

The Institute of Cancer Research

PHD STUDENTSHIP PROJECT PROPOSAL

PROJECT DETAILS

Project Title:	Pushing the limits of cryo-electron microscopy towards the lower molecular weight end: Investigating inhibitor binding to cancer targets in the 100-200kDa range
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Short Project Title:	Investigating inhibitor binding to cancer targets using cryo-EM
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SUPERVISORY TEAM

Primary Supervisor(s):	Dr Rob van Montfort
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Other supervisory team members:	Dr Fabienne Beuron Dr Catarina Rodrigues Dr Edward Morris
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DIVISIONAL AFFILIATION

Primary Division:	Cancer Therapeutics
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Primary Team:	Hit Discovery and Structural Design
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Other Division (if applicable):	Division of Structural Biology
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Other Team (if applicable):	Structural Electron Microscopy
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PROJECT PROPOSAL

BACKGROUND TO THE PROJECT

Recent developments in single particle cryo-electron microscopy (cryo-EM) are enabling structure determination of proteins and protein complexes at a near-atomic resolution ($\leq 3.5\text{\AA}$) previously only possible using X-ray crystallography. Moreover, cryoEM structures visualising small molecule ligands and inhibitors have been reported suggesting a potential application of cryoEM in structure-based drug discovery. However, the structures reported at this resolution range are typically of high molecular weight ($\geq 200\text{kDa}$) proteins or assemblies. Although many therapeutically relevant cancer targets such as protein kinases, molecular chaperones, histone methyl transferases, etc, have molecular weights considerably lower, they often form oligomers, and or complexes with interaction partners with sizes amenable to cryoEM. Additionally, conformational plasticity, which is a feature of many proteins that often prevents successful crystallisation, may be more readily investigated using cryoEM. Furthermore, the conformation of a protein observed in a crystal structure may not be relevant for productive inhibitor binding, but it may be accessed using the ability of cryoEM to sample different conformations within the same protein sample. Nevertheless, cryoEM is a time-consuming endeavour currently not compatible with the timelines of a structure-based drug discovery programme. Further developments in lowering the resolution and molecular weight boundaries, automation of data analysis and a decrease in structure elucidation time are needed to make routine and timely cryo-EM structure determination feasible as a method for structure-based drug discovery. This project aims to explore the applicability of cryo-EM in drug discovery for targets currently not amenable for crystallographic protein-inhibitor studies, and investigate ways of pushing the current resolution and molecular weight boundaries to enable the use of cryoEM to therapeutically relevant cancer targets. As an example we will study kinesin motor proteins, such as HSET/KIFC1 and others,

because of their relevance as cancers targets and the precedence of kinesin structural studies using cryoEM.

PROJECT AIMS

- Solve the cryoEM structure of full-length HSET, either on its own or in complex with tubulin
- Solve the cryoEM structure of full-length HSET in different nucleotide-bound states
- Investigate the molecular basis of HSET function in relation to its ATPase activity and nucleotide bound states
- Investigate the molecular basis of inhibitor binding to HSET using cryoEM
- Investigate expression systems and tags that could help HSET structure elucidation by cryoEM

RESEARCH PROPOSAL

DNA replication, followed by equal chromosome segregation, ensures the accurate transmission of the genetic information to daughter cells. Centrosome duplication is also tightly controlled and occurs simultaneously with DNA replication, thereby ensuring the generation of two functional centrosomes that form the poles of the mitotic spindle.¹ However, multiple centrosomes have been detected in virtually all human cancers and are implicated in multipolar mitosis, genomic instability and aneuploidy. Cancer cells with centrosome amplification overcome multipolar spindle formation during mitosis through centrosome clustering and formation of a bipolar spindle, allowing them to divide normally.² Therefore disrupting centrosome clustering in centrosome-amplified tumours could provide an attractive cancer therapy. HSET (also known as KIFC1) is a 73.7kDa protein required by tumour cells to cluster multiple centrosomes and belongs to the kinesin-14 family of motor proteins that transport organelles, protein complexes and mRNAs along microtubules in an ATP-dependent fashion.³

Kinesin motor proteins are composed of a catalytic motor domain, located at either the N- or C-terminal ends of the polypeptide chain. This motor domain is composed of ATP- and microtubule-binding subdomains and is connected to a coiled-coil stalk domain *via* a flexible linker region called the neck. In turn, the stalk is connected to a tail region, which interacts with cargo and adaptor proteins. The kinesin subfamily classification is based on the directionality of movement along the microtubules, and its respective domain and oligomeric organisation. Functionally, HSET is a microtubule minus-end directed motor kinesin, involved in sliding and crosslinking microtubules.⁴ Its motor domain is located close the C-terminus making it a C-type kinesin. Most of the kinesin 14 family members are homodimers, however, the functional oligomeric state of human HSET remains to be determined.

Crystal structures and electron microscopy studies are available for several kinesins⁵ with the plus-directed kinesin Eg5 being one of the best structurally characterised mitotic kinesins. A number of inhibitor-bound Eg5 motor-domain structures⁶ revealed two allosteric inhibitor-binding sites distinct from the nucleotide-binding site (Figure 1A), which demonstrated that interfering with the movements of the motor domain subdomains during the catalytic cycle is an effective way of inhibiting kinesin function. However, only one ADP-bound structure of the HSET motor-domain (PDB ID 2REP, Figure 1B) has been reported, in which the neck region, known to be important in translating the chemical energy from ATP-hydrolysis into mechanical movement of the motor domains, is not resolved. Furthermore, although a potent HSET inhibitor, AZ82, has been reported, no information about its exact binding mode is available despite extensive crystallographic experiments by the authors.⁷ The fact that its inhibitory effect can only be measured in an assay containing tubulin suggests that AZ82 binds either to an HSET-tubulin complex or to a conformation of HSET influenced by tubulin binding, both scenario's being difficult to confirm by X-ray crystallography.

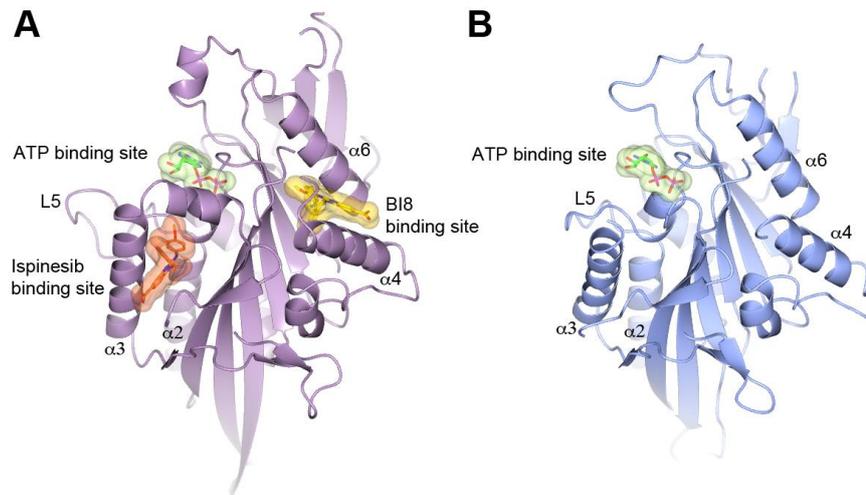


Figure 1 Kinesin small molecule binding sites. A) Structure of Eg5 (purple, PDB ID 3ZCW) bound to ADP (green) and showing the allosteric inhibitor binding sites: ispinesib (orange) and BI8 (yellow). Loop 5 (L5) is labelled in both structures. B) Crystal structure of HSET (blue, PDB ID 2REP) bound to ADP (green).

Most structural studies on kinesins have been carried out on plus-directed kinesins and the minus-directed kinesins are less well understood. Structural characterisation of full-length HSET and its interaction with tubulin would provide valuable insight not only in the mechanical mode of action of HSET, but in the minus-directed kinesins family. In light of recent advances in cryoEM⁸⁻¹⁰ and the literature precedence for electron microscopy studies on kinesins, the first aim of this project is to obtain a near-atomic resolution structure of a full-length HSET oligomer on its own and/or in complex with tubulin, or a tubulin fragment using cryoEM.

The project involves the expression of full-length and truncated versions of HSET and/or its fungal homologue Kar3 in the high quality required for cryo-EM studies. The project will use commercially available tubulin to form kinesin tubulin complexes for structural studies using the state-of-the art electron microscopy equipment the Division of Structural Biology can access. In addition, the project aims to investigate the use of special tags to restrict the orientation of protein samples for EM experiments and thus make smaller proteins more easily visible at higher resolution.

Site-directed mutagenesis and experiments using different nucleotides/nucleosides will be carried out to obtain different functional states of the kinesin motor domains to aid structural studies, and to obtain different structural snapshots of the kinesin catalytic cycle and mechanical states. It is envisaged that the obtained structural data will provide detailed insight in the coupling of ATP-hydrolysis with the mechanical mode of action of HSET and the broader class of minus-directed kinesin motor proteins.

Finally, the project aims to investigate the feasibility of visualising small-molecule binding by solving cryoEM structures of HSET in complex with AZ82 and other tool molecules.

All structural studies will be accompanied by biochemical and biophysical analyses to investigate nucleotide-, tubulin-, and inhibitor-binding to shed further light on HSET's mode of action.

The project builds on the extensive electron microscopy expertise within the team of Dr Ed Morris and the expertise in the production of full-length HSET, truncated HSET variants, and their biochemical,

biophysical, and crystallographic characterisation obtained by the team of DR van Montfort. It is expected that the student working on this project would spend the majority of his/her time in the Structural biology teams of Dr's Rob van Montfort and Ed Morris and work both at the Sutton and Chelsea ICR laboratories.

LITERATURE REFERENCES

- 1) Sharp DJ, Rogers GC, Scholey JM. Microtubule motors in mitosis. *Nature*. 407:41–47, (2000).
- 2) Ganem NJ, Godinho SA and Pellman D. A mechanism linking extra centrosomes to chromosomal instability. *Nature*. 460: 278-282, (2009).
- 3) Kwon M, *et al.* Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. *Genes & Dev*. 22:2189-203, (2008).
- 4) Cai S, *et al.* Proper organization of microtubule minus ends is needed for midzone stability and cytokinesis. *Curr. Biol*. 20:880–885, (2010).
- 5) Wang W, *et al.* Kinesin, 30 years later: Recent insights from structural studies. *Prot. Sci*. 24:1047-1056 (2015).
- 6) Yokoyama H, *et al.* Structural basis of new allosteric inhibition in Kinesin spindle protein Eg5. *ACS Chem. Biol*. 10:1128-1136, (2015).
- 7) Wu J, *et al.* Discovery and Mechanistic Study of a Small Molecule Inhibitor for Motor Protein KIFC1. *ACS Chem. Biol*. 8: 2201-2208, (2013).
- 8) Merk A, *et al.* Breaking Cryo-EM resolution barriers to facilitate drug discovery. *Cell* 165:1698-1707 (2016).
- 9) da Fonseca PCA and Morris EP. Cryo-EM reveals the conformation of a substrate analogue in the human 20S proteasome core. *Nat Commun*. 6, 7573 (2015)
- 10) Morris EP, da Fonseca PCA, High-resolution cryoEM proteasome structures in drug development. *Acta Crystallogr D* 73, 522-533 (2017).

CANDIDATE PROFILE

Note: the ICR's standard minimum entry requirement is a relevant undergraduate Honours degree (First or 2:1)

Pre-requisite qualifications of applicants:	BSc Hons; class 2.1 or above or MSc in Life Sciences
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Intended learning outcomes:

- 1) Expert in cloning and protein biochemistry
- 2) Expert in cryoelectron microscopy
- 3) Knowledge in X-ray crystallography
- 4) Expert in the use of biophysical methods complementary to structural biology (Tm-shift, or ITC or SPR)
- 5) Knowledge of the application of structural biology in structure-function relationships, chemical biology and drug discovery
- 6) Ability to present results in a coherent and analytical fashion both in person and in writing