HSA sample impact, either via direct competition or allostery, monastrol : protein interactions. While the exact concentration of fatty acids in HSA preparation 3 is unknown, it remains possible that if monastrol, similar to warfarin, binds HSA's Sudlow site I, then binding may be sensitive to fatty acid levels.

Additional analyses of the HSA / monastrol binding interaction (and / or binding site) will need to be performed in order to elucidate the compound's binding (and release) mechanism before the compound can be effectively used in anti-cancer therapies. For example, HSA : monastrol interactions may stimulate conformational changes within the protein that interfere with normal blood cell / serum albumin function and cause unelicited side effects. Further problems could arise if HSA binds monastrol irreversibly and as a result prevents the compound's anti-cancer activities. In opposition, if monastrol binds HSA reversibly, the ability of HSA to deliver exogenous compounds may be exploited and, it may be possible to utilize this interaction for delivery of monastrol to cancerous organs.

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Figure 5.1: ¹⁴C-Monastrol Binds BSA.

Binding assays were performed with 1 mg/ml of indicated protein and ¹⁴C-monastrol, either 0.9 mM (KLP61F and HsEg5) or 1 mM (BSA), and were subjected to size exclusion spin chromatography. Recovered monastrol : protein ratios were subsequently determined via liquid scintillation counting or Bradford protein assay, respectively. Average binding ratio \pm SEM are presented with the following n values for each protein: (BSA = 3), (KLP61F = 2), and (HsEg5 = 36).



Figure 5.2: ¹⁴C-Monastrol Binds BSA in a Concentration-Dependent Manner.

The binding of ¹⁴C-monastrol to a range of BSA concentrations (initial range 9 to 188 μ M) was evaluated. Protein (nmol) and ¹⁴C-monastrol (nmol) recovered from each experiment are plotted. Linear regression analysis determined the average ratio of ¹⁴C-monastrol binding to BSA over concentrations tested to be 0.7. Each point represents data from 1 column.



Figure 5.3: ¹⁴C-Monastrol Binds Three Different HSA Preparations.

Binding experiments similar to those presented in Figure 5.2 were performed with three different HSA preparations. HSA (1) data points are represented by black squares, HSA (2) with open triangles, and HSA (3) with grey circles. The initial concentration range is as follows: HSA (1) = 12 to 56 μ M, HSA (2) = 17 μ M to 72, and HSA (3) = 9 to 60 μ M. Linear regression analyses of each resulted in the following average binding ratios for each HSA preparation: HSA (1) 0.7, HSA (2) 0.6, and HSA (3) 0.1. Each data point represents the mean of 2 columns.

Chapter 6: Conclusions and Future Directions

The described work elucidates some specific characteristics of Kinesin-5 proteins that confer (or prevent) inhibition by small molecules, such as monastrol. We have determined the means by which some Kinesin-5 targeting compounds interact with HsEg5, resulting in alterations in the motor's ATPase activity or ability to interact with microtubules (MTs). NSC 622124, for example, through interactions that impact the MT binding site of kinesin motor proteins, may be useful in the future as a probe to further characterize the conserved kinesin MT-binding site. Further, we demonstrate the ability of this compound to inhibit HsEg5 activity by targeting a historically stimulatory-binding site. Additional experiments to probe a potential enhancement of HsEg5 activity may support the existence of an alternate mechanism to control motor function. Characterization of the effects of small molecules on HsEg5 and a monastrol-insensitive Kinesin-5, in the presence and the absence of MTs, showed that certain compounds, BSTLC in particular, affect these proteins differently under different conditions. Specifically, the presence of a natural allosteric regulator, such as MTs, may alter the interaction between Kinesin-5 proteins and small molecules.

We also show that amino acid mutations within the HsEg5 common small moleculebinding site, the L5 loop, can alter the effects such small molecules have on the protein. Further, the ability of ionic strength to control motor protein activity suggests the presence of structural elements, such as a salt bridge, that have not yet been described. In order to confirm the existence of such a salt bridge, site-directed mutagenesis on the amino acids potentially involved (E116 and R221) should be performed.

Lastly, the serendipitous discovery that monastrol binds bovine and human serum albumin in a concentration-dependent manner, may drastically impact medicinal use of this compound and its derivatives. Interactions between monastrol and human serum albumin (HSA) need to be characterized further in order to determine if this interaction will enhance or prevent monastrol's efficacy. For example, the monastrol binding site on HSA may be determined by crystallography, and analyzed in comparison to other compounds' HSA binding site (such as warfarin). If monastrol binds HSA in a manner similar to that of warfarin, it may be possible to exploit the interaction for a possible drug delivery system. The results presented here confirm that HsEg5 is a very complex motor protein with multiple levels of potential regulation. Our data agree with current literature that the L5 loop within the motor domain of HsEg5 serves as the binding site for a multitude of small molecule compounds and also functions in the absence of such compounds in motor ATPase activity. We additionally suggest that a novel electrostatic interaction exists within the L5 loop that facilitates these functions. Furthermore, our data advocates that, while numerous small molecules appear to specifically target HsEg5 and may produce a similar cellular phenotype (formation of a monoastral spindle), each compound is different, and acts on HsEg5 via a unique mechanism.

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