MASTER'S DISSERTATION TITLE

Biological Characterization of Myeloid-Epithelial-Reproductive Tyrosine Kinase (MERTK) Proteolysis Targeting Chimeras (PROTACs)

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SUMMARY

The advent of TPD has advanced the field of drug discovery, making it possible to degrade heretofore undruggable targets. PROTACs have extensively been applied to various targets including receptor tyrosine kinases. MERTK, a receptor tyrosine kinase has gained attention due to its central role in various pathologies and resistance mechanisms.

We sought to degrade this target with PROTACs hence this study aimed to characterize and understand their behavior at the molecular level. To achieve this, we combined *in-vitro* and *in-cellulo* assays. We developed proximity-based reporter assays based on the BRET system to monitor key events induced by these PROTACs in the cell.

We observed good penetration through the cell membrane and potent binding of compounds to MERTK, both *in-vitro* and inside cells. The PROTACs equally displayed strong binding to CRBN. Further, the PROTACs induced stable ternary complexes between MERTK and CRBN which had a strong positive correlation with the observed binding to MERTK. Ultimately, the PROTACs degraded MERTK in a concentration-dependent manner.

These detailed findings will provide insight for further optimizations and the development of degraders for MERTK. The established set of assays will allow a consistent, reproducible and efficient approach to the characterization of degraders.

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LIST OF ABBREVIATIONS

ALPHA: Amplified luminescent proximity homogeneous assay

ALS: Autophagy-lysosomal system

ATP: Adenosine triphosphate

ATTEC: Autophagosome tethering compound

AUTAC: Autophagy-targeting chimaera

AUTOTAC: AUTOphagy-TArgeting Chimera

AXL: AXL receptor kinase

BCA: Bicinchoninic acid

BLI: Bio-layer interferometry

BP: Band pass

BRET: Bioluminescent resonance energy transfer

BSA: Bovine serum albumin

CAPA: Chloroalkane penetration assay

CEIA: Capillary electrophoresis immunoassay

CETSA: Cellular thermal shift assay

CMA: Chaperone-mediated autophagy

CO-IP: Co-immunoprecipitation

CRBN: Cereblon

CTG: CellTiter-Glo®

DMEM: Dulbecco’s modified eagle medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's Phosphate-Buffered Saline

DSF: Differential scanning calorimetry

ECL: Enhanced chemiluminescence

EC_{50}: Half maximal effective concentration

EDTA: Ethylenediaminetetraacetic acid

FBS: Fetal bovine serum

FDA: Food and Drugs Administration

FLT: FMS-like tyrosine kinase

FP: Fluorescence polarization

FRET: Fluorescence energy transfer

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HDAC: Histone deacetylase

HECT: Homologous to the E6AP carboxyl terminus

HRP: Horseradish peroxidase

IAP: Inhibitor of apoptosis

ITC: Isothermal titration calorimetry

$K_{D:}$ Dissociation constant

LC-MS: Liquid chromatography-mass spectrometry

MERTK: Myeloid-epithelial-reproductive tyrosine kinase

MG: Molecular glue

MS: Mass spectrometry

MTS: Tetrazolium-based 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide

NLUC: NanoLuc luciferase

nM: Nanomolar

NMR: Nuclear magnetic resonance

PAMPA: Parallel artificial membrane permeability assay

PEG: Polyethylene glycol

pH: Potential of hydrogen

pM: Picomolar

POI: Protein of interest

PVDF: Polyvinylidene difluoride

RBR: RING-betweenRING-RING

RING: Really interesting new gene

RP: Retinitis Pigmentosa

RPM: Revolutions per minute

RTK: Receptor tyrosine kinase

SAR: Structure-activity relationship

SD: Standard deviation

SDS–PAGE: Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SEC: Size-exclusion chromatography

SPR: Surface plasmon resonance

TAM: TYRO3, AXL and MERTK

TBST: Tris-buffered saline with 0.1% Tween® 20 Detergent

T_M: Melting temperature

TPD: Targeted protein degradation

TR-FRET: Time-resolved fluorescence energy transfer

TUBE: Tandem ubiquitin-binding entities

TYRO3: TYRO3 tyrosine-protein kinase receptor

UPS: Ubiquitin- proteasomal system

VHL: Von Hippel-Lindau

XIAP: X-linked inhibitor of apoptosis protein

µM: Micromolar

°C: Degree Celsius

1. INTRODUCTION

1.1. TARGETED PROTEIN DEGRADATION

Targeted protein degradation (TPD) has emerged as an important field in drug discovery, allowing the degradation of proteins via the body's degradation machinery. This fast-evolving field employs small molecules to open the possibility of modulating the so-called undruggable part of the human proteome (1,2). This is especially important as amidst the advances in drug discovery campaigns, over 80% of proteins encoded by the human genome have been considered undruggable(3–6). These undruggable targets have proven difficult to modulate by traditional inhibitory mechanisms. Some have undefined binding pockets, flat and broad interfaces, shallow pockets, and few accommodating sites while others possess non-catalytic functions(1,7–9). These make the need for degradation strategies apparent.

Protein hemostasis is an important quality control mechanism relevant to the survival of organisms. In humans, the proteasomes and lysosomes ensure that damaged, malformed, or excess proteins are appropriately degraded to maintain a healthy proteome(10–13). TPD strategies have evolved to hijack these systems for the selective destruction of proteins, offering a refreshing perspective on drug discovery. In this context, the ubiquitin-proteasomal system (UPS) has been well explored over the autophagy-lysosomal system (ALS) to destroy proteins of interest (POI).

The UPS has been co-opted to drug intracellular and cell membrane proteins (14). The proteolysis targeting chimera (PROTAC) has been the most utilized strategy in TPD and has yielded with much success(15). These bivalent molecules have successfully targeted multiple proteins across many families. Molecular glues (MG) are another modality where small molecules also recruit the UPS for the destruction of POIs. They engage and change the recognition site of E3 ligases to induce degradation of the POI(2,14,16).

Extracellular proteins, which make up about 40% of the human proteome are important in many diseases(17), yet unreachable by the UPS. This has however been

salvaged by other TPD technologies through the ALS like the lysosome-targeting chimaeras (LYTACs)(18). LYTACs, just like PROTACs are bivalent molecules(16). Macroautophagy degradation targeting chimaera (MADTACs) have also emerged to destroy proteins, organelles, and pathogens via tendering them to autophagosomes (14). Modalities under this technology include Autophagy-targeting chimaera (AUTAC)(19), autophagosome tethering compound (ATTEC)(20) and more recently AUTOphagy-TArgeting Chimera (AUTOTAC)(21). In addition to the above, other bivalent technologies like Antibody-based PROTAC (AbTAC)(22), GlueTAC(23) and chaperone-mediated autophagy (CMA) based degraders (2,16,24) are rapidly emerging.

1.2. PROTEOLYSIS TARGETING CHIMAERAS (PROTACS)

The PROTAC technology has been a bedrock of TPD with over 1600 reported degraders acting on more than 100 targets from 60 different unique proteins(25,26). PROTACs evolved as important chemical biology tools and promising therapeutic modalities that are currently being tested in numerous clinical trials(27,28). Currently, PROTACs are directed towards cancers (29,30), infectious diseases(31), inflammatory and immune disorders(32) and neurodegenerative diseases(33)

PROTACs are heterobifunctional compounds which are made of 3 components: a ligand that recruits the E3 ligase, another ligand that binds to the POI and a linker connecting both ligands (34,35). Gradually over the last 2 decades, this technology has evolved and is broadly categorized into 3 groups based on the nature of their ligands. First an all-small molecule ligand PROTAC, an all-peptide-based ligand bioPROTAC and then a hybrid PROTAC containing both a small molecule and peptide(1). Based on this definition, the first PROTAC reported is a hybrid PROTAC. It contained a small molecule to target methionine aminopeptidase 2(MetAP-2) and phosphopeptide to recruit the E3 ligase complex F-box protein “β-transducin repeat-containing protein” ($SCF^{\beta TRCP}$)(36). The first all-small molecule PROTAC was reported seven years afterwards. This utilized nutlin-3a as the ligand for mouse double minute 2 homologue (MDM2) E3 ligase and flutamide derivative warhead for androgen receptor(37).

The mechanism of PROTACs is based on the process of polyubiquitinating substrates for eventual destruction via the proteosomes through a well-regulated cascade of events. This involves E1 activating ligase, E2 conjugating ligase and E3 ubiquitin ligases. Once inside the cell, the molecule first forms a binary complex with either the POI or E3 ligase. Afterwards, this complex forms a ternary complex, bringing the POI to the proximity of E3 ligase for polyubiquitination (34). The E1 ligase, forms a thioester bond with ubiquitin in an ATP-dependent manner. This is the beginning of the process to tag ubiquitin, an 8.6kDa to POIs post-translationally. Then through the process of trans-thioesterification, the activated ubiquitin is transferred to E2 ligase. Following this, tagging the POI with ubiquitin through a covalent amide bond is mediated by the E3 ligase, either directly or indirectly. The amide bond is formed on the C-terminal glycine of ubiquitin and a lysine residue of POI. Owing to the seven lysine residues of ubiquitin, multiple rounds of ubiquitination can be performed by the E3 ligase, resulting in a polyubiquitin chain on the POI for eventual destruction(14,28,38–40). Degradation is mainly by the 26S proteasome and to a lesser extent, the 20S proteasome (41,42). This mechanism is illustrated in Figure 1.1 below.

Unlike occupancy-driven small molecules, PROTACs act as catalysts that catalyze the transfer of ubiquitin to the POI (sometimes referred also as neo-substrate of E3 ligases)(28,43–45). Owing to this, PROTACs do not necessarily require high-affinity warheads(46) nor full occupancy of E3 ligase(47) for activity. In addition, targets with, feedback mechanisms or non-enzymatic functions like scaffold proteins can be drugged through complete degradation(43,48,49). Complete degradation of POIs reduces the overall levels of the malicious proteins, contributing to a more sustainable course of tackling the root cause of diseases(50). They also display sustained effects, even after washout or in the case of mutations over inhibitors, hence reducing the incidence of drug resistance (48). Excitingly, this technology allows us to alter and improve the selectivity of promiscuous warheads(34,49,51–53). Similarly, they permit interrogation of protein function through temporary control of their levels in a short time as compared to other techniques like gene editing(28,53). It has been shown that translation to in vivo experiments, both primate and non-primate specifies require no genetic modification, hence facilitating the drug discovery process(28,54–56).

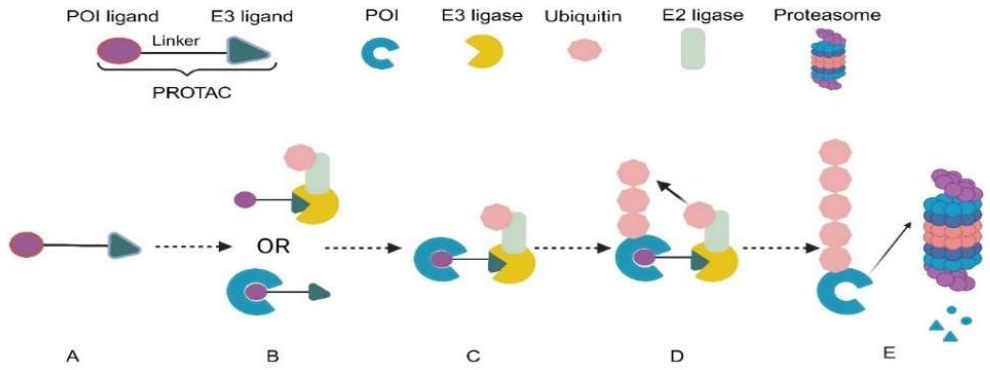

Figure 1.1: Schematic representation of PROTAC degraders. The PROTAC molecule (A) upon entering the cytosol forms a binary complex (B), then a ternary complex (C) before the POI is polyubiquitinated (D) leading to eventual proteasomal-mediated degradation (E). *The illustration was created with BioRender.com*

This technology, amidst its exciting advantages, is not without challenges. Their bulky nature presents with physiochemical and pharmacokinetic concerns hence deviating from properties that make them druglike for oral administration (57). Regardless of this profound challenge, orally available PROTACs have been reported(58). Also, the complex nature of PROTACs make them resource-intensive relative to traditional inhibitors. It requires multiple assays, varied techniques and different linkers (28,34,59). Ultimately, these strenuous processes affect time from the bench to the bedside, resulting in a negative impact on sustainability(50). Also, considering that complete degradation of unintended targets takes time to restore depending on the turnover rate, important biological functions could be compromised as a result (28,60). In addition, conventional pharmacological assays provide little clarity on these multi-step processes and mechanistic activity of PROTACs and off-target effects(60,61).

1.2.1. Components of PROTACs

1.2.1.1. E3 Ligases and Ligands

E3 ligases abound, with over 600 different ligases encoded in the human genome as compared to 2 for E1 and 30 for E2 ligases(62,63). E3 ligases can be categorized into the really interesting new gene (RING) and the homologous to the E6AP carboxyl terminus (HECT) classes. Notably, RING ligases, which make the bulk of the human E3 ligases (95%) have U-box fold catalytic domain which enhances direct transfer of ubiquitin to the POI. On the contrary, the 28-membered HECT ligases, by a conserved cysteine residue, form a thioester intermediate with ubiquitin before transfer to the POI (62–65).

Based on their specific structure and function, E3 ligases can be categorized into 4 groups: the HECT type, RING-finger type, U-box type and RING-betweenRING-RING (RBR) type(66). Also, based on characteristic domains and ubiquitin transfer mechanism, however, they can be categorized into the HECT, RING and RBR E3, the latter having about 12 members(67). Amidst these numerous E3 ligases in the human body, only a handful have been successfully employed in PROTAC design, which when expanded would be a key enabler for the technology to drug many other targets(68).

Another area of interest is the expression of specific E3 ubiquitin ligases in the body. This is because the presence of these ligases in specific tissues is relevant to the functioning of the PROTAC degrader in that tissue. The proteomic data of 81 different tissues indicated a wide spread of E3 ligases, 24 of which are in a minimum of 90% of the tissues. Interestingly about half of these widespread E3 ligases can be co-opted in TPD. Significant variations were however observed in some currently employed E3 ligases as almost all the tissues expressed MDM2 in higher detectable amounts (98.8%). Meanwhile CRBN (CRBN) had detectable levels of 76.5% and inhibitor of apoptosis (IAP) proteins BIRC2 and XIAP had levels below 50% (68,69).

Amidst the 1% of the E3 ligases employed in PROTACs, the CRBN and Von Hippel-Lindau (VHL) are mainly utilized. This is greatly motivated by the availability of drug-like small-molecule ligands for these proteins(28). Over 80 different ligands have

been used to recruit these E3 ligases according to the latest online database, PROTAC-DB 2.0 (70). The CRBN recruiting ligands, the so-called Immunomodulatory imide drugs (IMiDs), have been well accepted following the first CRBN-based degrader (71). These ligands are thalidomide and its analogues. Meanwhile, efforts to design VHL ligands were initiated around a decade ago (72)and yielded the first all small molecule VHL-based PROTAC(35,73). There is no clear advantage of one over the other currently. However, reports from kinase targeting PROTACs(52), histone deacetylase (HDAC) PROTACs(74) and homo-PROTACs(75) suggest the VHL-based PROTACs seem to have a slight edge over CRBN. Also, in some specific instances, the side effect of CRBN ligands hinders their function (76).

Before these ligases, $SCF^{\beta TRCP}$ was first recruited by phosphopeptide yet its dependency on phosphorylation was limiting (35,36). Later the first all small molecule PROTAC recruited MDM2 with nutlin compound (37) and has in recent times resurfaced for PROTAC discovery(77). In addition, methyl bestatin ligands were employed in the report of the first cellular inhibitor of apoptosis protein 1 (cIAP1)/ BIRC2 PROTAC(78). These ligases however seem less preferred due to their auto destruction and inhibition mechanisms(28). Also, aryl hydrocarbon receptor (AhR) (79), Kelch-like ECH-associated protein 1(KEAP1) (80), DDB1- and CUL4-associated factor 15 (DCAF15) (81) have been co-opted through previously reported ligands(25). Meanwhile small molecule ligands for DCAF16(47), DCAF11(82), RING finger protein 4 (RNF4)(83), RFN114(84) and Fem-1 Homolog B(FEM1B)(85) have also been discovered (25,86).

1.2.1.2. Linkers

The choice of a linker can substantially influence the physicochemical properties impacting degradation efficiency, membrane permeability, solubility and pharmacokinetic properties(1,30,87,88). This is also true for the linker attachment point (43). It has been demonstrated that PROTACs with the same ligands for E3 ligase and POIs could have different selectivity profiles. This relies on the linker attachment point and composition as it influences the plastic conformation of the POI and E3 ligase(89). This insight is consistent with other studies(90). Also, changes in linker length for the same ligands exhibited different degradation abilities (43,48,52,74,90).

An important decision is the length of the PROTACs as too short linkers may lead to steric clashes preventing ternary complex formation. In contrast, longer ones may affect the induced proximity, hence the current approach is to vary the length from the shortest to the longest possible(91). While shorter linkers are generally recommended (89), lessons from promiscuous ligands reveal different PROTACs work better with different linker lengths (46,52,74). This implies that decisions should be on a case-by-case basis.

Currently, most linkers employed in PROTAC design are flexible linkers. These allow more degrees of freedom to maximize the number of low-energy conformations in solution. It also aids the molecules in exhibiting hydrophobic collapse, a phenomenon called chameleonicity. This ability to fold reduces the polar surface area by forming intramolecular hydrogen bonds, hence masking its available hydrogen bond donors and acceptors to increase cell permeability (88,92–94).

Most of the flexible linker motifs employed in PROTACs are made of polyethylene glycol (PEG) and alkyl chains. In a study involving over 400 degraders, PEG was about 55% and 30%, made of alkyl chains (30,95). Even though flexible linkers are the starting point for linker design, their clinical application is limited. To put this in perspective, only one amongst the PROTACs in clinical trials has a flexible linker(96), solidifying the role of rigid linkers. Indeed, insights from linker studies show that the rigidity of linkers is essential in the potency of the molecule, hence a positive association between rigid linkers and degradation. This is evident from the short and rigid linkers amongst PROTACs in clinical trials (38,92). The more favourable properties of rigid linkers in the clinic could be attributed to their enhanced water solubility, permeability, pharmacokinetic properties and chemical stability towards metabolic enzymes (88,93,94). A current approach is to establish the optimal linker length with flexible linkers, and then replace it with rigid linkers of the same length (92,97). (Hetero)cycles, alkynes and spirocycles are common rigid linkers reported in the literature(92). In addition to these broad classes, clickable and photoswitchable linkers are also being employed in the technology(30,92).

1.2.1.3. Ligands for POI

The ligands that bind to the POI as part of the PROTAC mechanism are mainly responsible for the selectivity of the molecule (52,74). Many existing small molecule ligands hold the potential to be utilized for PROTAC development, targeting proteins from various classes for degradation. Excitingly, pharmacologically ineffective ligands developed in the past can be used for PROTAC development to induce the degradation of their binding partners(98). This is an exciting observation to rescue drugs that fail in clinical trials due to lack of efficacy. Thus far, ligands from a wide range of target families have been developed. Notably, PROTACs currently in clinical trials have ligands that target mostly nuclear receptors and kinases(99). This provides insight into the susceptibility of kinases to the PROTAC modality. In this study, PROTACs with ligands directed against MERTK are of interest, to characterize their degradation pathway.

1.3. PROTEIN KINASES

Protein Kinases are part of the four privileged families of targets in drug discovery. They account for 44% of all disease targets and are responsible for 70% of all approved small molecules(100). About 2% of the entire human genes code for the over 500 kinases in the body. These are involved in phosphorylation, a basic mechanism for key cellular processes like cell growth, metabolism, and cell cycle regulation (101–103). However, mutations and misfunctioning of these proteins are responsible for numerous diseases including cancers, making them attractive targets to the drug discovery community. Protein kinases can be categorized based on the amino acid residue they phosphorylate. These amino acids are heterogenous with the majority on serine (85%), followed by threonine (11.8%), and tyrosine (1.8%) residues (104–106). In addition, there are kinases which phosphorylate both serine/threonine and tyrosine, the dual specific kinases(107).

Fast forward, there are 72 FDA-approved small molecule kinase inhibitors (at the end of 2022) and around 200 in various phases of clinical trials(108–111). These inhibitors are broadly classified as reversible or irreversible, with the latter binding covalently to the active site(112). Despite this progress, kinase inhibitors are faced with challenges, justifying the need for other technologies. A major challenge has been the

low selectivity of inhibitors resulting in undesired off-target effects and toxicity(113,114). On the contrary, PROTACs have demonstrated enhanced selectivity amongst kinases(52). Another significant obstacle is the phenomenon of kinome re-wiring, where inhibition leads to compensatory reprogramming of the pathways, leading to resistance(115,116). The strategy of using PROTAC degraders has the potential to destroy the POI, hence preventing resistance mechanisms faced by kinase inhibitors(48).

1.3.1. MER Tyrosine Kinase

Myeloid-epithelial-reproductive tyrosine kinase (MERTK) is a member of the TYRO3/AXL/MER (TAM) family of receptors hence relevant targets considering their multiple roles in disease pathology. These transmembrane kinases have been involved in multiple haematological and solid malignancies, given their presence in immune cells and contributions to compromising host anti-tumour immunity (117,118). Aside from the primary malignancies, these kinases have been reported to contribute to metastasis and chemoresistance of existing agents(119). Amidst their widely observed expression in multiple malignancies, they are non-essential kinases with predicted reduced toxicity hence a favorable therapeutic index(118).

Several small molecules and antibodies at various phases of drug discovery have been reported in the literature to target these agents. A clear advantage of the antibodies has been their selectivity for MERTK. Indeed, most small molecules act on other TAM and related kinases due to the conserved domains and structural similarities. Hence, the small molecule inhibitors in the clinic and clinical trials are not MERTK selective (119,120). Regardless, attempts at selectivity for MERTK are being made with several chemical modifications(120–124),

Despite this impressive progress in modulating MERTK, there have been cases of successfully blocking MERTK immune functions but not the kinase activities. This implies that traditional inhibitors may not sufficiently interrupt all the oncogenic functions, hence the role of degraders becomes apparent(119). By binding to the intracellular domain of the kinase, PROTACs reduce the levels of intact receptors by

tagging them for proteasomal degradation. Several MERTK inhibitors could serve as a basis for PROTAC development (125). Until now, there have been only two reports of MERTK targeting PROTACs with only one selective for the TAM family(46,126).

This study seeks to explore the degradation pathway and characterize selective MERTK PROTACs. This series of PROTACs is based on demonstrated highly selective inhibitors of MERTK(120). This is of great relevance to the drug discovery community considering the relevance of the target in diseases. Also, the PROTAC technology seeks to overcome current challenges with MERTK inhibitors as mentioned earlier. Excitingly, little has been reported on MERTK PROTACs hence this work promises to provide insightful novel knowledge on this target.

1.4. CHARACTERIZATION OF PROTACS

The PROTAC technology as a new modality requires accurate and insightful characterization of the degraders, often combining multiple complementary biochemical and biophysical technologies. This step of characterization is essential in providing scientists with relevant information about the molecule, and aid in the lead optimization step in drug discovery. Indeed, it is recommended that each step of the PROTAC mechanism, from cell penetration to target degradation be assessed. These include but are not limited to; i. Affinity and cell permeability ii. Binary complex formation iii. Ternary complex formation iv. Ubiquitination v. Target degradation and vi. Phenotypic manifestation of the degradation, as illustrated in Figure 1.2 (127,128).

1.4.1. Cell penetration

The ability of the PROTAC to enter the cytosol is crucial to its function considering the UPS is located inside the cell. Seemingly, these bulky molecules would present some concerning challenges to cell penetration and other properties, yet they seem to act astonishingly normal in some cases(129). Regardless, these beyond the “Rule of 5” (bRo5) compounds are threatened by poor cell permeability(130). In the case of PROTACs however, it has been demonstrated that satisfactory degradation is achievable albeit the compromised permeability (131). Amidst all these interesting findings, deliberate efforts are being implemented to improve the cell permeability of

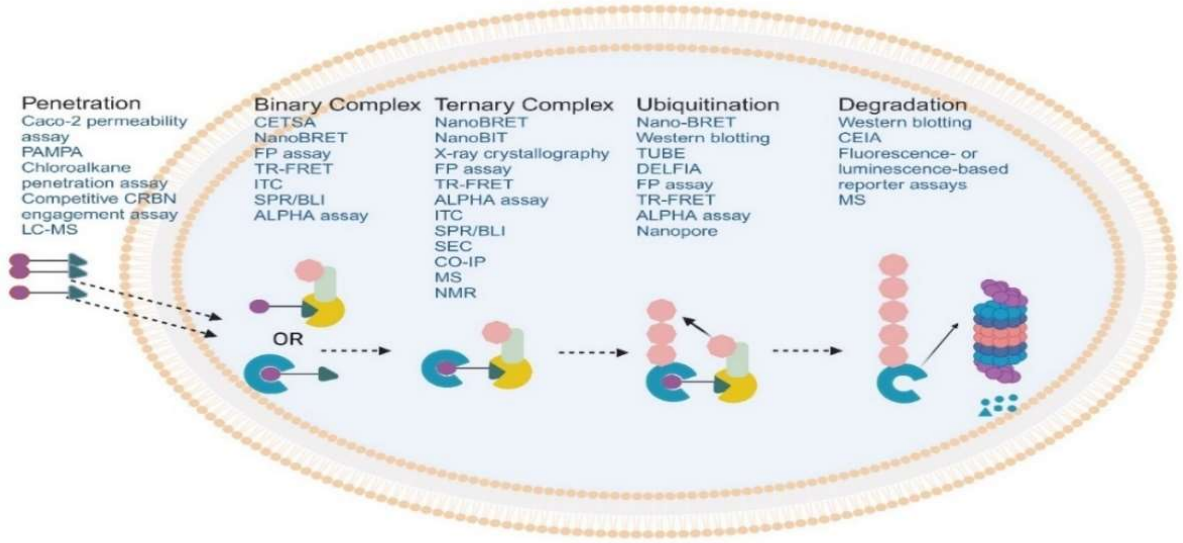

Figure 1.2: Schematic illustration of various technologies and assays for PROTAC characterization along the degradation pathway. *The illustration was created with BioRender.com and adapted from Liu et al. Future Medicinal Chemistry (2020)*(128).

PROTACs. For instance, the reduction of amides(132) and amide to ester substitution(133) successfully improved cell permeability. In addition to chemical modifications, other strategies like nanocarriers, prodrugs and cell-penetrating peptides are being explored to enhance the permeability of PROTACs(134).

Equally important are the techniques to determine this property of the molecule. The Caco-2 permeability assay, a well-accepted assay in *in-vitro* drug investigation has been largely employed in studying PROTACs. The use of the human colon epithelia cancer cell line under the appropriate conditions stimulates the enterocyte lining of the small intestine for these studies(135). Hence are relevant to approximate permeability, especially involving mechanisms like active transport, paracellular permeation and passive transmembrane diffusion, applicable to PROTACs(134,136,137). To this end, this assay has been well employed in the field of

PROTAC characterization for its reliability, simplicity, and translatability in in-vivo models (138–140).

Parallel artificial membrane permeability assay (PAMPA) is another technique employed in such studies offering cheap, quick and high throughput permeability information of drug molecules(134). This bio-mimetic method has a flaw of being only applicable to passive permeation methods and is influenced by factors like pH(137). This makes it less preferred compared to Caco-2 method(141). A more effective approach however is the combination of this method with others. For instance, by combining PAMPA and lipophilic permeability efficiency, insights into PROTAC permeability and its relation with structural changes were obtained by Klein et al (131).

The chloroalkane penetration assay (CAPA), a Halotag-based assay has recently been employed to offer high throughput quantitative information on drug permeation. In addition, it provides information on cytosolic localization(142). In the pioneering work of employing CAPA in PROTAC permeation studies, labelled molecules based on their permeability were categorized quantitatively. Also, a structure-permeability relationship was obtained from the PROTACs and individual components(143). A limitation however is its tagged nature, hence PROTACs without a good derivatization site may be difficult to characterize(128). Also, the influence of the chloroalkene tag on permeability may be different from the parent PROTAC, and in instances of tag cleavage, results would be influenced(128,137). Other techniques like liquid chromatography-mass spectrometry (LC-MS) (128) and competitive CRBN engagement assay(144) have been employed in PROTAC permeation.

1.4.2. Complex formation

The first step after entering the cytosol is the formation of a binary complex with either the POI or E3 ligase and then ternary with the other component. The formation, stability and co-operativity of the ternary complex is of much relevance to its activity, relative to the binary complex formation(46,61,145). The cellular thermal shift assay (CETSA), a type of differential scanning calorimetry (DSF) helps monitor interactions between ligands and proteins. This is done by heating to denature, cell lysis and ligand-

bound protein detection. DSF techniques have the potential to help determine non-functional PROTAC binding(146,147). This thus has successfully been employed to monitor POI:PROTAC and E3 ligase:PROTAC interactions (148,149)

Some other techniques to characterize binary complex formation apply to ternary complex formation. This trimer can be studied either in live cells, lysed cells or through structural determination (150). The Nanobioluminescent resonance energy transfer (NanoBRET) technique, amongst other uses, has been applied to studying ternary complex formation in PROTACs. It is based on the principle of fluorescence transfer between a donor and acceptor fluorophore, based on their proximity(128,150). This technology has been a reliable ternary complex characterization assay considering the reliability of live cell results to the others(61,151–153). To its advantage, NanoBRET can be applied to characterizing every stage of the degradation pathway. In addition, is the NLuc Binary Technology (NanoBiT®), which essentially tags the POI and E3 ligase with NanoBIT subunits to detect the interaction between them(148). This has successfully been applied in various PROTAC characterization studies(154–156). Further, various imaging techniques have been applied to ternary complex formation, and though uncommon, are expected to proliferate in the coming years (157–159). Structural techniques, most commonly X-ray crystallography give well-resolved information on the complex formation including the bind poses and have been applied in studying PROTACS (61,138,145,160,161).

The time-resolved fluorescence energy transfer (TR-FRET) and Amplified luminescent proximity homogeneous assay (ALPHA), two similar proximity-based assays are applicable in this field. They measure energy transfer between donor and acceptor to yield insight into ternary complex formation and the concentration of binding(128,148). Other *in-vitro* techniques like Fluorescence polarization (FP) have been applied to study ternary complexes(138,161).

Isothermal titration calorimetry (ITC) is a direct and label-free assay to study important binding parameters like the thermodynamics of ternary complexes. Albeit a low throughput technique, it can be employed to guide the design of more effective

PROTAC, by forming stable ternary complexes for degradation(138,162). Surface plasmon resonance (SPR) and bio-layer interferometry (BLI) have also been employed to characterize the aspects of the affinity, kinetics and thermodynamics of PROTAC ternary complexes(163–165). Further, other complementary techniques like size-exclusion chromatography (SEC)(166,167), co-immunoprecipitation (CO-IP)(168,169), MS (170,171) and nuclear magnetic resonance (NMR) (172,173) have been useful in ternary complex studies.

1.4.3. Ubiquitination

This post-translational modification step is a necessity for effective target degradation and hence warrants characterization for better insight into the function of the molecule. Available assays permit the studies in both live and lysed cells with the former more desired as it uses native cell machinery. Nano-BRET assay has also been applied to these studies in live cells(61,153). Also, the tandem ubiquitin-binding entities (TUBE) technology allows ubiquitination study in live cells. This ubiquitin-binding peptide-based technology allows the characterization of the ubiquitin chain, even at very low levels and with high throughput, hence superior to other methods. TUBE offers chain selectivity and has successfully been used to characterize PROTAC-induced ubiquitination and in addition, establish the potency of PROTACs in rank order(174,175).

Further, the use of ubiquitin-specific antibodies in western blotting analysis following immunoprecipitation (IP) of the POI offers a simple and more direct approach to characterizing ubiquitination. In addition, the dissociation-enhanced lanthanide fluoroimmunoassay and enzyme-linked immunosorbent assay add a more quantitative perspective to the detection(176). MS amongst its many functions has been employed not only to profile protein ubiquitination but also to give information on the ubiquitination type and size(177–180).

For this purpose of characterizing ubiquitination, FP, ALPHA and TR-FRET assays have been employed also (128). Excitingly, an engineered Cytolisin A nanopore technology offers a fast and label-free approach to studying protein ubiquitination. An

interesting addition to existing assays is the real-time study of the ubiquitination process this technology offers. Also, it gives insight into the endpoint and ability to differentiate between mono and polyubiquitinated proteins(181).

1.4.4. Target degradation

Proteasomal degradation of POI is the desired endpoint of the cascade, yet this is not always certain nor predictable. It is of great relevance then to characterize the degradation behaviour of the molecule and how this manifests phenotypically. Western blotting has emerged as a widely applied assay to detect POI degradation after PROTAC application. This simple and easy assay, however, relies heavily on the quality and specificity of antibodies to yield accurate results. In addition, it is semi-quantitative, has low sensitivity and is unable to help account for degradation failures(128,137,148).

A similar yet much less time-consuming method of capillary electrophoresis immunoassay (CEIA) has also been used in such characterizations(170,182). Also, fluorescence- or luminescence-based reporter assays have been fast and reliable for detecting PROTAC-induced degradation of POIs. These however suffer the challenge of background interference which may have a bearing on sensitivity and dynamic range, (144,153,162). Proteomic studies with MS also offer a tag and antibody-free alternative for studying protein degradation and are especially suitable for probing the selectivity across the proteome (46,138,170,180,183).

Considering the exhaustive investment in designing degraders, it is especially important to assess the phenotypic outcome of the degradation. In the cases of POIs with known inhibitors, it guides the decision as to whether degradation ultimately has superiority over inhibition(148). For this purpose, the HaloPROTAC system has been designed to degrade the HaloTAG7 fusion protein by using VHL E3 ligase. This is a way of characterizing the phenotype of degradation with the help of antibodies(184,185). Another system, the degradation tag (dTAG) technology degrades the mutant form of $FKBP12^{F36V}$ to offer a fast study on the phenotypic manifestation of POI degradation (54). This can be merged with fluorescence- or luminescence-based assays for phenotypic characterization in live cells, which is more desirable (148,186).

1.4.5. Cytotoxicity

Cytotoxicity studies can be employed to study possible damage caused by the PROTAC to the cell. It is desired that PROTACs selectively degrade the desired target while sparing the cells. The cell viability assays, amongst the many cytotoxicity assays, have been used in studying PROTACs. These include the CellTiter-Glo® (CTG) Luminescent Cell Viability Assay (187), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) (188) and tetrazolium-based 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays(189). These studies demonstrated desirable discrimination of PROTACs between the cancer cells and normal cells studied (187,188). The CTG assay is based on adenosine triphosphate (ATP) measurement as an indication of cell viability. This is the fastest and most sensitive approach to test the viability of cells(190).

2. OBJECTIVES

To develop a potent and selective degrader of MERTK, we synthesized 9 novel PROTACs, bearing thalidomide derivatives as CRBN recruiting ligand and a structural motif of UNC5293 as the warhead recruiting MERTK. The synthesized PROTACs varied in length and chemical composition of linkers connecting both ligands. To understand the processes and actions at the molecular level, there was a need to characterize them. The main objective of this thesis was to characterize these PROTACs using a set of *in-vitro* and *in-cellulo* assays. Notably, some of these cell-based assays are based on contemporary NLuc/HaloTag-based technologies.

To realize this, we performed a preliminary *in-vitro* screening using DSF to evaluate binary complex formation. Also, to ensure the compounds have a safe cytotoxicity profile a cell viability study was conducted. In addition, we aimed to develop and establish *in-cellulo* assays to account for the interactions and dynamics of the PROTACs in live cells. The developed NLuc/HaloTag-based assays were used to probe the formation of binary complexes between PROTAC and CRBN or MERTK, ternary complex and ubiquitination of MERTK. To evaluate the degradation efficiency of the PROTACs, we used conventional western blot analysis to monitor the depletion of endogenous MERTK.

3. MATERIALS AND METHODS

3.1 DIFFERENTIAL SCANNING CALORIMETRY (DSF)

Purified MERTK kinase domain (5 μM) was mixed with SYPRO Orange fluorescent dye at 1000-fold dilution. A 19.5 μL protein-dye mixture was added into each well in a defined order. 0.5 μL of compounds were added to the wells. The plate was sealed and spanned at 1000 rpm for 2 minutes with a Labnet Mini plate spinner. Filters for excitation and emission of SYPRO Orange fluorescent dye were set to 465 and 590 nm, respectively. The temperature was increased from 25°C to a final temperature of 99°C with 1°C/min. Data was collected and processed using the MxPro program (Agilent Technologies). Samples were measured in technical triplicates.

3.2 BICINCHONINIC ACID (BCA) ASSAY

Standard solutions of the BCA assay were prepared using the bovine serum albumin (BSA) provided by the manufacturer (Thermo Fisher Scientific) by diluting them into 8 concentrations from 2000 μg/mL to 25μg/mL and a blank solution. 25 μL of standards and blank together with the same volume of samples were pipetted into a 96-well plate in duplicates.

Pierce™ BCA Protein Assay Kit (Thermo Fisher # 23225) working solutions were made by adding 50 parts of BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) to 1 part of BCA reagent B (CuSO4, 4%), as per manufacturer instructions. A volume of 200 μl of the working solution was quickly added to the standards and samples to achieve an 8:1 ratio. This was gently mixed on a plate shaker for a minute. The plate was covered and incubated for 30 minutes at 37°C and 5% CO_2. This was then equilibrated at room temperature before measurement, and absorbance was measured at 562 nm on a PHERAstar FSX plate reader (BMG Labtech). The average absorbance was deducted from the blank and the quantity of unknown samples was determined from the standard curve from the standard solutions.

3.3 CELL CULTURE

HEK293T, Hela, HuCCT1 and U20S cells were cultured in DMEM medium (Gibco) which was supplemented with 10% (v/v) FBS (Atlanta Biologicals), and 1% Penicillin/Streptomycin. All cells were incubated at 37°C and 5% CO_2.

3.4 WESTERN BLOT

3.4.1 Western blot for cell line comparison

To determine the expression of MERTK, 2.5 $\times 10^5$ cell/ml of HEK293T, Hela, HuCCT1 and U20S cells were seeded into a 6-well plate and allowed to be attached in the incubator for 24 hours at 37°C and 5% CO_2. Whole cells were harvested by aspirating the medium and adding cold DPBS (Gibco). 1% NP40 cell lysis buffer at pH 7.4 (50 mM Tris, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P40 (NP40), 0.02% NaN3) was added to the plates at 4°C. The cells were scrapped, allowed to incubate for 30 minutes (at 4°C) and centrifuged at 17.000g for 15 minutes at 4°C.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 25 µg of lysate after BCA assay was mixed with 4× Laemlli buffer, and heated at 95°C for 5 minutes. This was loaded per well and run at 200v for 1 hour with NuPAGE gradient gel (4–12%,Invitrogen NP0321PK2). The gel is transferred to a PVDF membrane, using a Trans-Blot Turbo Transfer System (BioRad) at 4°C. The wet transfer was done at 0.3A for 3 hours. The membrane was blocked at room temperature for 1 hour with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween® 20 Detergent (TBST). Afterwards, it was treated overnight at 4°C with MERTK monoclonal primary antibody (Invitrogen, Thermo Fischer Scientific) with a 1:1000 dilution. Following this, the membrane was washed in TBST and incubated with HRP-linked secondary anti-mouse antibody (Cell Signaling #7074) for 2 hours at room temperature with a 1:5000 dilution. Clarity Western ECL Blotting Substrate (BioRad) was added after a second wash to detect HRP following the manufacturer's protocol and chemiluminescent images were captured using Biorad ImageDoc.

3.4.2 Western blot determination of NLuc MERTK

Full-length MERTK were obtained as plasmids cloned in frame with a terminal NLuc-fusion for this experiment. The plasmid was transfected into HEK293T cells using FuGENE HD (Promega, E2312) and transfection carrier DNA. Proteins were allowed to express for 20 hours at 37°C and 5% CO_2 and cells were seeded in a 6-well plate. The processes of cell harvesting, SDS PAGE, transfer, antibody treatment and HRP detection were carried out as described above.

3.4.3 MERTK degradation

2.5 $\times 10^5$ cell/ml of HEK293T were seeded in 2 mL of DMEM in 6 well plates overnight for cells to attach. Cells were treated with 2 uL of different concentrations of compounds which are dissolved in DMSO. The treatments were done for different time points while cells were incubated at 37°C and 5% CO_2. The processes of cell harvesting, SDS PAGE, transfer, antibody treatment and HRP detection were carried out as described above.

3.5 CELL VIABILITY ASSAY

To determine the cytotoxicity of the compounds, 10 µl of HEK293T cells were seeded in 384-well plates (Greiner 784075) at 2000 cells/well. This was incubated for 24 hours at 37°C and 5% CO_2. Compounds were dispensed with an Echo acoustic dispenser (Labcyte) and the plate was incubated for another 24 hours at 37°C and 5% CO_2. 10 µl of Ultra-Glo Recombinant Luciferase (CellTiter-Glo® 2.0 Reagent, PROMEGA G9241) was added and covered. This was shaken in a centrifuge at 100 g for 3 minutes and incubated at room temperature for 10 minutes. The PHERAstar FSX plate reader (BMG Labtech) was used in reading the luminescence and data were evaluated in GraphPad Prism 9.

3.6. NANOBRET TARGET ENGAGEMENT ASSAY

3.6.1. MERTK binary assay

Full-length MERTK were obtained as plasmids cloned in frame with a terminal NLuc-fusion for this experiment. The plasmid was transfected into HEK293T cells using FuGENE HD (Promega, E2312) and transfection carrier. Proteins were allowed to express for 20 hours at 37°C and 5% CO_2. 10 µl of the transfected HEK293T cells were seeded in 384-well plates (Greiner 784075) at a density of 2×10^5 cells/ml after trypsinization and resuspending in Opti-MEM without phenol red (Life Technologies).

The cells were treated with NanoBRET tracer (K10) and compounds (at concentrations which have been previously determined) with an Echo acoustic dispenser (Labcyte) and the plate was incubated for another 2 hours at 37°C and 5% CO_2 to equilibrate. Before BRET measurement, 5 µl of NanoBRET NanoGlo Substrate (Promega) was added in a 1:200 ratio with Opti-MEM without phenol red (Life Technologies). The filtered luminescence was measured on a PHERAstar FSX plate reader (BMG Labtech) equipped with a luminescence filter pair (450 nm BP filter (donor) and 610 nm LP filter (acceptor)). GraphPad Prism 9 software using a normalized 3-parameter curve fit with the following equation: *Y = 100/(1 + 10^(X-LogIC50)),* was used to process the competitive displacement data. Afterwards, cells were lyzed by treating with 25 nL of 10 µM digitonin and luminescence was measured after 5 minutes.

3.6.2. CRBN binary assay

Full-length CRBN were obtained as plasmids cloned in frame with a terminal NLuc-fusion for this experiment. The transfection process and seeding into wells were carried out as described above. A CRBN NanoBRET tracer (Promega) and compounds were treated with an Echo acoustic dispenser (Labcyte). The plate was incubated, treated with substrate and measured as described above. Afterwards, cells were lyzed by treating with 10 µM digitonin and luminescence was measured after 5 minutes.

3.6.3. Ternary complex formation assay

MERTK NLuc and CRBN HaloTag were transfected into HEK293T cells using FuGENE HD (Promega, E2312) and transfection carrier DNA. Proteins were allowed to express for 20 hours at 37°C and 5% CO_2. 10 µL of the transfected HEK293T cells were seeded in 384-well plates (Greiner 784075) at a density of 4×10^5 cells/mL after trypsinization and resuspending in Opti-MEM without phenol red (Life Technologies). 40 nL HaloTag® NanoBRET™ 618 Ligand (PROMEGA) was added with an Echo acoustic dispenser (Labcyte) and the cells were further incubated for 20 hours at 37°C and 5% CO_2. With the Echo acoustic dispenser (Labcyte), compounds were titrated 2 hours before measurement and made to equilibrate at 37°C and 5% CO_2. Before BRET measurement, 5 µL of NanoBRET NanoGlo Substrate (Promega) and Extracellular NLuc InHiBiTor (Promega, N2540) were added in 1:200 and 1:500 ratios respectively with Opti-MEM without phenol red (Life Technologies). The filtered luminescence was measured on a PHERAstar FSX plate reader (BMG Labtech) equipped with a luminescence filter pair (450 nm BP filter (donor) and 610 nm LP filter (acceptor)). GraphPad Prism 9 software using a normalized 3-parameter curve fit with the following equation: *Y = 100/(1 + 10^(X-LogIC50)),* was used to process the competitive displacement data.

3.6.4 Ubiquitination assay

To investigate polyubiquitination, HEK293T cells were transfected with MERTK NLuc and ubiquitin HaloTag. The remaining steps of the assay were carried on just as described above for the ternary complex assay.

4. RESULTS

4.1. DIFFERENTIAL SCANNING CALORIMETRY

In this DSF experiment, a purified kinase domain of the target expressed with *E. coli* expression medium was tested for their binding and stability with all 9 PROTACs and parent inhibitor (**HA119**). The melting temperature shift (T_M shift) observed was 8°C for **HA119** with all but **HA139** and **HA143** having comparable values as shown in Table 4. 1. The DMSO controls did not have any influence on the T_M shift. The curve of the first derivative of the fluorescence per temperature increase of DMSO 2 and **HA119** is shown in Figure 4.1, while the others are in the supplementary information (SI Figure 1).

4.2. CELL VIABILITY ASSAY

In this experiment, HEK293T were treated with all compounds and digitonin as a control. This was in 10 different concentrations from 25 nM to 20 µM. The findings after 24-hour treatment are shown in Figure 4.2 and Table 4.2, with all but digitonin demonstrating significant cell viability.

4.3. WESTERN BLOT FOR CELL LINE COMPARISON

Having established the preliminary binding of compounds to the purified binding domain of MERTK, it was necessary to establish other characterization assays in human cell lines. MERTK is not ubiquitously expressed in the body hence it was necessary to choose a cell line for further cellular assays. To resolve this, the western blot technique was performed on 4 in-house cell lines, which were HEK293T, Hela, HuCCT1 and U20S. Cells were seeded for 24 hours for adequate attachment and expression of proteins. As shown in Figure 4.3, only HEK293T expressed MERTK.

4.4. WESTERN BLOT DETERMINATION OF NLUC MERTK

To proceed with further assays *in-cellulo*, a western blot assay was done as a confirmatory test for the expression of NLuc transfected with the cells. The transfected cells were incubated for 24 hours before making lysates for the blot. The outcome was a thick band stretching to around 200kDa indicative of overexpression of MERTK (Figure 4.4).

Table 4.1: Thermal shift values for compounds

Compound	T_M shift (°C)
HA136	9
HA137	8
HA138	7
HA140	8
HA143	4
HA144	8
HA145	7
HA199	8

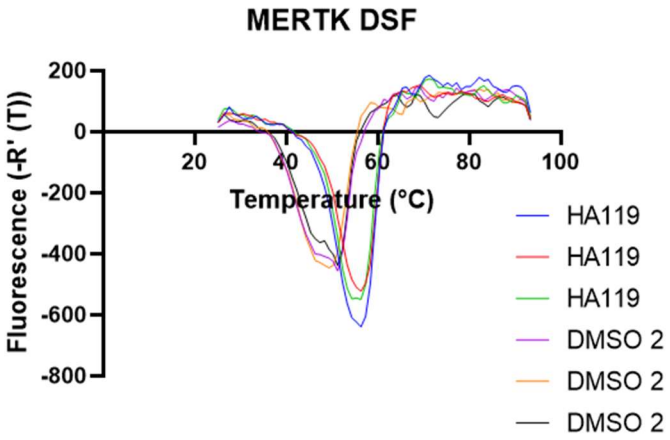

Figure 4.1: DSF result showing protein melting curves of purified MERTK kinase domain treated with DMSO and inhibitor (HA119). The curves are individual replicates.

Table 4.2: EC_{50} of Cell Viability Assays

Compound	EC_{50}(μM)
HA136	243 ± 0.00
HA137	592 ± 409
HA138	551 ± 0.00
HA141	589 ± 803
HA143	475 ± 51
HA144	334 ± 346
HA145	468 ± 479
HA119	303 ± 272
Digitonin	0.0000127± 0.0000172

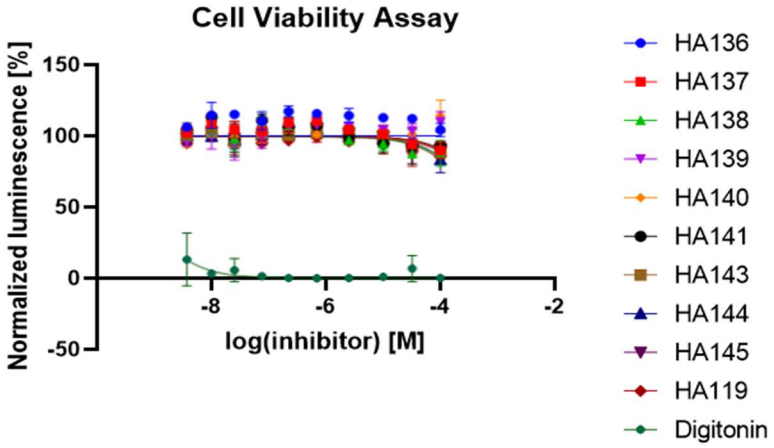

Figure 4.2: CTG assay showing the percentage viability of cells after 24 hours of treatment with compounds. Digitonin (green) distinctly is cytotoxic, unlike compounds. Data were measured in biological replicates with error bars expressing the SD (n = 2).

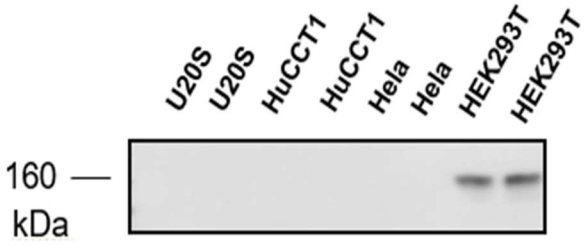

Figure 4.3: Comparison of MERTK expression in cell lines with western blot technique. Only HEK293T cells expressed MERTK. Blots were measured in biological replicates.

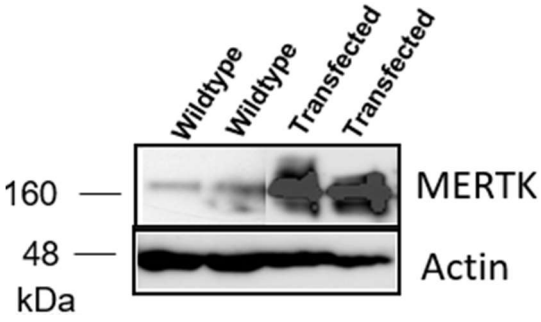

Figure 4.4: Comparison of MERTK expression in wildtype and cell transfected with NLuc with western blot technique in biological replicates. MERTK expression in transfected cells was enhanced compared to the wild type. Blots were measured

4.5. MERTK BINARY ASSAY

From the assay, all compounds had good cell penetration and binding to the target. In the intact mode, **HA136** had the best outcome with EC_{50} of 250 nM, even more than **HA119**, which was the inhibitor (320 nM). Compounds **HA137**, **HA138**, **HA141** and **HA144** were also in the nanomolar ranges with respective EC_{50} values of 490 nM, 890 nM, 390 nM and 850 nM. The remaining compounds had values in the micromolar ranges. **HA139** has 8.19 μm, 12.3 μM for **HA140**, 7.27 μM for **HA143** and 1.29μM for **HA145**.

In the lyzed mode, all but **HA143** had EC_{50} values in the nanomolar ranges as shown in Table 4.3. An interesting observation was that unlike the others **HA143** and

Table 4.3: Affinity data measured for binary complex formation.

Compounds	MERTK			CRBN		
	Intact EC_{50} (μM) [a]	Lyzed EC_{50} (μM) [a]	Penetration Ratio [b]	Intact EC_{50} (μM) [a]	Lyzed EC_{50} (μM)[a]	Penetration Ratio[b]
HA136	0.25 ± 0.01	0.14 ± 0.02	1.74	0.85 ± 0.01	1.25 ± 0.03	0.68
HA137	0.49 ± 0.09	0.26 ± 0.04	1.89	0.88 ± 0.21	2.07 ± 0.13	0.43
HA138	0.89 ± 0.06	0.53 ± 0.03	1.68	1.39 ± 0.31	3.53 ± 0.12	0.4
HA139	8.19 ± 1.57	0.49 ± 0.04	16.64	21.60 ± 7.39	1.04 ± 0.16	20.81
HA140	12.30 ± 0.50	0.91 ± 0.05	13.45	10.60 ± 3.04	0.70 ± 0.03	15.22
HA141	0.39 ± 0.04	0.31 ± 0.04	1.23	0.07 ± 0.02	0.66 ± 0.02	0.11
HA143	7.27 ± 1.37	7.92 ± 2.00	0.92	-	-	-
HA144	0.85 ± 0.01	0.47 ± 0.04	1.81	0.25 ± 0.04	1.13 ± 0.08	0.22
HA145	1.29 ± 0.10	0.83 ± 0.20	1.55	0.67 ± 0.43	2.51 ± 0.37	0.1
HA119	0.32 ± 0.06	0.59 ± 0.07	0.54	-	-	-

[a] Affinity data were measured in biological replicates of technical duplicates.

[b] Penetration ratio is the ratio of intact EC_{50} to Lyzed EC_{50}.

HA119 had a better binding in intact mode. This was reflected in the penetration ratio as well, which yielded 0.92 and 0.54 for the respective compounds. The others had results in the reverse direction. The assays had Z-factors of 0.69 and 0.72 for both the intact and lyzed modes (Figure 4.5). The assay windows were also 2.03 and 2.01. In both modes, modification was made for **HA143** due to the significant interference of its colour in the assay.

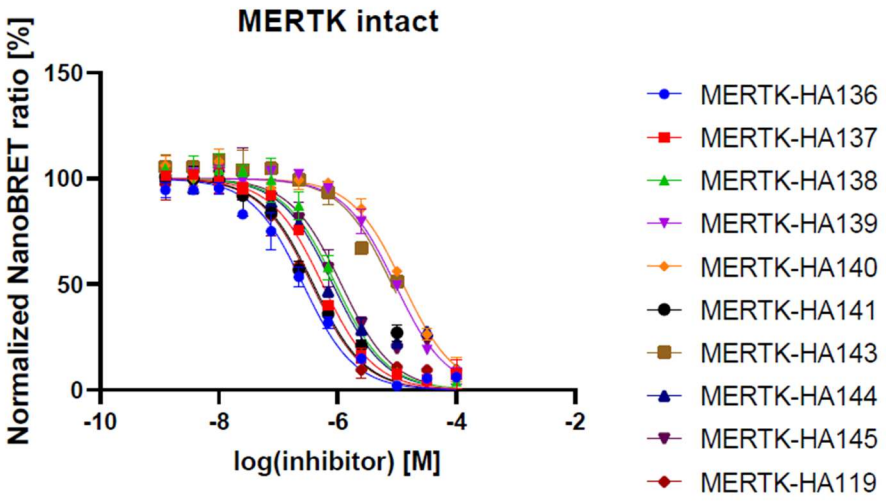

Figure 4.5: MERTK target engagement studies with NanoBRET dose-response curves of compounds in intact cells. Displacement of tracer K10 binding to the POI results in a decrease of the BRET signal. Data were measured in biological replicates with error bars expressing the SD (n = 2).

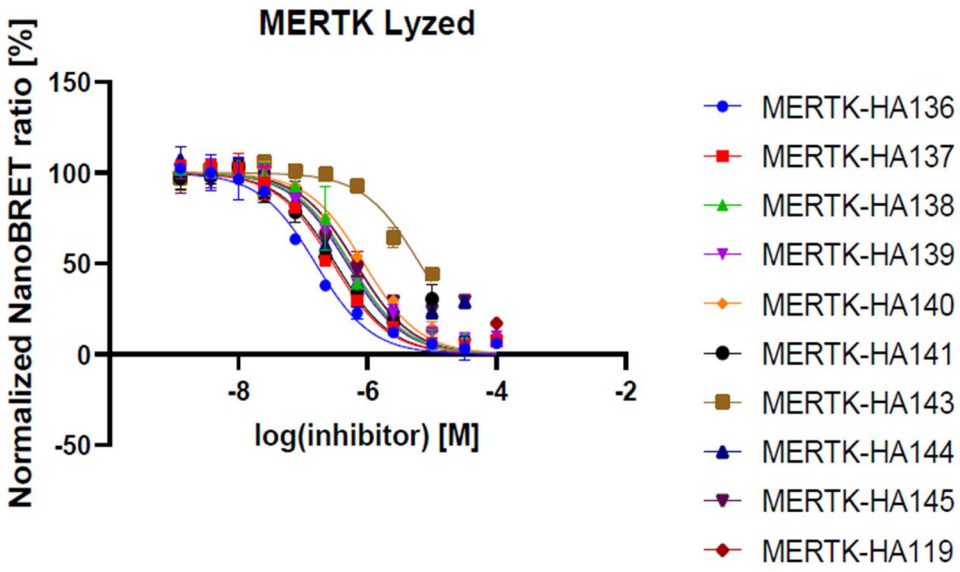

Figure 4.6: MERTK target engagement studies with NanoBRET dose-response curves of compounds in lyzed cells. Displacement of tracer K10 binding to the POI results in a decrease of the BRET signal. Data were measured in biological replicates with error bars expressing the SD (n = 2).

4.6. CRBN BINARY ASSAY

Aside binding to the neosubstrate, PROTACs are required to have good binding to the E3 ligases as part of their mechanism of action. In this assay, the binding of the compounds to CRBN was ascertained. As shown in Table 4.3, all but **HA138**, **HA139** and **HA140** had nanomolar EC_{50} intact. When lyzed, it was interestingly observed that the compounds except for **HA139** and **HA140** had reduced EC_{50} values of 1.04 μM and 0.69 μM respectively. This is reflected in the penetration ratio of 20.81. The Z-factor and assay window for the intact assay were 0.65 and 2.2 while that of the lyzed assay were 0.66 and 2.2.

4.7. TERNARY COMPLEX ASSAY

From this study, amidst the decent binding of all the compounds, **HA136**, **HA137**, **HA138**, **HA144**, **HA143** and **HA145** were observed to induce ternary complexes between MERTK and CRBN. The ternary K_D were interestingly all in nanomolar ranges as shown in Table 4.4. The assay had a Z-factor of 0.8 and an assay window of 2.69.

4.8. UBIQUITINATION ASSAY

From this assay, the ubiquitination of compounds was not obvious with mostly flat lines (SI: Figure 3.1). A follow-up competitive assay of selected compounds with thalidomide had a similar outcome (SI: Figure 3.2), while a repetition of the general ubiquitination assay had flat lines only (SI: Figure 3.3). The assays had an average Z-factor of 0.67 and an assay window of 2.54.

4.9. WESTERN BLOT FOR DEGRADATION OF MERTK

Western blot analysis was performed at varying concentrations of compounds and time points. Treatment of 50 nM with all compounds and DMSO control showed no obvious degradation after 20 hours as shown in Figure 4.10. Actin was employed to normalize protein levels. In Figure 4.11, cells which were treated with. compound concentrations of 500 nM and 2.5 μM for 6 hours showed degradation from some compounds. At 24 hours, selected compounds in concentrations of 50 nM, 500 nM,

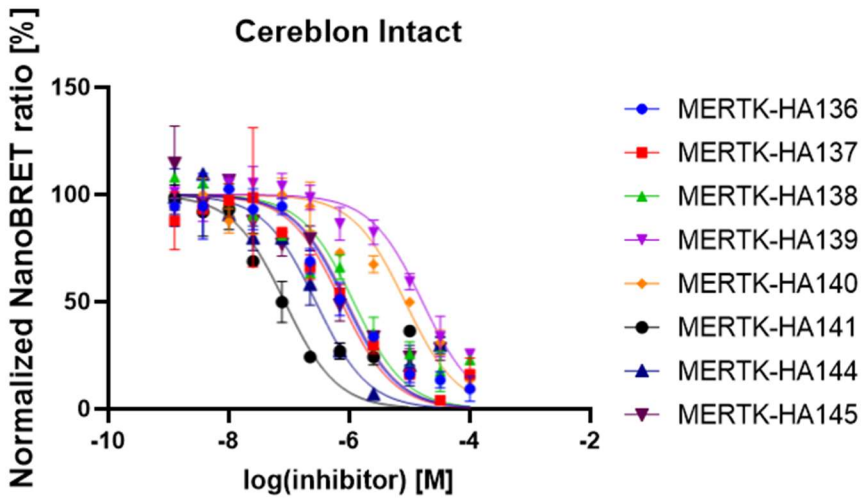

Figure 4.7: CRBN target engagement studies with NanoBRET dose-response curves of compounds in intact cells. Displacement of CRBN tracer binding to the CRBN results in a decrease of the BRET signal. Data were measured in biological replicates with error bars expressing the SD (n = 2).

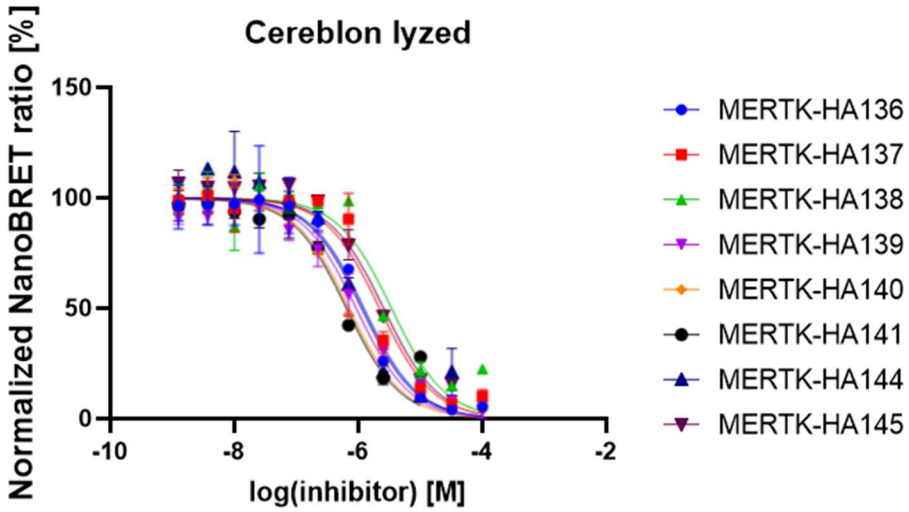

Figure 4.8: CRBN target engagement studies with NanoBRET dose-response curves of compounds in lyzed cells. Displacement of CRBN tracer binding to the CRBN results in a decrease of the BRET signal. Data were measured in biological replicates with error bars expressing the SD (n = 2).

Table 4.4: Affinity data measured for ternary complex formation

Compounds	Apparent K_D (nM)[a]
HA136	22.12 ± 0.21
HA137	17.42 ± 4.00
HA138	15.30 ± 4.56
HA141	4.64 ± 0.76
HA143	64.72 ± 36.10
HA145	54.72 ± 41.16

[a] Affinity data were measured in biological replicates of technical duplicates.

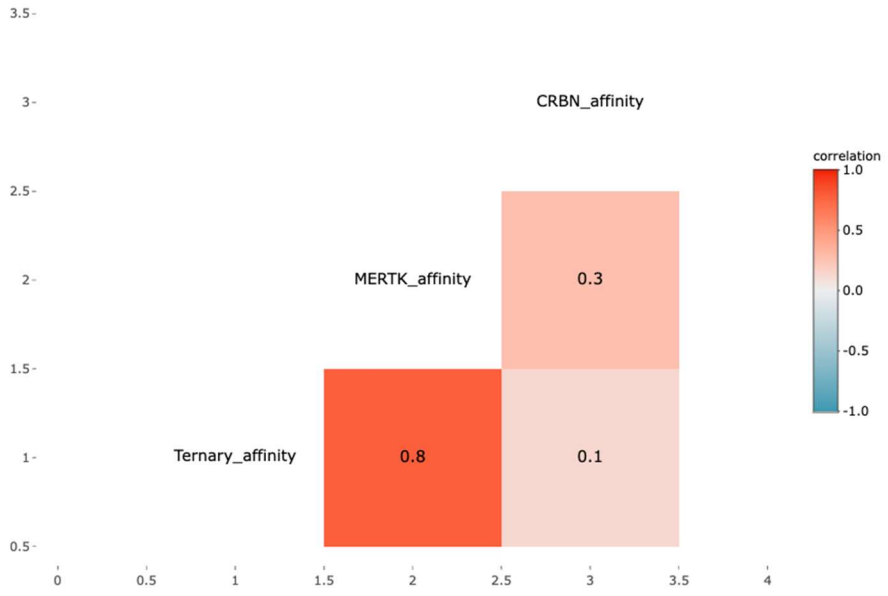

Figure 4.10: A plot of correlation between the measured affinity data for compounds which induced ternary complexes. Affinity for the formation of

ternary complexes correlated strongly with binding to MERTK and weakly to CRBN. The plot was generated with R software.

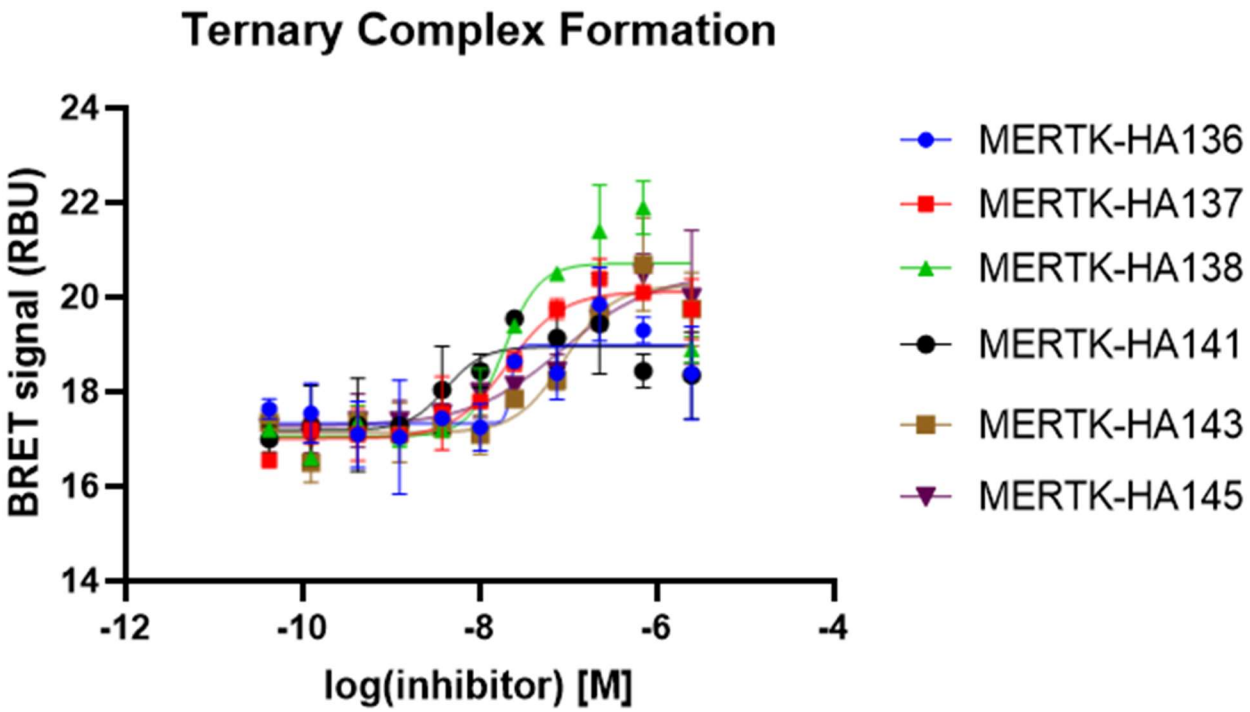

Figure 4.10: Ternary complex affinity determination of selected compounds using the established NLuc/HaloTag reporter assay. Data were measured in biological replicates with error bars expressing the SD (n = 2).

and 5 µM showed various levels of degradation as well (Figure 4.12). GAPDH was used in the last two blots.

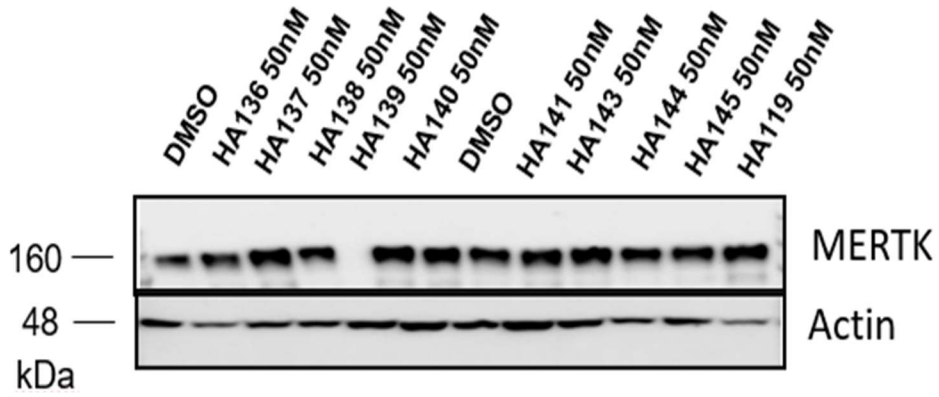

Figure 4.11: Western blot analysis of MERTK expression in HEK293T cells after 20 hours of 50nM treatment with all compounds in the study.

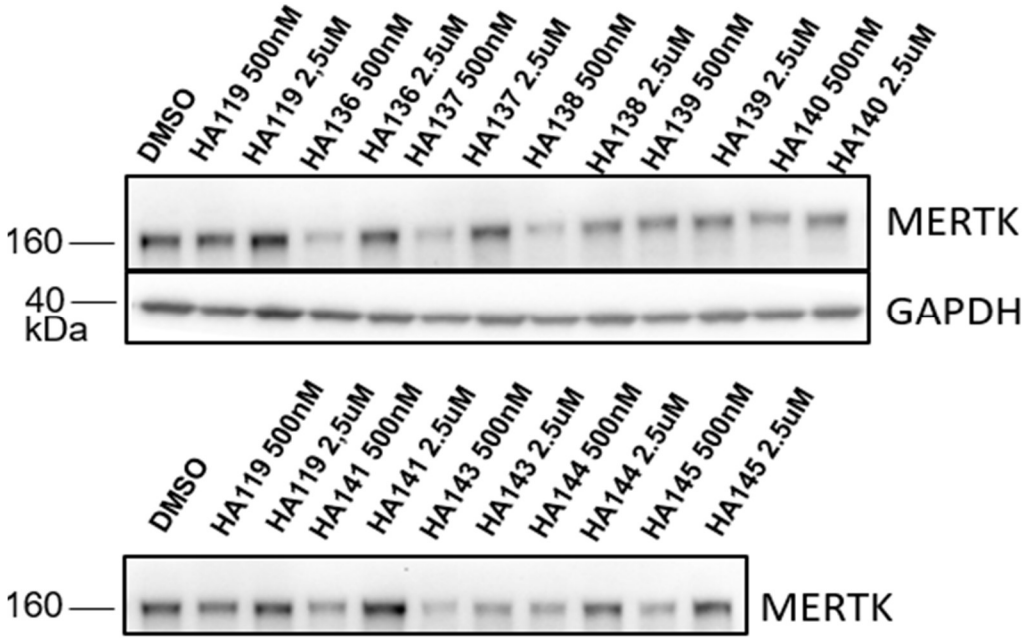

Figure 4.12: Western blot analysis of MERTK expression in HEK293T cells after 6 hours of 500nM and 2.5μM treatment with all compounds in the study.

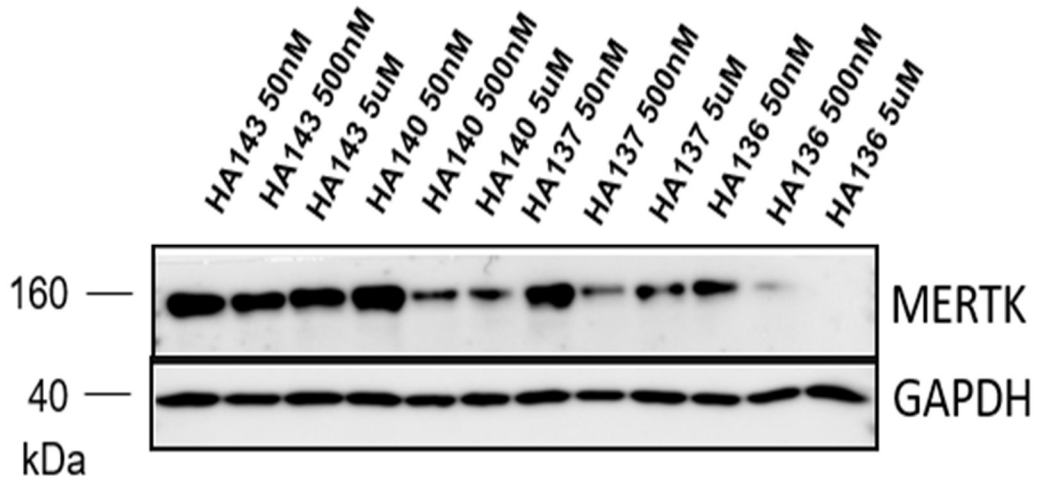

Figure 4.13: Western blot analysis of MERTK expression in HEK293T cells after 24 h of 50 nM, 500 nM and 5 µM treatment with selected compounds in the study.

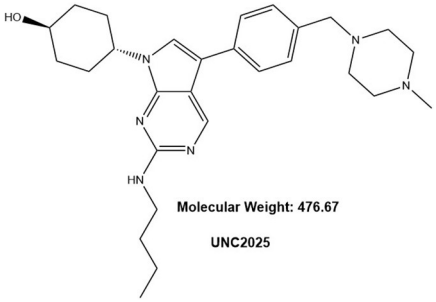

Figure 5.1: Chemical structure of UNC2025

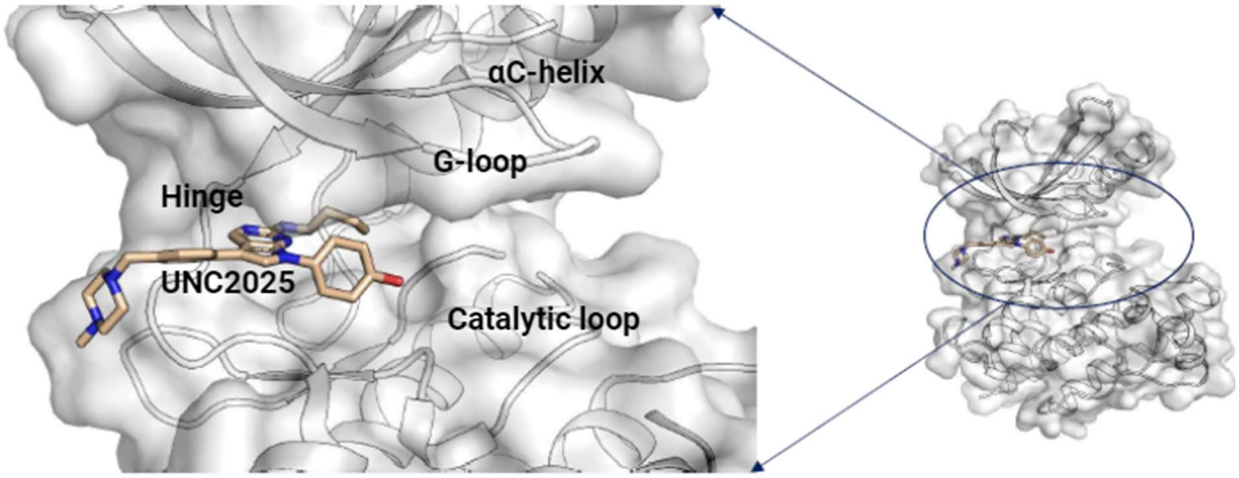

Figure 5.2: Crystal structure UNC2025 bound to MERTK kinase domain

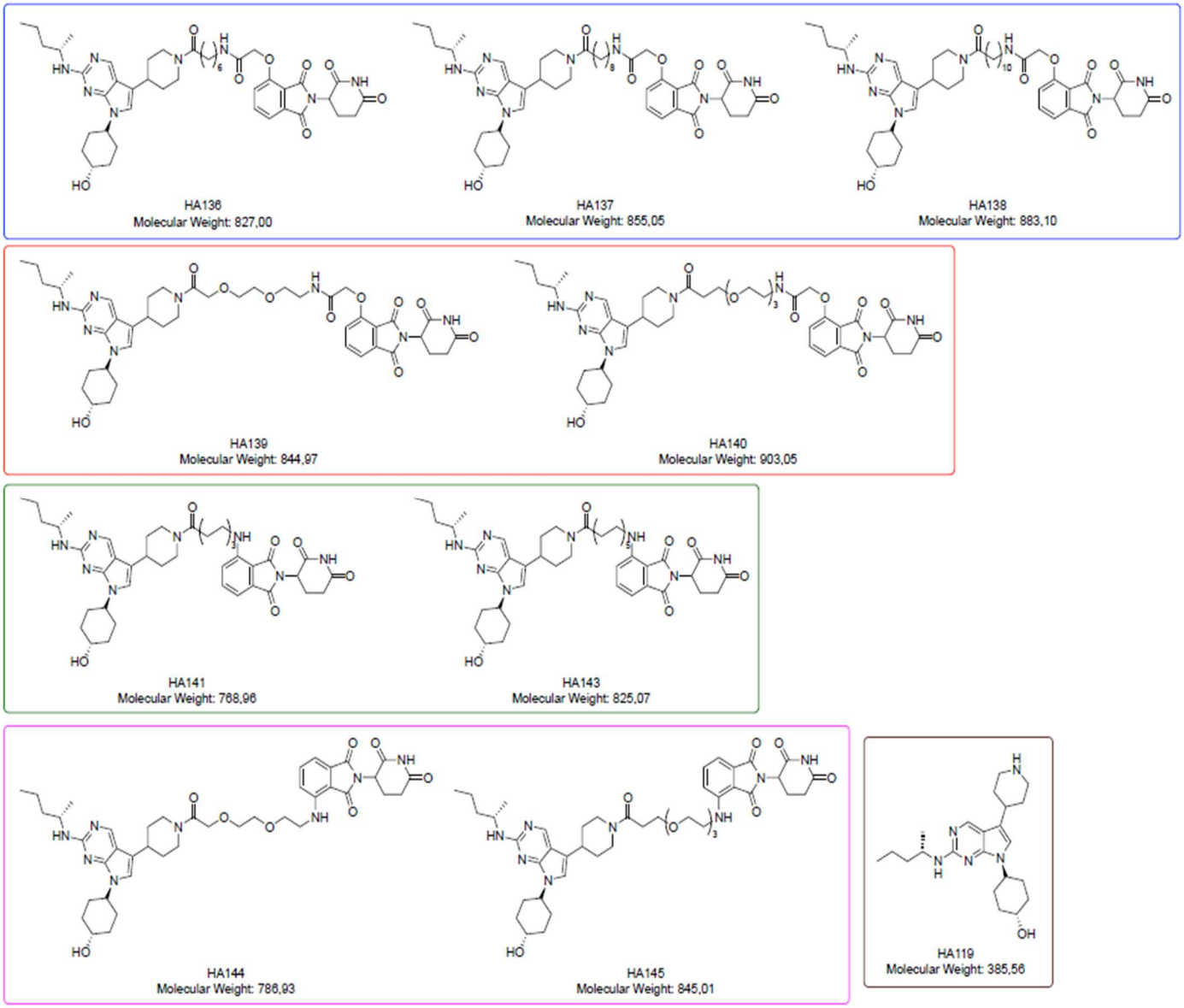

Figure 5.3: Structures of compounds used this study. In blue- alkyl linkers and amide attachment. Red- PEG linkers and amide attachment. Green - alkyl linkers and amine attachment. Purple – PEG linkers and amine attachment. Brown – Inhibitor.

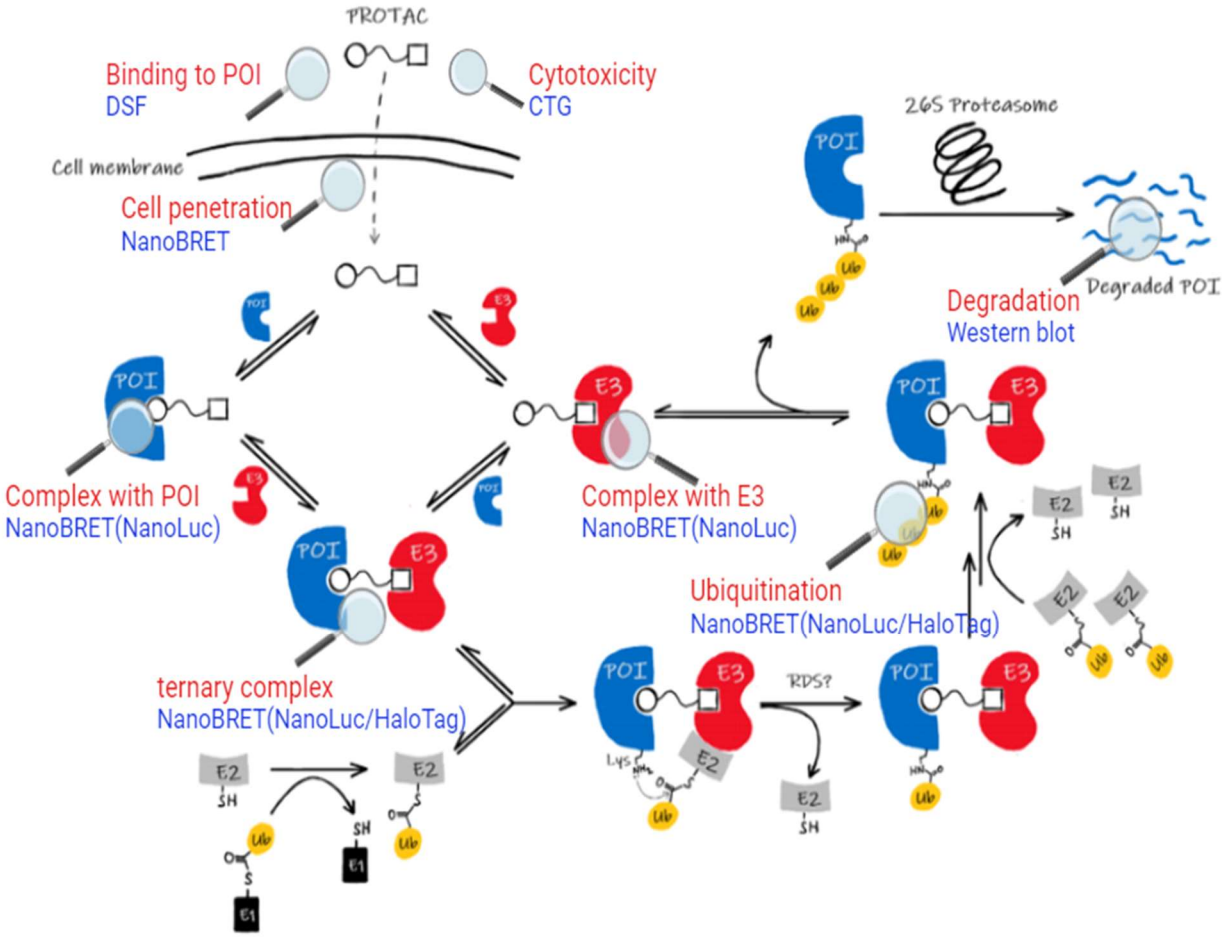

Figure 5.4: Schematic representation of protein degradation pathway induced by PROTACs and the assays employed in this study.

5. DISCUSSION

5.1. PROTACS BIND TO MERTK KINASE DOMAIN

MERTK has become an attractive target in drug discovery due to its involvement in several pathologies. This target is a single-pass transmembrane protein, with 2 immunoglobin-like domains, 2 fibronectin type III domains at the extracellular portion, a transmembrane domain and a kinase domain in the cytoplasm(191,192) Attempts have previously been made to modulate the kinase domain of this target with small molecule inhibitors. One of these, UNC2025 (Figure 5.1, Figure 5.2), was shown to have potent binding to MERTK. UNC2025 is a dual MERTK/FLT3 inhibitor hence was further modified to achieve selectivity for MERTK. By introducing a piperidine ring at C5 and a methyl group at C2, UNC5293, an inhibitor with high selectivity for MERTK was achieved (120). Using this warhead (Figure 5.3), 9 PROTACs with PEG and alkyl linkers of varying lengths and attachment points to thalidomide were synthesized (*by Adrian Haag*). Considering the cytoplasmic location of the kinase domain of MERTK, the idea of PROTAC degraders is justifiable which was characterized in this study.

The development of PROTACs is a non-trivial process. Currently, we do not have a general and reliable way to predict ternary complexes (which are central to successful PROTAC-induced degradation) that would allow the prediction of molecular structures providing potent PROTACs. Therefore, we rely on extensive experimental efforts involving syntheses of often large series of potential PROTAC molecules with various linkers, POI and E3 recruiting warheads. In addition, characterization of PROTACs requires a set of assays due to their complex mechanism of action. MERTK being a transmembrane receptor presents peculiar challenges for this technology given the UPS machinery is in the cytosol. Although there have been previous successes in degrading this class of targets (126), there have not been extensive reports on these degraders at the cellular level. This warrants the combination of *in-vitro* and cell-based assays to study the path of MERTK through the ubiquitin proteasomal cascade (Figure 5.4).

The warhead, **HA119**, has good binding to the MERTK in literature (120), yet it was necessary to establish this in-house and compare this to that of the PROTACs.

Hence the DSF technique was applied, with a purified MERTK kinase domain for all compounds and DMSO as control. The resulting T_m shift of 8°C of the warhead was indicative of a strong binding at the kinase binding domain, hence a strong stabilization. In one report, six compounds against MERTK had T_m shifts greater than 4°C relative to the average and these correlated with good IC_{50} values(193). Hence the observed results could have promising implications subsequently. The PROTACs also had comparable T_m shifts to the inhibitor, with values from 4°C to 9°C, indicating comparable binding. Similar values have also been reported for some PROTACs(194). Interestingly **HA139** and **HA141** showed strange curves hence T_m were not calculated. The cause of this, however, was unknown since all compounds were subjected to the same experimental conditions. In all, this preliminary screening was a good indication for further investigations.

5.2. PROTACS ARE NOT CYTOTOXIC TO CELLS

The overall aim of the TPD modality is to selectively degrade POIs without harming the cells. Cytotoxicity observed in PROTACs could be a limiting factor and may not warrant further investigations. This is because the degradation assay needs to be done under conditions where the cell is not dying or at the edge of cell death. In such cases, the depletion of protein could be caused by a dying cell via a crude cytotoxic effect rather than PROTAC-induced degradation. Therefore, this assay helps to identify and prioritize compounds with a good window of toxicities at relevant concentrations and time points for subsequent studies.

The CellTiter-Glo® assay, an ATP-based cell viability assay was used for this study. This assay is the fastest, most sensitive and most suited for high-throughput studies, making it reliable (190). Cells were treated with concentrations from 25 nM to 20 µM for 24 hours, to allow adequate activity. All test compounds were not cytotoxic, with EC_{50} values in micromolar ranges. The EC_{50} values for **HA136**, **HA139** and **HA140** could not be calculated due to the nature of the curves. On the contrary, the control

compound, digitonin, exhibited high cytotoxicity even at the lowest dose of 25nM with an EC_{50} of 12.7pM.

5.3. HEK293T AS CELL LINE FOR CELL-BASED ASSAYS

A preliminary review of the literature revealed most reported studies on PROTACs utilized endpoint and biochemical assays in characterization. These are usually done with recombinant targets to interact with the compounds outside the cell (195). They have drawbacks being time and resource intensive. Also, they are faced with artefact hits and false positives hence often require a combination of assays to establish a binding(196). In instances of good binding also, some disappointingly do not exhibit the required biological activity(197). This is explained by the complexity and dynamism of the biological environment, like natural competitors and enzymes, the behaviour and size of full-length proteins and post-translational modifications. The introduction of cell-based assays greatly helps this challenge by reporting the actual behaviour of the compounds in cells.

NanoBRET target engagement assay was employed in this study. MERTK is not ubiquitously expressed in the body and is mostly found in myeloid, epithelia, reproductive, immune and cells of various pathologies like cancers(125). Hence there was a need to choose an appropriate cell line with a good expression of MERTK for this study. Four cell lines HEK293T, Hela, HuCCT1 and U20S were investigated with western blot. HEK293T was found to express the target and hence suitable for these assays. In addition, this cell line has the advantage of easy transfection to express desired reporters to study biological activities (198), which was necessary for this study.

5.4 PROTACS PENETRATE AND FORM BINARY COMPLEXES WITH MERTK

NanoBRET is a proximity-based assay based on the principle of BRET to study protein-protein interactions and other molecular events in cells, even at low levels like some physiological interactions. By utilizing a small (19kDa), exceptionally bright and ATP-independent luciferase (NLuc) as an energy donor and fluorophore-tagged acceptor (tracer), we can measure biological interactions in <10 nm proximity (199–201).

The expression of NLuc by the HEK293T cells was the first and crucial step for these assays to proceed. A commercially acquired construct, including a C-terminal protein fusion vector, was obtained for this step and the cells were transfected with it for 20 hours. The choice of the construct was due to the intracellular location of MERTK's C-terminus, unlike the N-terminus which is in the extracellular region (125). To ensure a successful transfection, a western blot was done, with untransfected HEK293T cells as control. The positive outcome of overexpressed MERTK detected around 200kDa was indicative of this expectation.

The penetration of compounds and formation of binary complexes with MERTK is necessary for the subsequent processes of protein degradation. Considering their bulky nature, membrane permeability is typically compromised. Therefore, we decided to evaluate also *in-cellulo* target engagement, in addition to *in-vitro* binary binding (DSF assay). A NanoBRET assay was established for this investigation in live and lyzed cells, and both were used to analyze binary affinity. As reported for some PROTAC series, a ratio of their EC_{50} values will inform on their cell penetration also(194). This assay is based on the compounds' abilities to displace a tracer (ligand for MERTK linked to NanoBRET590 dye). The NLuc and tracer in proximity generate a BRET signal at 590–610 nm hence a dose-dependent loss of signal when displaced(199). In this study, tracer K10, a kinase tracer was used(202). Also, all the assays conducted had recommendable assay windows of >2 and Z-factors of >0.5, making them very robust (203,204).

All compounds had good binding to the target in the live cell with EC_{50} values in nanomolar and micromolar ranges. The parent MERTK ligand, **HA119** had a better binding with an apparent affinity of 320 nM. Interestingly, **HA136** had the most potent binding, with 250 nM. This is consistent with the DSF results. An interesting observation was with that of **HA143** as its intense colour significantly affected the outcome of the experiment. Owing to the principle behind the assay, compounds that absorb or emit light energy around the wavelength of 460 nm could affect the outcome of the experiment. This observation was due to a change in the luciferase signal through the compound's absorption of light (205) and was intense at higher concentrations.

The influences of the type of linkers, their length, and the attachment point on the activity of PROTACs are well-established in the literature(206,207). We observed that reduction in BRET signal decreased with increasing linker length, regardless of the type or attachment of linkers. Implying the potency of PROTACs with shorter linkers in displacing tracer K10. Within compounds connected to the phthalimide ring of thalidomide through an amide linkage, the alkyl linkers had better binding than PEG linkers. This is in the order of **HA136**> **HA137**> **HA138**> **HA139**> **HA140** as shown in Table 4.3. This observed binding of **HA139** and **HA140** in micromolar ranges is likely due to the different solubility or permeability influences of the linkers.

On the contrary, we observed inconsistencies within the different linkers of PROTACs attached through an amine on the phthalimide ring. The order was **HA141**> **HA144**> **HA145**> **HA143,** with no clear advantage of the linker type.

In the lyzed mode, the compounds had better binding as expected. Surprisingly, **HA119** and **HA143** had a better affinity in the intact mode and were consistent in all replicates. This was pronounced for the former with about twice a better affinity in live cells. The affinity in the lyzed mode affirmed the superiority of **HA136**'s binding. However, six other compounds had a better affinity than **HA119** indicating slight restrictions on their entry through the cell membrane. With less than twice the affinity in lyzed cells, the compounds demonstrated acceptable penetration ability. Notably, **HA139** and **HA140** had significantly less penetration ratio reaffirming the influence of the PEG and attachment point on these PROTACs.

An alternative to this assay is HiBiT-CETSA, a combination of thermal shift assay and HiBiT. Through the principle of protein stabilization by HiBiT, while increasing temperature, binding can be assessed in live cells, hence requiring no tracers nor dose titration and in cases of weak interactions. However, the increase in temperature can compromise membrane integrity hence misleading results. Also, it provides only relative affinity, in contrast to NanoBRET, making NanoBRET a preferred and more reliable cell-based binary assay over HiBiT-CETSA

5.6 PROTACS BIND TO CRBN

Aside from the complex formation with the POI, a binary complex with the E3 ubiquitin ligase is equally important. Recruiters of these ligases should have adequate affinity to form stable interactions with the ligase. The compounds in this series have CRBN ligands and were expected to bind stably with CRBN. By employing a NanoBRET binary assay, this aspect of the study was investigated.

The compounds had a good binding affinity to CRBN in the intact mode, mostly in the nanomolar ranges (Table 4.3). **HA141** demonstrated the best binding with EC_{50} of 70 nM while **HA139** had the least binding with 21.6 μM. Consistent with their binding to MERK, we observed that decreasing linker length reflected in the ability to displace the CRBN tracer, hence reduction in the BRET signal. An exception to this was with PEG linkers attached via amide bond where **HA140> HA139.** The affinity of **HA143** was not measured due to the strong interference it had with the assay.

In the lyzed mode, the affinity towards CRBN was compromised hence seemingly giving the impression that the compounds bind better in intact cells. This can be accounted for by the disintegration of the CUL4-RBX1-DDB1-CRBN complex of which CRBN is the substrate recognition component (208,209). Strangely, **HA139** and **HA140** had a better affinity in the lyzed state reflecting in a reverse penetration ratio.

The affinity for CRBN has crucial implications on the degradation process as too high an affinity may lead to hook and off-target effects. It is noted that a higher CRBN affinity with a slow off-rate compared to POI could increase the chances of degradation (1). However, a high E3 affinity is not a guarantee for the degradation of the POI (210). Regardless, PROTACs with a fairly balanced affinity for both proteins also induce efficient degradation.

5.7. PROTACS FORM TERNARY COMPLEXES

Next to forming dimers is the crucial step of forming trimers, the so-called ternary complexes. This is possibly the rate-limiting step of the PROTAC-induced degradation pathway, and a stable ternary complex is thus required for subsequent events leading to degradation. The success of this event depends on the synergistic binding compatibility of both MERTK and CRBN and how they cooperate with the PROTACs(61). While most

ternary complex studies have been based on biophysical assays, a cell-based assay was developed to study MERTK/PROTAC/CRBN interactions in this work. A BRET pair based on a luciferase reporter tagged to MERTK and a HaloTag acceptor tagged to CRBN was established for this assay.

A C-terminal HaloTag fusion vector was transfected and labelled with the fluorescent HaloTag® NanoBRET® 618 Ligand to serve as the energy acceptor for this assay. The outcome of this study (SI Figure 2.1) seemingly suggested no PROTAC-induced ternary formation. The C-terminal domain of CRBN has been found to contribute to the functionality and stability of the CUL4-RBX1-DDB1-CRBN complex by binding to the adaptor protein, DDB1. Also, the presence of pseudouridine synthase and archaeosine transglycosylase in the domain acts as a site of interaction for thalidomide (211,212). Hence, it was hypothesized that tagging the C-terminal of CRBN with the HaloTag either disrupted the CUL4-RBX1-DDB1-CRBN complex formation or the binding of thalidomide.

An N-terminal HaloTag fusion vector was employed with compound treatment concentrations 1.2 nM and 99 μM. This resulted in curves which suggested that binary complexes outcompeted ternary complex formation leading to the hook effect (SI Figure 2.2). Hence concentrations were reduced to the range of 41 pM to 2.5 μM. Excitingly, the outcome revealed ternary complex formation guiding the apparent ternary complex K_D value determination. With a robust Z factor of 0.8, compounds **HA136**, **HA137**, **HA138**, **HA141**, **HA143** and **HA144** had optimal ternary complex curves with respective apparent K_D values of 22.12 nM, 17.42 nM, 15.29 nM, 4.64 nM, 64.72 nM and 54.72 nM. The ternary complex graphs are represented in Figure 4.10 while the entire series is in SI Figure 2.3.

We observed that almost all compounds which formed ternary complexes had alkyl linkers. The trend in intensity of the BRET signal correlated with increasing linker length amongst the amide attachment series hence **HA136**< **HA137**< **HA138**. In contrast, those with amine attachment favoured the shorter linker **HA143 < HA141.** Also from this observation, **HA145** was the only PEG linker compound that induced

ternary complex formation. Hence, this study showed that alkyl linkers were superior to PEG linkers in generating the BRET signal as a measure of ternary complexes.

Based on the affinities determined from the assays, there is a coherence between the developed assays and the measured activity of the compounds. This shows a general strongly positive correlation (0.8) with binary binding to MERTK (Figure 4.9). In the case of CRBN binding affinity, a weak correlation was observed (0.1).

5.8. UBIQUITINATION ASSAY WAS NOT ESTABLISHED FOR PROTACS

We investigated the step of ubiquitination using a commercially acquired HaloTag-Ubiquitin construct from Promega. This assay was carried out with the MERTK NLuc as the energy donor. The outcome gave a mild impression of ubiquitination induced by compounds **HA137**, **HA138**, **HA143** and **HA144** (SI Figure 3.1). Although not convincing, these compounds in the previous assay formed ternary complexes hence the need for further investigation to establish this outcome.

To realize this, thalidomide was employed as a co-treatment with the selected compounds. Being a CRBN ligand, thalidomide is expected to compete for binding with CRBN over the PROTACs in a higher concentration (10 mM). This is expected to give a flat line due to the diminished signal, and rightly so, this was observed (SI Figure 3.2). The ubiquitination assay was repeated to clarify the doubts about the assay, but the initial findings were not reproducible (SI Figure 3.3). Hence the assay could not be established to study the process of ubiquitination of these compounds.

Querying this challenge points to a likely general problem in establishing this assay in the field. Only two studies, both on BRD2 and BRD4 with MZ1 PROTACs, which recruit VHL ligase (153,213) have successfully reported this assay. However, the human VHL has 3 lysine residues compared to 28 of CRBN, hence ideally, the possibility of ubiquitination in the latter (214). Yet, this evidence suggests these assays work for VHL-induced ubiquitination only.

5.9. PROTACS DEGRADE MERTK

The desired endpoint of this study is to successfully degrade MERTK with the developed PROTACs. To investigate this, the western blot technique was employed at varying times and concentrations. A treatment at 50 nM concentration for all compounds was first investigated. Even though the molecular weight of MERTK is 110kDa, it was detected at approximately 160kDa in this study. This is due to the NH2-linked glycosylation site at the extracellular domain, which increases the molecular weight up to 205kDa for full glycosylation(215). At the 20-hour time point, no obvious degradation was observed from the compounds (Figure 4. 11). This is not uncommon, as a lot of reported PROTACs degrade at higher concentrations.

Further investigations were conducted on all compounds, this time at a shorter time point. For 6 hours, cells were treated with 500 nM and 2.5 μM of compounds (Figure 4.12). Detection of GAPDH was done to ensure normalized protein concentration across samples. The DMSO control and inhibitor (**HA119**) did not show any reduction in MERTK expression. At 500 nM, all PROTACs induced degradation with the most potent from **HA136**, **HA137**, **HA138** and **HA143**, reaffirming the superiority of alkyl linkers. Surprisingly, **HA140** and **HA144** achieved slight degradation of MERTK even though they did not induce detectable ternary complexes. This is possible since the BRET signals in the ternary complex assay are dependent on the orientation and proximity of the sensors, not necessarily the association of the complex (61). Unexpectedly, only **HA143** degraded at 2.5μM concentration. This consistent observation of hook effect in all the other PROTACs warrants further investigation.

At 24 hours, selected compounds were investigated at concentrations of 50 nM, 500 nM and 5 μM (Figure 4.13). **HA136** emerged as the most potent degrader in a concentration-dependent manner, from slight degradation to complete degradation at 5 μM. Comparing the outcome of the 50nM treatment at 20 hours and 24 hours shows the time-dependent degradation also. **HA137** and **HA140** degraded almost equally at 500 nM and 5 μM. Also, no obvious degradation was realized for **HA143** at all time points, which is quite inconsistent with the findings at 6 hours. Further investigations to establish this inconsistency are therefore necessary.

While western blot has been a well-established assay and most employed to assess the degradation capabilities of PROTACs, it has been shown to have major flaws. These include limited dynamic range and lower reproducibility depending on factors like the cell line. Also, this endpoint determination does not offer information on the rate of proteasomal-mediated degradation. The HiBiT assay which is made of the split NLuc sensor system can therefore be employed in this study, to offer comprehensive characterization of PROTAC-induced degradation, even in live cells. In addition, this system offers more accurate and reproducible data with high throughput(27,61). Another concern is the selectivity of these compounds. This can be investigated with proteomic analysis or western blots using antibodies for closely related kinases like AXL, TYRO3 and FLT3.

6. CONCLUSION

This study sought to characterize MERTK PROTACs through the ubiquitin-proteasomal-mediated cascade. To achieve this, a combination of *in-vitro* and cell-based assays were employed. It is a common practice to characterize degraders with biophysical and biochemical assays, however, these do not account for the complexities of the cellular environment, hence sometimes giving misleading results. To this end, proximity-based assays based on the BRET system were developed to study key steps in the cascade such as membrane penetration, binary complexes and ternary complexes.

The compounds demonstrated satisfactory binding to the target through preliminary in-vitro screening with DSF and had favourable cytotoxicity profiles on cells. Further, they exhibited potent binding to both MERTK and CRBN and with a good penetration across the cell membrane. This binding increased with decreasing the length of linkers. Also, alkyl linkers influenced potent binding to MERTK while PEG linkers, to CRBN.

The established assay was extended to study ternary complexes induced by the PROTACs. Stronger ternary complex affinities were observed with increasing the length of alkyl linkers for compounds with amide attachment while the opposite was observed in those with amine attachment. Alkyl linkers were superior to PEG linkers in this series. The ternary complex affinities also strongly correlated with the binding affinities for MERTK positively. However, this assay could not detect ubiquitination, a seemingly general challenge in the field.

Finally, the degradation of MERTK was achieved with these PROTACs in a concentration-dependent manner. This potency of degradation favoured structures with alkyl linkers and in the order of decreasing linker length. **HA136** had the most potent degradation with slight degradation at 50 nM to complete degradation at 5 μM in 24 hours.

These results and knowledge of the SAR of the linkers will guide subsequent lead optimization campaigns. Owing to their progressive performances in all the assays,

PROTACs with alkyl linkers could be prioritized for this purpose. PROTACs with different exit vectors or CRBN ligands with these linkers could be designed for further studies.

The study, however, had a few limitations. The established assays were based on the orientation and distance of the tagged sensors. Hence, as in the case of **HA140** and **HA144**, some active degraders may not have noticeable ternary complex formation, leading to false negatives. Also, structural insights, which would be a good complement to the assays for studying ternary complexes were lacking in this study. Considering the close relation of MERTK to other RTK, proteomic studies to establish the selectivity and off-target profile of these compounds would be helpful. Also, the use of the HiBiT system to study the degradation of the target would help outdo the inconsistencies observed in western blot analysis.

Notwithstanding, the successful establishment and application of these assays would help in studying events induced by MERTK PROTACs while accounting for the cellular environment. The consistency within and between the assays, including the superiority of compounds with alkyl linkers confers credibility for general applicability. These assays due to their efficiency, reproducibility and throughputness will contribute to sustainable assays for early drug discovery campaigns.

7. SUSTAINABILITY ASSESSMENT

Incorporating sustainability in drug discovery is gradually gaining the necessary attention and has led to innovative approaches to meeting our current needs without compromising the future. This study aimed to degrade MERTK, which plays an important role in multiple unmet medical needs like cancers, neurological, cardiovascular disorders and retinitis pigmentosa (RP)(216,217). Hence, characterizing the degradation of MERTK is a part of many campaigns to address these unmet medical needs.

The use of the PROTAC modality in this work adds to sustainability by addressing the underlying cause of various diseases. The inhibitor strategy thus far has been challenged with the inability to block all downstream effects in MERTK activation(119). Complete degradation of this target nullifies all these concerns hence addressing its underlying role in disease pathology which adds to sustainable treatment(50).

The assays established in this study were largely cell-based. While *in-vitro* assays mostly use purified recombinant proteins, the assays established here happen with full-length proteins in cells. They consider the complexity of cellular mechanisms like post-translational modifications and natural competitors and their actual influence on the compound. These are more applicable, giving reliable insight into the behaviours of compounds. In addition, compared to methods like ITC and SPR, the assays employed here demanded little resources and were highly reproducible. These efficient and economical assays add relevant sustainable aspects to drug discovery.

However, the PROTAC modality has a complex design process with a high risk of non-degrading compounds. To save time and resources, the concept of clickable PROTACs could be adopted. This allows the preliminary screening of the compounds through self-assembly in cells bypassing some challenges of current PROTACs. This can lead to prioritizing projects informatively, being efficient, saving resources and enhancing a lean discovery process.

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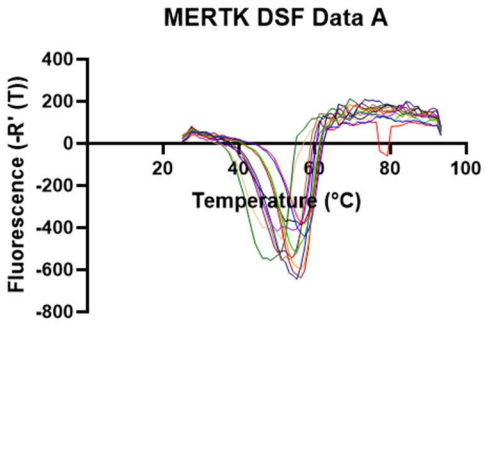
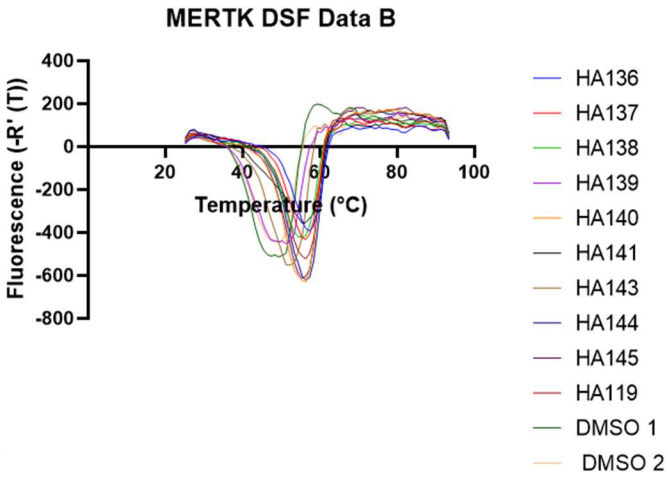
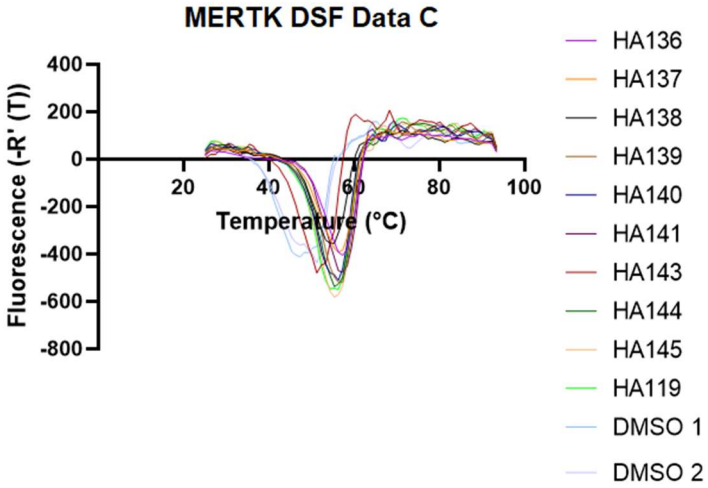
9. SUPPLEMENTARY INFORMATION



SI Figure 1.1

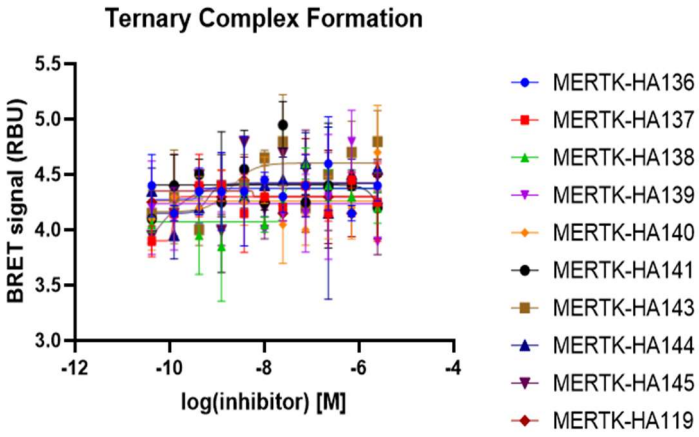


SI Figure 1.2



SI Figure 1.3

SI Figure 1.1 to 1.3: DSF result showing protein melting curves of purified MERTK kinase domain treated with DMSO and all compounds.



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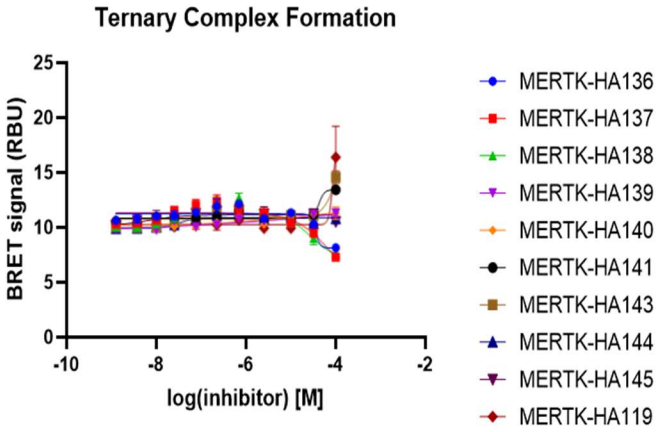

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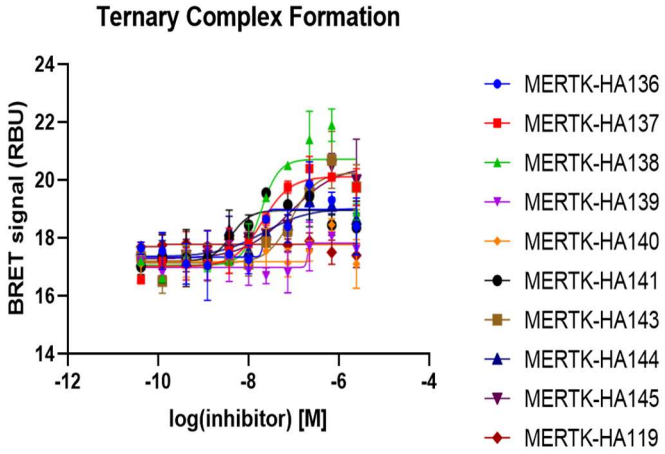


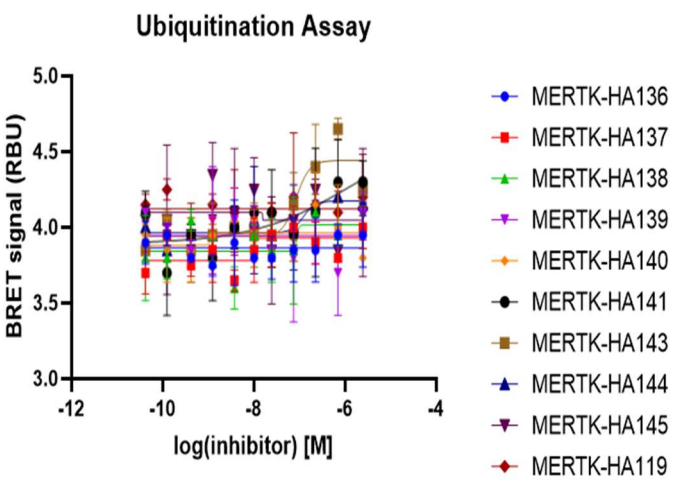
SI Figure 2.3

SI Figure 2.1: Ternary complex affinity determination of all compounds using the NLuc/ C-terminal HaloTag reporter assay. Data were measured in biological replicates with error bars expressing the SD (n = 2).

SI Figure 2.2: Ternary complex affinity determination of all compounds using the established NLuc/ N-terminal HaloTag reporter assay. Compound concentrations were from 1.2nM and 99µM. Data were measured in biological replicates with error bars expressing the SD (n = 2).

SI Figure 2.3: Ternary complex affinity determination of all compounds using the established NLuc/ N-terminal HaloTag reporter assay. Compound concentrations

were from 41pM to 2.5 µM. Data were measured in biological replicates with error bars expressing the SD (n = 2).



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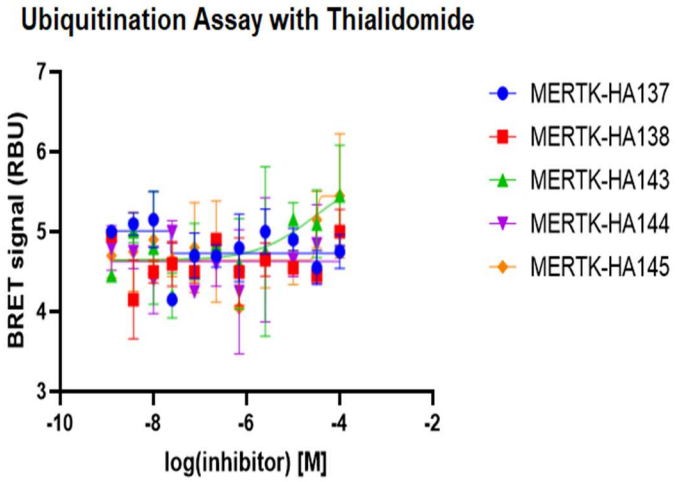
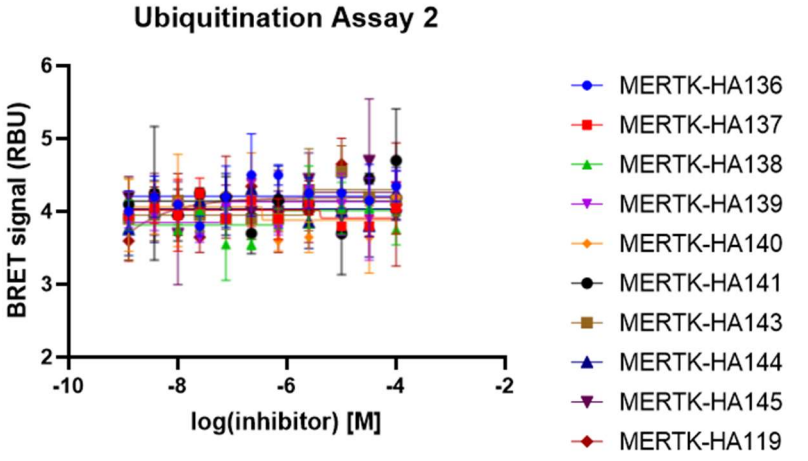


SI Figure 3.2



SI Figure 3.3

SI Figure 3.1: Results of the first ubiquitination assay of all compounds using the NLuc/ ubiquitin HaloTag reporter system. Data were measured in biological replicates with error bars expressing the SD (n = 2).

SI Figure 3.2: Results of the ubiquitination assay of selected compounds using the NLuc/ ubiquitin HaloTag reporter system. These were treated with 10mM

thalidomide. Data were measured in biological replicates with error bars expressing the SD (n = 2).

SI Figure 3.3: Repetition ubiquitination assay of all compounds using the NLuc/ ubiquitin HaloTag reporter system. Data were measured in biological replicates with error bars expressing the SD (n = 2).