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MASTER'S THESIS

**Novel therapeutical strategies targeting NAMPT and
BRD4 in oncology**

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LIST OF ABBREVIATIONS

4T1	Mouse Mammary Carcinoma Cell Line
A2780	Human Ovarian Carcinoma Cell Line
AKT	Protein Kinase B
ALK	Anaplastic Lymphoma Kinase
ANOVA	Analysis Of Variance
AR	Androgen Receptor
BALB/c	Albino Laboratory Mouse Strain
BD1 / BD2	Bromodomain 1 / Bromodomain 2
BET	Bromodomain And Extra-Terminal
BRD2 / BRD3 / BRD4	Bromodomain-Containing Protein 2 / 3 / 4
BRDT	Bromodomain Testis-Specific Protein
BT549	Human Breast Carcinoma Cell Line
C57BL/6	Inbred Laboratory Mouse Strain
Caov-3	Human Ovarian Adenocarcinoma Cell Line 3
CBP	CREB-Binding Protein
CCL5	C-C Motif Chemokine Ligand 5
CCR5	C-C Chemokine Receptor Type 5
CD8	Cluster Of Differentiation 8
CDK9	Cyclin-Dependent Kinase 9
CRBN	Cereblon
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRL4	Cullin-RING Ligase 4
CTD	C-Terminal Domain
DCAF15 / DCAF16	DDB1- And CUL4-Associated Factor 15 / 16
DC₅₀	Degradation Concentration 50
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E2 / E3	Ubiquitin-Conjugating Enzyme / Ubiquitin Ligase
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial-To-Mesenchymal Transition
eNAMPT	Extracellular Nicotinamide Phosphoribosyltransferase
EO771	Mouse Mammary Adenocarcinoma Cell Line
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
ET	Extra-Terminal
FBS	Fetal Bovine Serum
FK866	NAMPT Inhibitor (Daporinad)
GLUT1	Glucose Transporter 1
GrzB	Granzyme B
HCC	Hepatocellular Carcinoma
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HEXIM1	Hexamethylene Bis-Acetamide Inducible 1
HGSOC	High-Grade Serous Ovarian Carcinoma
HL60	Human Promyelocytic Leukemia Cell Line
HRP	Horseradish Peroxidase
HSP70	Heat Shock Protein 70
HyT	Hydrophobic Tagging
IBG	Intramolecular Bivalent Glue
ID8	Mouse Ovarian Surface Epithelial Cell Line
IDO1	Indoleamine 2,3-Dioxygenase 1
IFNγ	Interferon Gamma
IKZF1 / IKZF3	IKAROS Family Zinc Finger 1 / 3
IL-6 / IL-8	Interleukin 6 / Interleukin 8
IMDM	Iscoe's Modified Dulbecco's Medium
iNAMPT	Intracellular Nicotinamide Phosphoribosyltransferase

JQ1	Reference BET Bromodomain Inhibitor
K562	Human Chronic Myelogenous Leukemia Cell Line
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
L02	Human Normal Hepatic Cell Line
LC3	Microtubule-Associated Protein 1A/1B Light Chain 3
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MCF-10A	Human Non-Tumorigenic Mammary Epithelial Cell Line
MD-2	Myeloid Differentiation Factor 2
MDA-MB-231	Human Triple-Negative Breast Cancer Cell Line
MEM	Minimum Essential Medium
mTOR	Mammalian Target Of Rapamycin
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
MYC	Myelocytomatosis Oncogene
NAD⁺	Nicotinamide Adenine Dinucleotide
NAMPT	Nicotinamide Phosphoribosyltransferase
NAPRT	Nicotinate Phosphoribosyltransferase
NF-κB	Nuclear Factor Kappa B
NMN	Nicotinamide Mononucleotide
NMNAT	Nicotinamide Mononucleotide Adenylyltransferase
NR	Nicotinamide Riboside
NRK	Nicotinamide Riboside Kinase
PAK4	P21-Activated Kinase 4
PARP	Poly(ADP-Ribose) Polymerase
PBEF	Pre-B-Cell Colony-Enhancing Factor
PBS	Phosphate-Buffered Saline
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PDGF-BB	Platelet-Derived Growth Factor BB
PI3K	Phosphoinositide 3-Kinase
PIC	Protease Inhibitor Cocktail
PIM1	Proviral Integration Site For Moloney Murine Leukemia Virus 1
PMSF	Phenylmethylsulfonyl Fluoride
POI	Protein Of Interest
PROTAC	Proteolysis-Targeting Chimera
P-TEFb	Positive Transcription Elongation Factor B
RANTES	Regulated On Activation, Normal T Cell Expressed And Secreted
RBM39	RNA Binding Motif Protein 39
RelA	Transcription Factor P65, NF-κB Subunit
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RRID	Research Resource Identifier
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error Of The Mean
SIRT1	Sirtuin 1
SKOV3	Human Ovarian Adenocarcinoma Cell Line
STAT3	Signal Transducer And Activator Of Transcription 3
TGF-β	Transforming Growth Factor Beta
TLR4	Toll-Like Receptor 4
TNBC	Triple-Negative Breast Cancer
TNF	Tumor Necrosis Factor
TPD	Targeted Protein Degradation
TR-FRET	Time-Resolved Fluorescence Resonance Energy Transfer
U42	NAMPT-Targeting PROTAC
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau
ZEB1	Zinc Finger E-Box Binding Homeobox 1

SUMMARY

Background: Nicotinamide phosphoribosyltransferase (NAMPT) is the rate-limiting enzyme of the NAD⁺ salvage pathway, functioning both intracellularly (iNAMPT) to support the metabolic demand of proliferating cells and extracellularly (eNAMPT) as a cytokine within the tumor microenvironment. The BET chromatin reader bromodomain-containing protein 4 (BRD4) fuels oncogenic transcriptional programs but lacks catalytic activity and has therefore long resisted conventional inhibitor design. Both proteins are overexpressed in aggressive tumors and both are difficult to drug by occupancy-based approaches, making them attractive candidates for targeted protein degradation.

Objectives: Here we investigated targeted protein degradation as a pharmacological approach to NAMPT and BRD4 in high-grade serous ovarian carcinoma (HGSOC) and triple-negative breast cancer (TNBC). The objectives were to: 1) characterize the expression and secretion of NAMPT in ovarian cancer cell lines and evaluate the novel PROTAC U42 as a degrader of both forms of the enzyme; 2) screen a library of newly synthesized compounds targeting BRD4 in TNBC and determine whether their antiproliferative activity was due to true engagement of BRD4.

Materials and Methods: In vitro models included murine (ID8) and human (Caov-3, SKOV3) ovarian cancer cell lines and murine TNBC cell line 4T1. Cell viability was measured by MTT assay. Protein levels of iNAMPT, eNAMPT and BRD4 were measured by Western blotting. Concentrated conditioned media were used to measure eNAMPT. Target engagement for the BRD4-directed compounds was assessed using a cell-free TR-FRET binding assay.

Results: iNAMPT was expressed by all ovarian cancer cell lines, with the highest levels found in Caov-3. eNAMPT secretion was highly cell-line-dependent and almost undetectable in SKOV3. PROTAC U42 decreased cell viability to less than 50% only in ID8 cells and caused the parallel degradation of iNAMPT and eNAMPT with a DC₅₀ below 0.1 μM, and a clear hook effect. The human cell lines were refractory both to degradation and to cytotoxicity. Screening of two series of BRD4-targeting compounds in 4T1 cells led to the identification of several molecules more cytotoxic than JQ1, the molecular glue SMA-545 being the most active of the library. However, neither of the designed degraders decreased BRD4 protein levels, and none of the compounds displaced the acetylated ligand from the bromodomain module, whereas JQ1 behaved as expected in the same assay.

Conclusions: U42 is an effective degrader of intracellular and extracellular NAMPT and represents a promising pharmacological tool in ovarian cancer, although its efficacy is strictly dependent on the cellular background. The new BRD4-targeting series, based on the JQ1 scaffold, was cytotoxic without binding to its intended target. The conclusion was reached only from the target engagement experiments, highlighting the significance of these experiments in the evaluation of novel degraders.

1. INTRODUCTION

1.1 Novel therapeutic strategies in cancer

Classical small-molecule inhibitors

For many years, drug discovery in oncology has focused on small-molecule inhibitors of the catalytic activity of proteins that promote tumor growth. They are classified by their mode of interaction with the target enzyme: competitive inhibitors bind the active site and compete with the natural substrate, while non-competitive inhibitors bind a distinct allosteric site and cause a conformational change that leads to decreased catalytic activity, irrespective of the substrate concentration (1, 2).

FK866 is a highly specific, non-competitive inhibitor of Nicotinamide phosphoribosyltransferase (NAMPT) identified through high-throughput screening, which induces tumor cell apoptosis through gradual depletion of the intracellular coenzyme NAD⁺ (3). A first-in-human phase I study subsequently evaluated FK866 as a continuous 96-hour infusion in patients with advanced, treatment-refractory solid tumor malignancies, to characterize its maximum-tolerated dose, toxicity profile, and pharmacokinetics (4).

Limitations of the occupancy-driven paradigm

Most NAMPT inhibitors, such as FK866, are dose-dependent toxic and have poor pharmacokinetic profiles, and no NAMPT inhibitor has been approved for clinical use as of yet (5). Most enzyme inhibitors bind reversibly, and thus require high drug levels to continuously occupy the active site, a paradigm known as occupancy-driven pharmacology. There is no way to address the non-enzymatic scaffolding functions that many disease-associated proteins, including NAMPT, perform independently of their catalytic activity (6, 7).

Dual inhibitors

A way to overcome these limitations has been the design of dual inhibitors that target NAMPT and a second protein simultaneously, also solving the pharmacokinetic problems that arise from combining separate molecules. KPT-9274 (ATG-019) is a dual NAMPT and p21-activated kinase 4 (PAK4) inhibitor that reduces nuclear β -catenin and its downstream targets cyclin D1 and c-Myc, reduces invasion and migration, induces apoptosis in renal cell carcinoma models, with dose-dependent tumor growth inhibition and no apparent toxicity in xenografts (8). STF-31, a dual NAMPT/GLUT1 inhibitor, retains tumor-suppressive activity at low concentration,

unlike other GLUT-selective inhibitors (9). A dual NAMPT/IDO1 inhibitor showed potent antitumor efficacy without significant toxicity in a taxol- and FK866-resistant non-small cell lung cancer model, while increasing sensitivity to taxol (10).

Toward event-driven pharmacology: targeted protein degradation

Unlike traditional drug discovery, which aims at the direct regulation of protein activity, targeted protein degradation (TPD) removes disease-related proteins by hijacking the cell's own destruction machinery, with PROTACs and molecular glue degraders as its most promising modalities (Fig. 1) (1). It has provided therapeutic opportunities for previously undruggable targets, such as transcription factors (MYC, STAT3), mutant oncoproteins (KRAS G12C) and scaffolding molecules without canonical binding pockets (11). Degraders eliminate the target rather than inhibit part of its function, thus decreasing resistance due to long-term selection pressure and acting substoichiometrically at low concentrations (1).

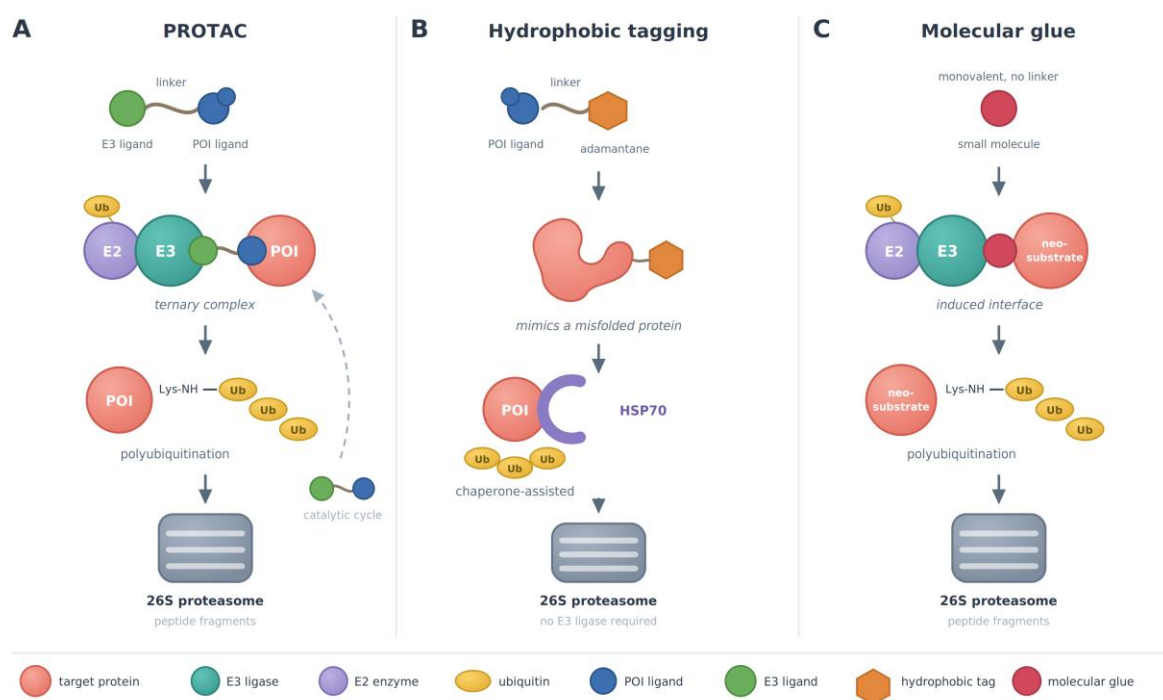


Figure 1. Comparison of three targeted protein degradation strategies. (A) PROTACs are bifunctional molecules comprising a warhead that binds to the protein of interest (POI) and an E3 ligase ligand that are connected by a flexible linker. The formation of the POI–PROTAC–E3 ternary complex allows the E2–E3 machinery to polyubiquitinate the target on a lysine residue and direct it to the 26S proteasome. Because the PROTAC is released intact, one molecule can induce multiple rounds of degradation. (B) Hydrophobic tagging involves coupling a POI-binding ligand to a hydrophobic group (e.g., adamantane) mimicking a misfolded protein surface, which recruits chaperones such as HSP70 and directs the target to proteasomal degradation without the requirement of a specific E3 ligase. (C) Molecular glues are monovalent small molecules that do not possess a linker and operate at the interface between an E3 ligase and a neosubstrate, stabilizing an induced protein–protein interaction that leads to ubiquitination and degradation. Original schematic prepared for this thesis.

PROTAC (proteolysis-targeting chimera)

PROTAC molecules consist of an E3 ligase recruiting ligand, a POI targeting warhead and a flexible linker connecting both. Induction of a POI-PROTAC-E3 ternary complex leads to ubiquitination and subsequent proteasomal degradation of the POI, hijacking the machinery that usually regulates protein turnover in the cell (1, 12). Among the many known E3 ligases, cereblon (CRBN) and von Hippel-Lindau (VHL) remain the most utilized (1). The first PROTAC to enter the clinic was ARV-110 (bavdegalutamide), which selectively degrades wild-type androgen receptor and most clinically relevant AR mutants in castration-resistant prostate cancer; the most advanced compound to date is ARV-471 (vepdegestrant), an oral PROTAC that degrades the estrogen receptor through CRBN recruitment and has advanced through phase III clinical evaluation (1).

HyT (hydrophobic tagging)

HyT has advantages over PROTACs such as lower molecular weight, no teratogenic risk associated with thalidomide-derived CRBN ligands, and a mechanism independent of the recruitment of specific E3 ligases (13). It mimics a misfolded protein form causing unfolded protein response, promoting recruitment of molecular chaperones and subsequent proteasomal degradation (13). Classical HyT molecules consist of a ligand that selectively binds the POI and a hydrophobic tag mimicking the misfolded conformation; only a few tags have been identified, including adamantane, fluorene and pyrene (14).

Norbornene was recently identified as a new hydrophobic tag and conjugated to an ALK ligand to generate the degrader Hyt-9, which reduced ALK levels through Hsp70 and the ubiquitin-proteasome system (14); norbornene- and bornane-based tags were later developed to overcome E3 ligase downregulation resistance (15).

Molecular glue degraders

Unlike the linker-based approach, molecular glue degraders induce or stabilize the interaction between a POI and an E3 ubiquitin ligase without a linker connecting two separate binding moieties, instead interacting with either one of the two proteins, usually the E3 ligase (1). The first molecular glue degraders identified, thalidomide, lenalidomide, and pomalidomide, were approved for the treatment of different tumors before the understanding of their mechanism of action, and were later found to induce the interaction of the transcription factor substrates IKZF1 and IKZF3 with CRBN, leading to their degradation (1).

Another class of molecular glues, the anticancer aryl-sulfonamides, was discovered by a different mechanism. They induce proteasomal degradation of the splicing factor RBM39 by promoting direct binding of RBM39 to DCAF15, a substrate receptor of the CRL4 E3 ligase complex, through extensive protein–protein contacts that compensate for the relatively low affinity of the sulfonamide itself (16). Indisulam has demonstrated activity in preclinical models of neuroblastoma and acute myeloid leukemia and has a favorable clinical safety profile (16).

All three modalities have since been applied directly to the two therapeutic targets addressed in this thesis, NAMPT and bromodomain-containing protein 4 (BRD4), and the corresponding degraders are described in detail in the following sections.

Comparing novel degradation strategies with classical inhibitors

Classical inhibitors require continuous engagement of a binding site of a target, whereas PROTACs, HyT and molecular glue degraders all induce or stabilize a single molecular event that re-directs the target protein to the cell's own degradation machinery (1). Importantly, PROTAC efficacy depends on the expression of a compatible E3 ligase and is thus vulnerable to E3 ligase downregulation as a resistance mechanism. HyT circumvents this limitation by utilizing the endogenous protein quality control machinery (13, 15). PROTAC selectivity can also be improved by tumor microenvironment-responsive functionalization, as in a glutathione-responsive PROTAC that stays inactive until reactivated by the high glutathione levels of tumor cells (17).

Molecular glue degraders do not require a synthetic linker or hydrophobic tag and work through the intrinsic or induced affinity of the target for an E3 ligase, either through relatively high-affinity ligands such as the interaction of CRBN with IKZF1/3 exploited by thalidomide analogs, or large protein–protein interfaces that compensate for low affinity of binding, as demonstrated for indisulam and DCAF15 (1, 16). They lack a linker and have a lower molecular weight than for PROTACs, which may be advantageous for pharmacokinetics but considerably complicates rational design (1).

1.2 Therapeutic targets in cancer

1.2.1 Nicotinamide phosphoribosyltransferase (NAMPT)

Nicotinamide phosphoribosyltransferase (NAMPT) is a pleiotropic protein that acts as the rate-limiting enzyme in the mammalian NAD⁺ salvage pathway and is essential for the regulation of cell metabolism, reprogramming, aging and apoptosis (Fig. 2) (18).

NAMPT is a homodimeric type II phosphoribosyltransferase with two active sites located at the interface of the two monomers (19, 20). The enzyme is present in many tissues including bone marrow, liver and muscle and is produced by immune cells, cardiomyocytes, neurons and cancer cells (18). The enzyme exists in two different functional forms: intracellular NAMPT (iNAMPT) and extracellular NAMPT (eNAMPT) (18, 20).

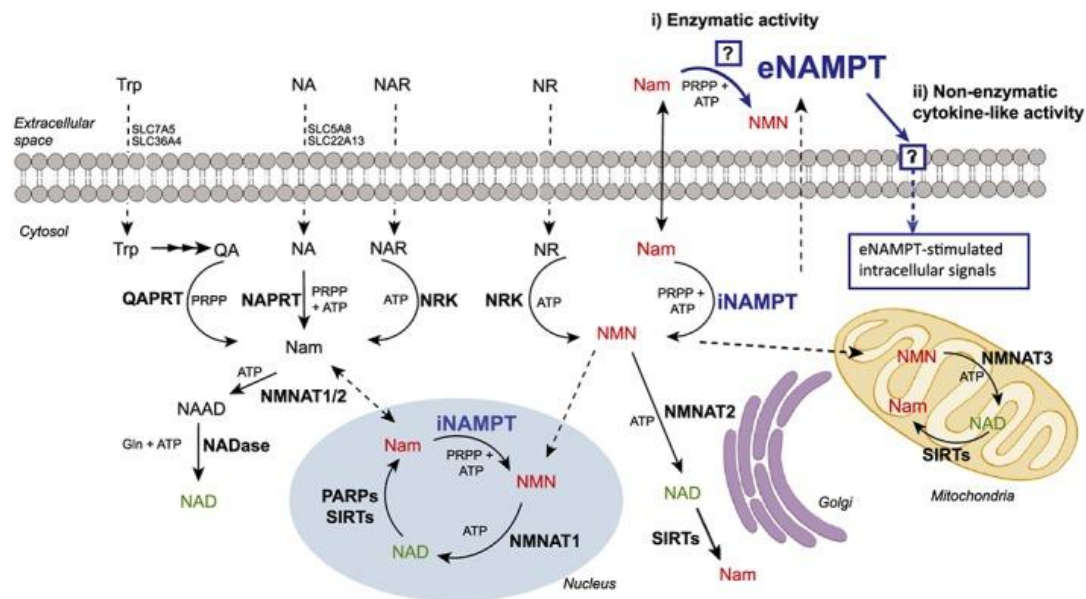


Figure 2. NAD⁺ biosynthesis pathways and NAMPT localization. NAD⁺ is generated by three converging pathways: the *de novo* pathway from tryptophan, the Preiss–Handler pathway from nicotinic acid via the nicotinate phosphoribosyltransferase (NAPRT) and the salvage pathway from nicotinamide via NAMPT, the rate-limiting enzyme. Nicotinamide riboside (NR) enters the same pathway via the NR kinases (NRK). Intracellular NAMPT (iNAMPT) shuttles between cytosol, nucleus and mitochondria, where NMNAT1–3 complete NAD⁺ synthesis. eNAMPT has a dual function as an ectoenzyme that produces NMN and as a cytokine-like factor that induces intracellular signaling. Reproduced from Grolla et al. (21), under a CC BY-NC-ND 4.0 license.

Physiological roles of iNAMPT

iNAMPT is a ubiquitous protein mainly localized in the cytoplasm, nucleus and mitochondria of most mammalian tissues, with high expression in brown adipose tissue, liver and kidney (18, 20). Its main role is enzymatic, as it directly regulates intracellular NAD⁺ concentrations and indirectly regulates the activity of NAD⁺-dependent enzymes, such as sirtuins and poly(ADP-ribose) polymerases (18, 19). Transient autophosphorylation of histidine 247 tightly controls catalytic efficiency by markedly increasing the affinity for nicotinamide, while stabilizing the interface between the two monomers of the active dimer (19).

The role of iNAMPT in cancer

iNAMPT overexpression is a prominent feature of malignant cells to satisfy their rapid proliferation and metabolic requirements, an important aspect of tumor metabolic reprogramming that promotes the Warburg effect and high NAD⁺ turnover (18, 22).

Under physiological conditions, iNAMPT is predominantly cytosolic, but genotoxic and oxidative stress characteristic of cancer cells facilitates its interaction with glyceraldehyde-3-phosphate dehydrogenase and the formation of a stable complex that translocates iNAMPT to the nucleus, where local production of NMN rescues NAD⁺ and counteracts the activation of NAD⁺-consuming enzymes such as PARPs and sirtuins (23).

While its overexpression in cancer presents a possible therapeutic target, it is also associated with acquired resistance to a number of chemotherapeutic agents (24). Sauriol and colleagues showed that tumoral iNAMPT is a key player in drug resistance in high-grade serous ovarian carcinoma (HGSOC), one of the tumor models used in this thesis (25). Since the salvage pathway is the main route of NAD⁺ synthesis in these cells, iNAMPT-mediated production is crucial to preserve PARP1 activity during the DNA damage response, and its pharmacological inhibition has been proposed to deplete intracellular NAD⁺ and sensitize HGSOC cells to PARP inhibitors (25).

Physiological roles of eNAMPT

eNAMPT pleiotropically functions as an enzyme, cytokine and growth factor linking metabolic regulation and immune responses (18). Its historical names, pre-B-cell colony-enhancing factor (PBEF) and visfatin, reflected the circumstances of its discovery (18, 26).

Physiologically, secretion of eNAMPT is not due to cell lysis or death, but is an active process stimulated by cellular stress, nutritional cues and inflammatory signals (27), and circulating levels show a diurnal rhythmicity that drives circadian oscillations in hypothalamic NAD⁺ levels, locomotor activity and energy expenditure (28). eNAMPT lacks the signal peptide required for classical ER-Golgi secretion and is thought to be secreted via a non-classical pathway insensitive to brefeldin A and monensin (27). One proposed trigger is SIRT1-mediated deacetylation of lysine 53 on iNAMPT, which primes it for packaging into extracellular vesicles and release, predominantly from adipose tissue (29).

In terms of signaling, eNAMPT interacts with several cell-surface receptors, two of which are considered major. CCR5 is overexpressed in cancers such as breast cancer and melanoma.

eNAMPT is a natural antagonist of this receptor and competes with the endogenous ligand CCL5 and inhibits RANTES mediated activities (30). Conversely, eNAMPT acts as an agonist of TLR4 because of its structural similarity to MD-2, the co-receptor required for LPS-induced TLR4 activation, and activates pro-inflammatory NF- κ B signaling in the absence of bacterial infection (31). A controversial insulin-mimetic activity has also been proposed (26) but has not been reconfirmed in subsequent independent studies (32). These functions are mediated through complementary mechanisms as an ectoenzyme converting extracellular nicotinamide to NMN and as an adipocytokine involved in systemic metabolic homeostasis (27), as a cytokine or damage-associated molecular pattern activating NF- κ B, MAPK and STAT3 to promote immune cell chemotaxis (27, 31) and as a pro-angiogenic factor that stimulates endothelial proliferation and migration through PI3K/AKT/mTOR and MAPK signaling, upregulating VEGF and VEGF receptor 2 and matrix metalloproteinases (27).

The role of eNAMPT in cancer

Elevated circulating eNAMPT levels are well recognized in solid tumors and hematological malignancies where eNAMPT contributes to tumor progression via multiple mechanisms (27). Obesity-related cancers such as breast, endometrial and colorectal cancer are characterized by overexpression, generally associated with worse prognosis and advanced stage. Circulating levels correlate positively with tumor size, stage and lymph node invasion and negatively with overall and disease-free survival, making eNAMPT a candidate diagnostic and prognostic biomarker (27). Preclinically, eNAMPT promotes proliferation of cancer cells through PI3K/AKT, MAPK/ERK and Notch1/NF- κ B signaling pathways and protects malignant cells from apoptosis and oxidative DNA damage (24), enhances migration and invasion via TGF- β - and NF- κ B/Snail-driven EMT and induces VEGF expression (24, 27). It also favors an immunosuppressive tumor microenvironment through M2 macrophage polarization and mediates therapy resistance by enhancing the antioxidant capacity of tumor cells (27).

In triple-negative breast cancer (TNBC), one of the tumor models used in this thesis, eNAMPT has been shown by Moro and colleagues to act as a potent chemoattractant for pericytes and synergize with platelet-derived growth factor-BB (PDGF-BB) to activate them via NF- κ B, resulting in abnormal, heavily pericyte-covered vessels (33), and neutralization using a selective monoclonal antibody decreased tumor growth and metastatization in a murine TNBC model (33, 34). eNAMPT also promotes the immune evasion of TNBC via PD-1/PD-L1 axis (34). Together, these data show that the enzymatic and cytokine-like activities of NAMPT

converge to drive tumor growth, chemoresistance and immune evasion, providing a strong rationale for targeting both iNAMPT and eNAMPT.

1.2.2 Bromodomain-containing protein 4 (BRD4)

BRD4 is a member of the Bromodomain and Extra-Terminal (BET) family, a family of paralogous, evolutionarily conserved chromatin readers that also includes BRD2, BRD3 and the testis-specific protein BRDT. Each of them contains two tandem bromodomains that recognize acetylated lysine residues on histones and therefore function as chromatin-targeting modules that interpret the pattern of histone acetylation to regulate transcription during cell proliferation and differentiation (Fig. 3) (35).

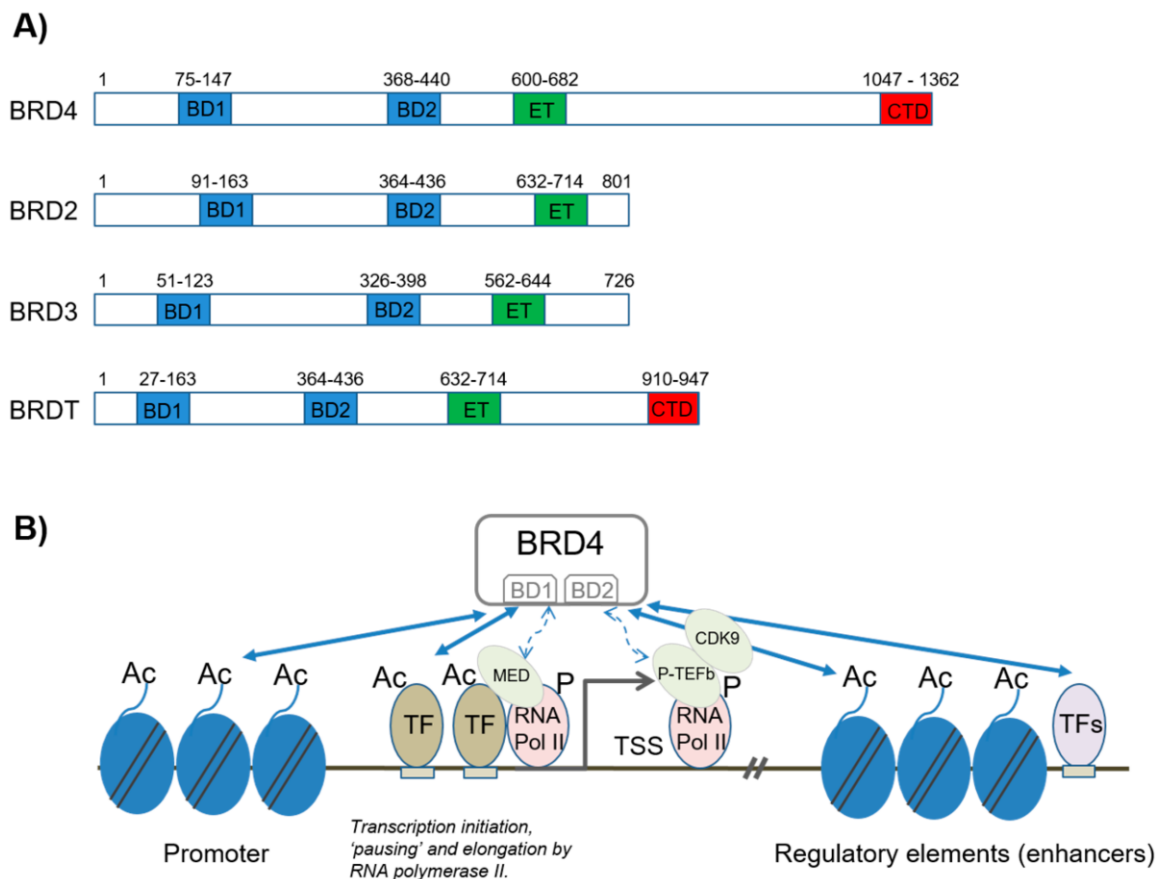


Figure 3. Domain architecture and transcriptional function of the BET family. (A) Domain organization of BRD4, BRD2, BRD3 and BRDT. Two tandem bromodomains (BD1, BD2), the extra-terminal (ET) domain and the C-terminal domain (CTD) only present in BRD4 and BRDT. (B) BRD4 is recruited to acetylated histones at promoters and enhancers where it interacts with Mediator complex and positive transcription elongation factor b (P-TEFb), whose catalytic subunit is cyclin-dependent kinase 9 (CDK9), to drive RNA polymerase II phosphorylation and transition from transcriptional pausing to productive elongation. Reproduced from Hajmirza et al. (36), under a CC BY 4.0 license.

Physiological roles of BRD4

The bromodomains of BRD4 bind to acetylated chromatin and recruit P-TEFb, a heterodimer of CDK9 and a cyclin T subunit, through its C-terminal domain. P-TEFb phosphorylates the C-terminal domain of RNA polymerase II and thus enables productive elongation at target promoters (36, 37). The stability of this complex is regulated by the 7SK/HEXIM1 ribonucleoprotein complex that sequesters and inactivates P-TEFb (37).

In addition to transcriptional elongation, BRD4 is also bound to chromatin throughout the cell cycle and involved in DNA damage repair, checkpoint activation, and telomere maintenance during mitosis (38), as well as the maintenance of embryonic stem cell identity and the regulation of osteoblast differentiation, adipogenesis, hematopoiesis, and immune and nervous system development postnatally (39).

The role of BRD4 in cancer

Dysregulated BRD4 activity is implicated in several disease states including viral infection, chronic inflammation and cancer due to its central role in transcriptional control (39). In cancer, BRD4 is present as three splice isoforms with opposite functions: a long isoform (BRD4-L) with tumor-suppressive activity and two short isoforms, BRD4-S(a) and BRD4-S(b), acting as oncogenic drivers (40).

BRD4 also binds acetylated lysines on non-histone proteins such as RelA, the p65 subunit of NF- κ B. The p300/CBP complex acetylates RelA at lysine-310, creating a docking site for the BRD4 bromodomains; BRD4 is then recruited to NF- κ B target promoters, where it engages CDK9 to phosphorylate RNA polymerase II, maintaining constitutively active NF- κ B signaling and the release of pro-inflammatory cytokines such as IL-6 and TNF that contribute to inflammation, angiogenesis and metastasis (41). Consistently, treatment of TNBC cells with a BRD4 inhibitor disrupting this interaction decreased IL-6 and IL-8 expression, modulated the EMT markers Snail1, Vimentin and ZEB1 and led to senescence, DNA damage and decreased invasive capacity (42).

Another oncogenic process linked to BRD4 activity is related to c-Myc, a master regulator of cell proliferation (43, 44). The activation of c-Myc drives transcriptional programs that control cell division, metabolic adaptation and survival (43, 44). In TNBC, c-Myc is often overexpressed and supports histone lysine acetylation, reinforcing BRD4 recruitment to chromatin and amplifying its transcriptional output (43). In addition to breast cancer, focal and

recurrent amplification of the BRD4 gene characterizes a subgroup of high-grade serous ovarian carcinomas that sustain an oncogenic transcriptional program and sensitivity to BET inhibition (45).

This provided a strong rationale for the pharmacological targeting of BRD4 in cancer, but BRD4 has no intrinsic catalytic activity and thus remained a difficult target for conventional inhibitor design until the discovery that thienodiazepine-based compounds could bind its bromodomains led to JQ1, the first selective BET bromodomain inhibitor (46). NAMPT is a metabolic enzyme and BRD4 is an epigenetic reader; they are in different functional classes but convergent evidence places them on a single regulatory axis in the tumors studied in this thesis. In TNBC, NAMPT was found to be a dependency gene whose expression is driven by a tumor-specific super-enhancer located approximately 40 kb downstream of its promoter, and CRISPR-mediated deletion of a single constituent enhancer abolished NAMPT induction and blocked tumorigenesis and metastasis in xenograft models (47). Since BRD4 is the major reader implicated in super-enhancer organization and oncogene transcription (38), the transcriptional machinery responsible for NAMPT expression may represent an indirectly targetable vulnerability for BET-directed strategies. Indeed, functional convergence has been described in models of hepatocellular carcinoma (HCC), in which BRD4 inhibition leads to downregulation of NAMPT, and concomitant NAMPT and BRD4 blockade inhibits NAD⁺ generation via the salvage and Preiss–Handler routes, respectively, a rationale that led to the first dual NAMPT/BRD4 inhibitors (48).

1.3 Novel therapeutic strategies applied to NAMPT and BRD4

1.3.1 NAMPT as a therapeutic target: novel strategies

As discussed above, NAMPT is a promising therapeutic target in cancer through alteration of NAD⁺ metabolism. Therefore, targeting the vulnerability of NAMPT, especially in combination with other therapies, has the potential to overcome resistance to current therapies and improve treatment efficacy. A number of complementary pharmacological approaches, from classical small-molecule inhibitors to targeted protein degradation and antibody-based neutralization, have been developed to exploit this vulnerability (18).

Small-molecule NAMPT inhibitors

Small-molecule NAMPT inhibitors have been primarily designed to target occupancy-based inhibition of the enzyme's catalytic activity, on the assumption that depletion of intracellular

NAD⁺ leads to metabolic failure and cell death (6). The first potent non-competitive inhibitor to be characterized in detail was FK866, which displayed high affinity for the nicotinamide-binding pocket and efficiently induced apoptosis in a variety of cancer models (3); however, clinical development was hampered by dose-limiting thrombocytopenia and gastrointestinal distress, a pattern subsequently observed with the pyridine cyanoguanidine inhibitor CHS828/GMX1778, which was withdrawn from phase I trials due to gastrointestinal bleeding (5).

Specifically for ovarian cancer, several NAMPT inhibitors showed sub-nanomolar antiproliferative activity toward A2780 cells and potent tumor growth inhibition in xenograft models. Triazole-based compounds designed by structure-activity relationship studies showed nanomolar antiproliferative and anti-inflammatory activity in the same line, although they often need further pharmacokinetic optimization (49). The main disadvantage of these series is still the systemic depletion of NAD⁺ in healthy blood cells (5).

Targeted protein degradation: PROTACs and HyT

Targeted protein degradation (TPD) approaches represent a paradigm shift in NAMPT-directed drug discovery to overcome the limitations of classical NAMPT inhibitors, as they eliminate the target protein via an event-driven mechanism rather than relying on continuous occupancy of its catalytic site (1).

Among NAMPT-targeting PROTACs, the Sheng group developed the first NAMPT-directed PROTAC, PROTAC A7, which degrades iNAMPT at low nanomolar concentrations and concomitantly reduces eNAMPT levels, thus targeting both the intracellular enzymatic and the extracellular cytokine-like functions of the protein, and which suppresses the expansion of myeloid-derived suppressor cells to improve antitumor immunity in murine models of colorectal cancer (50). A subsequent compound, PROTAC B3, showed a half-maximal degradation concentration of less than 0.17 nM and maximal degradation of greater than 90% in A2780 ovarian cancer cells, with significant tumor growth inhibition in a xenograft model and no evidence of toxicity (51). This substoichiometric catalytic mode allows a single molecule to mediate multiple rounds of degradation, and in therapy-resistant malignancies such as HGSOC and TNBC complete removal of the protein scaffold may help to overcome compensatory resistance that persists despite enzyme inhibition alone (51). PROTACs still suffer from the “hook effect” where high concentrations paradoxically decrease degradation

efficiency as excess drug forms unproductive binary complexes rather than the required ternary complex (1).

Further NAMPT-targeted PROTACs have been rationalized on this scaffold for particular applications such as a photoswitchable version that enables reversible light-controlled NAMPT degradation with less toxicity, a fluorescent theranostic PROTAC that enables real-time monitoring of degradation in live cells, and a drugtamer-conjugated PROTAC that enhances tumor-selective delivery (52, 53, 54). NAMPT has been also targeted through the autophagy-lysosome pathway. Autophagosome-tethering compounds (ATTECs) were obtained by conjugation of a NAMPT inhibitor with ispinesib, an LC3-binding ligand. The optimized compound showed significant NAMPT degradation and potent antiproliferative activity in ovarian cancer cells, through lysosome-mediated autophagy instead of the ubiquitin-proteasome system (55).

An alternative TPD strategy for NAMPT is hydrophobic tagging, where a POI-binding ligand is conjugated to a hydrophobic tag mimicking a misfolded protein state to recruit chaperones and proteasomal machinery, avoiding both the higher molecular weight and the teratogenic risk of thalimide-derived CRBN ligands (13). The first HyT-based NAMPT degraders were obtained by tethering adamantane and norbornene to known NAMPT inhibitors; the most potent, NH-11, induced dose- and time-dependent degradation of NAMPT via the ubiquitin-proteasome system selectively in the leukemia lines K562, HL60 and Jurkat, with significant antiproliferative activity, apoptosis and S-phase arrest in K562 cells and no significant cytotoxicity toward the normal lines L02 and MCF-10A (56).

Antibody-mediated neutralization of eNAMPT

The targeting of eNAMPT with monoclonal antibodies to neutralize its cytokine-like signaling has emerged as a promising biologic approach for blocking extracellular NAMPT without the systemic toxicities of intracellular NAD⁺ depletion (34).

Mechanistically, eNAMPT neutralization simultaneously impacts several hallmarks of cancer. The anti-eNAMPT antibody C269, developed in the Grolla laboratory, counteracts T-cell immune evasion by downregulating the PD-1/PD-L1 axis: eNAMPT directly induces PD-L1 expression on tumor cells and PD-1 on T lymphocytes, an effect reverted by C269, which restores anti-tumoral immunity through activated CD8⁺IFN γ ⁺GrzB⁺ cytotoxic T cells and a reduced immunosuppressive phenotype of regulatory T cells; in two syngeneic TNBC models,

4T1 cells in BALB/c mice and EO771 cells in C57BL/6 mice, biweekly administration significantly decreased tumor size, spleen weight and lung metastases (34). C269 also abrogated the pericyte-recruiting, pro-angiogenic activity of eNAMPT in the tumor vasculature (33).

Aqualung Therapeutics has produced a humanized anti-eNAMPT monoclonal antibody (ALT-100) for inflammatory lung diseases in which persistent tissue injury is mediated by eNAMPT/TLR4 signaling; a first-in-human phase 1 study in healthy volunteers showed that single intravenous doses were well tolerated, with dose-proportional pharmacokinetics and no serious adverse events (57). Taken together, these approaches demonstrate that NAMPT can be targeted at three different levels: inhibition of catalytic activity, removal of the protein itself and neutralization of its extracellular signaling.

1.3.2 BRD4 as a therapeutic target: novel strategies

Since BRD4 accumulates as a compensatory response to long-term treatment with classical BET inhibitors, some research groups have explored PROTAC-based degradation as an alternative strategy to physically remove the protein rather than just blocking its bromodomains (58).

Most BRD4-targeting PROTACs use a triazoloazepine warhead derived from JQ1 (46) or the related clinical inhibitor OTX015 and differ mainly in the E3 ligase-recruiting ligand (59). The first VHL-based BET degrader, ARV-771, achieved pan-BET degradation with a DC_{50} below 1 nM in castration-resistant prostate cancer models while inhibiting AR signaling and c-Myc more potently than BET inhibition alone, producing tumor regression in a xenograft model (59). ARV-825, obtained by conjugating OTX015 to pomalidomide, induced rapid and sustained BRD4 degradation in Burkitt's lymphoma cell lines with more durable c-Myc suppression and greater antiproliferative activity than JQ1 or OTX015 alone (58), and in acute myeloid leukemia models the CRBN-based degrader dBET1 likewise induced BRD4 degradation, downregulated MYC and PIM1 and reduced tumor size (60).

In TNBC, the other tumor model investigated in this thesis, both MZ1, a VHL-based degrader preferentially targeting BRD4 over BRD2 and BRD3, and ARV-825 showed strong antiproliferative activity in MDA-MB-231 and BT549 cells in 2D and 3D culture, with similar efficacy in a JQ1-resistant MDA-MB-231 subline (61). BETd-246, a CRBN-based degrader designed specifically for this tumor type by linking BETi-211 to thalidomide, achieved almost

complete depletion of BRD2, BRD3 and BRD4 in TNBC cell lines within one hour, with markedly stronger growth inhibition and apoptosis than BETi-211 alone, an effect further validated *in vivo* (62).

Besides linker-based degraders, the molecular glue modality has been also applied to BRD4. Intramolecular bivalent glues (IBGs) degrade BRD2 and BRD4 by a mechanism distinct from PROTACs: instead of recruiting target and E3 ligase in trans, they bind to two adjacent bromodomains of BRD4 in cis, which improves its intrinsic affinity for the E3 ligase substrate receptor DCAF16 (63). The lead compound IBG1 selectively degrades BRD2 and BRD4 over the paralogue BRD3 in a CRL4-DCAF16-dependent and DCAF15-independent manner, and structural characterization of the ternary complex guided the design of improved degraders with low picomolar potency (63). A growing pipeline of PROTACs using both CRBN and VHL ligases is now under clinical evaluation in solid and hematologic malignancies and autoimmune conditions (1).

2. OBJECTIVE OF THE THESIS

The main aim of this thesis was to study targeted protein degradation as a pharmacological approach against two proteins that have been challenging to target with conventional methods, NAMPT and BRD4, in two aggressive tumor models: high-grade serous ovarian carcinoma and triple-negative breast cancer.

The first part of my work was aimed at extending the investigation of the NAMPT pathway to ovarian cancer, a tumor known to be highly dependent on NAD⁺ metabolism for its growth and survival. Since this is a relatively new area of research in our laboratory, my first aim was to establish a baseline profiling of NAMPT expression and secretion in a panel of mouse (ID8) and human (Caov-3 and SKOV3) ovarian cancer cell lines, distinguishing between the intracellular and the extracellular form of the enzyme. With this characterization, my second objective was to test a new pharmacological strategy against NAMPT using PROTAC technology. Specifically, I tested U42 on ovarian cancer cells, a novel degrader previously generated and validated in our laboratory in breast cancer, to evaluate its cytotoxic and degradation activity.

In the second part of my thesis, I switched from a validated degrader to a library of newly synthesized molecules and focused on BRD4, a chromatin reader without any catalytic activity and therefore long resistant to traditional inhibitor design. Together with the collaboration with a medicinal chemistry group in Czech Republic, I evaluated the antiproliferative activity of a panel of new BRD4 compounds based on classical JQ1 inhibitor on the 4T1 murine TNBC model. The identification of the most active molecules was intended to give a rational starting point for their future development into degraders.

3. RESULTS AND DISCUSSION

3.1 Investigation of NAMPT as a therapeutic target in ovarian cancer

As mentioned in the introduction, NAMPT is up-regulated in several cancers, including ovarian cancer (22). Our laboratory showed that in TNBC eNAMPT, as a cytokine, drives the formation of an aberrant, pericyte-rich vasculature and supports immune evasion through the PD-1/PD-L1 axis (33, 34). Because ovarian cancer is heavily dependent on NAMPT-mediated energy pathways, targeting it has become a major area of research.

We started by determining basal expression of iNAMPT and eNAMPT in a murine ovarian cancer cell line (ID8) and two human ovarian cancer cell lines (Caov-3 and SKOV3) (Fig. 4). Western blot analysis of cell lysates showed that all three cell lines express iNAMPT, with Caov-3 cells expressing the highest levels and SKOV3 and ID8 expressing the protein to a broadly similar extent (Fig. 4A). Conversely, a different pattern was found for the secreted form: whereas Caov-3 cells released the protein strongly into the conditioned medium, eNAMPT levels were drastically lower in ID8 and almost undetectable in SKOV3 (Fig. 4B). These findings confirm that all assessed ovarian cancer cells express iNAMPT and its secretion is cell-line-dependent. This is consistent with the observations of Zhu and colleagues who found that all tumor cell lines expressing iNAMPT released the extracellular form, but that their eNAMPT secretion capacity was significantly different between models (7).

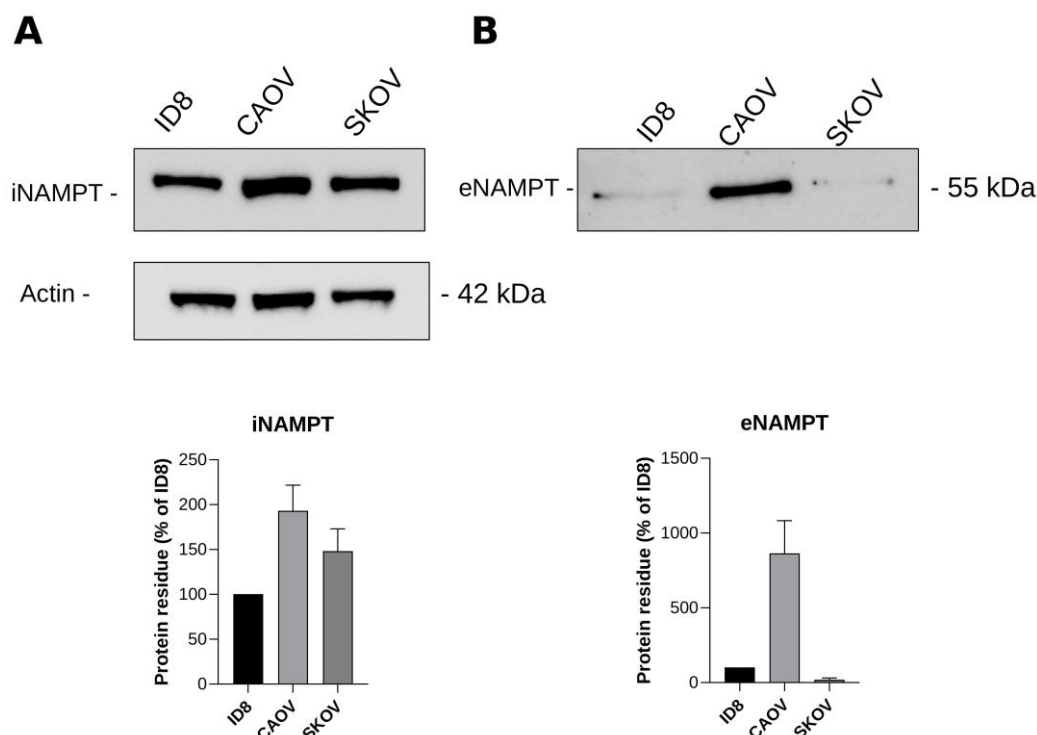


Figure 4. Basal expression of iNAMPT and secretion of eNAMPT in various ovarian cancer cell lines. (A) Representative Western blot image (top) and densitometric quantification (bottom) of basal iNAMPT protein expression levels in mouse (ID8) and human (Caov-3, SKOV3) ovarian cancer cell lines. (B) Representative Western blot image (top) and corresponding quantification (bottom) of extracellular NAMPT (eNAMPT) in conditioned media of the respective cell lines. Quantitative data are expressed as a percentage of the ID8 control line and are shown as mean $\pm$ SEM of at least 3 independent experiments.

Using this characterization, we then asked whether NAMPT might also serve as a therapeutic target in ovarian cancer. The laboratory has already obtained significant data in breast cancer: together with the medicinal chemistry group at our department led by Prof. Pirali, a class of NAMPT-directed PROTACs was developed starting from a previously characterized NAMPT inhibitor, and structure-activity relationship studies identified U42 as the optimized degrader (64). U42 was found to possess low nanomolar antiproliferative activity in breast cancer cells, where it binds to NAMPT, inhibits its function and induces its proteasomal degradation via recruitment of VHL, resulting in a reduction of NAD^+ levels (64). Interestingly, U42 was effective in promoting degradation of both intracellular and extracellular forms of the enzyme, although a long treatment protocol was needed to achieve a complete eNAMPT depletion. U42 showed a long-lasting efficacy in mammosphere cultures, reducing both the amount and the surface area of three-dimensional colonies (64). On the basis of these promising data, we decided to implement this approach in ovarian cancer.

First, we assessed the cytotoxicity of U42 in the three cell lines. Cells were treated with increasing concentrations of the compound (0.03 μM to 1 μM) and cell viability was determined by MTT assay after 72 hours of treatment (Fig. 5). ID8 cells were the most sensitive model with viability dropping below 50% at the highest concentrations tested (0.6 μM and 1 μM) (Fig. 5A). Conversely, neither Caov-3 nor SKOV3 cells showed any significant decrease in viability across the entire concentration range, demonstrating a clear difference in drug response between the murine and human models (Fig. 5B–C).

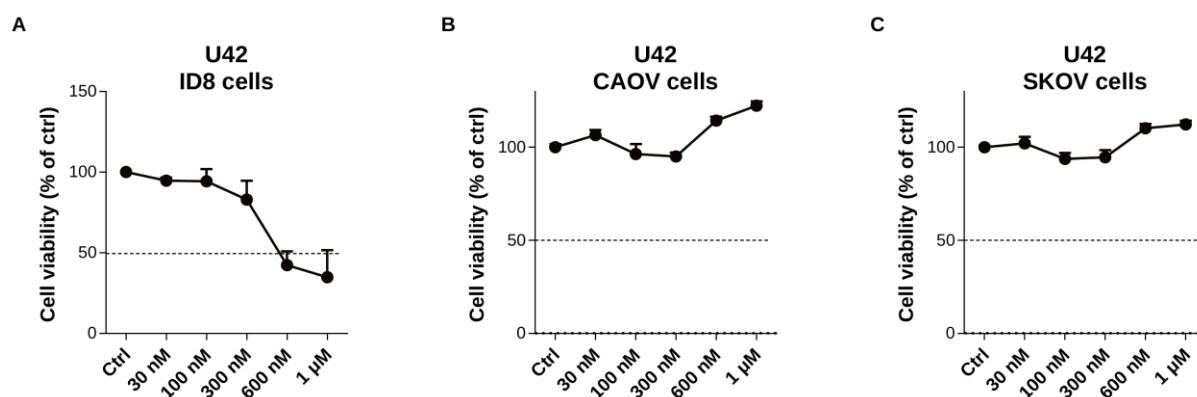


Figure 5. Effect of PROTAC U42 on ovarian cancer cell viability. Dose-response curves to assess the viability of (A) ID8, (B) Caov-3, and (C) SKOV3 cell lines by MTT assay 72 h after treatment with increasing concentrations of U42 (0.03 μM to 1 μM). Data are mean % of control $\pm$ SEM.

We then examined whether the decreased viability observed in ID8 cells was attributable to NAMPT degradation. For iNAMPT depletion, cells were treated with U42 at the indicated concentrations (0.03 μM to 1 μM) for 18 hours and Western blot analysis was performed on the cell lysates (Fig. 6). Among the three models, ID8 was the most responsive model. iNAMPT levels decreased significantly, and the maximum degradation occurred at 0.1 μM and 0.3 μM . However, the effect gradually weakened at 0.6 μM and 1 μM , showing a clear hook effect (Fig. 6A). In the two human lines, U42 showed little to no degradative activity and no comparable depletion was obtained (Fig. 6B–C). The parallelism between degradation and viability indicates that the resistance of Caov-3 and SKOV3 is upstream, due to failure in degrading the target rather than downstream. A possible explanation can be due to the presence of another E3 ubiquitin ligase system or the possibility that these cells can depend more on another NAD-dependent pathway. Indeed, NAMPT has a twin enzyme called NAPRT which is responsible for NAD production via the nicotinic acid precursor.

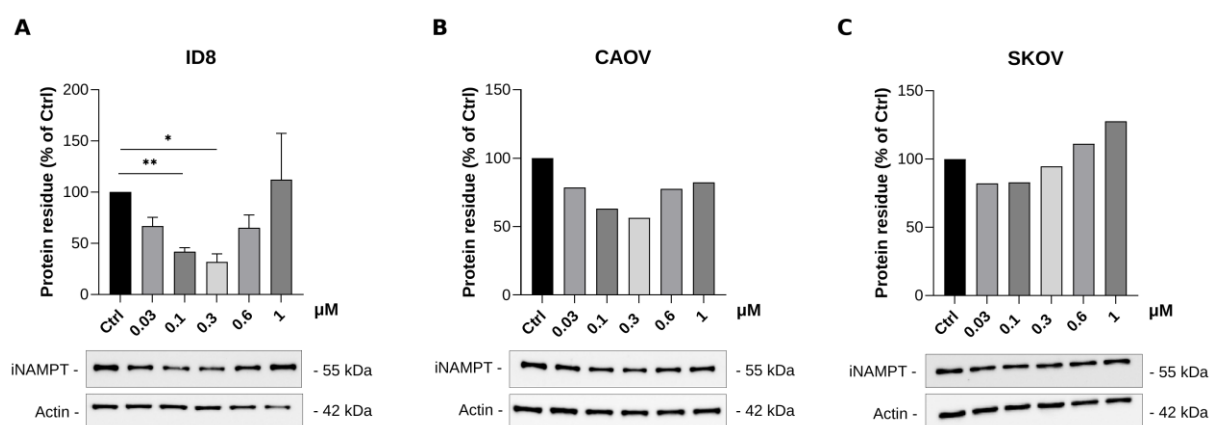


Figure 6. iNAMPT degradation by U42 in different ovarian cancer cell lines. Representative Western blot images and the relative densitometric quantification of iNAMPT protein levels in (A) ID8, (B) Caov-3, and (C) SKOV3 cells after treatment for 18 h with increasing concentrations of PROTAC U42 (0.03 μ M to 1 μ M). Actin was used as loading control. Data are presented as mean protein residue (% of control) $\pm$ SEM from independent experiments.

The nature of this resistance deserves further consideration. The formation of a productive ternary complex does not only depend on the affinity of the warhead for the target, but also on the abundance of the recruited E3 ligase and on the geometry of the resulting protein-protein interface, so that the same degrader can behave very differently in two cell lines expressing comparable amounts of the target protein (1). U42 induces degradation via recruitment of VHL (64), and either reduced expression of this ligase or preferential reliance of Caov-3 and SKOV3 on a different ubiquitin ligase system would be enough to abolish degradation while leaving the binding of the compound to NAMPT unaffected. It is important to point out that this is unlikely to be a species-specific phenomenon, as structurally analogous NAMPT-directed PROTACs achieve efficient degradation in A2780 cells, which are human in origin (51). This demonstrates that human ovarian cancer cells are not intrinsically refractory to this modality, and that the behavior observed here is a property of these two particular models. The easiest way to discriminate among these possibilities would be to measure the levels of VHL in the three cell lines by Western blot and compare U42 with a NAMPT degrader recruiting a different E3 ligase (e.g., cereblon) in the same panel of cells. Such an experiment would also tell whether the resistance is absolute or can be overcome by increasing the time of exposure, because a slower rate of formation of the ternary complex would mean that the loss of the protein is delayed but not prevented.

Finally, we tested if U42 could also modulate the extracellular enzyme in the responsive ID8 line. A simple treatment of 18 hours as conducted for intracellular form of NAMPT was not

sufficient to observe eNAMPT degradation (not shown). Therefore, based on the literature, cells were treated for 18 hours in complete medium and then kept for another 24 hours in serum-free medium (necessary condition for technical issues in western blot analysis of a culture medium), after which both lysates and conditioned media were collected. Under these conditions, U42 caused parallel degradation of both iNAMPT (Fig. 7A) and eNAMPT (Fig. 7B) within the same concentration range, with a DC_{50} below 0.1 μ M. Both forms of the enzyme showed the most depletion at 0.3 μ M, confirming the ability of U42 to degrade the iNAMPT form even with this optimized protocol for extracellular eNAMPT and showing the degradation of eNAMPT in these conditions. Once again, a gradual recovery of protein levels was observed from 0.6 μ M onward, a clear hook effect confirming the typical PROTAC mechanism that strictly requires the formation of a ternary complex to induce protein degradation.

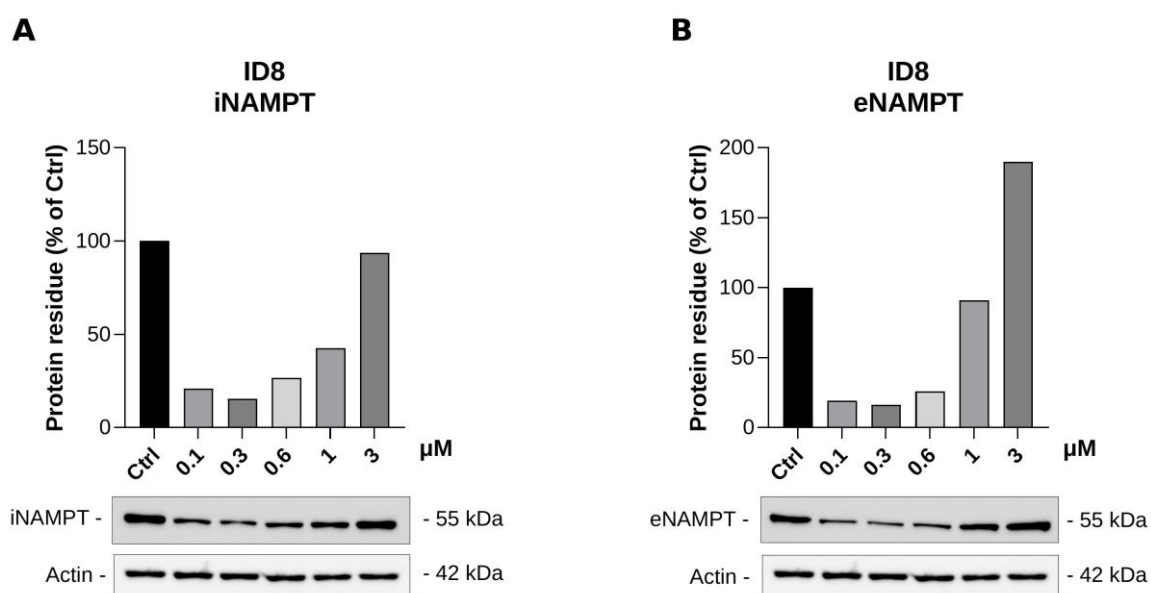


Figure 7. Dose-dependent degradation of iNAMPT and eNAMPT by U42 in ID8 cells. Representative Western blot images and densitometric quantifications of (A) iNAMPT and (B) eNAMPT protein levels in ID8 cells. Cells were treated with increasing concentrations of U42 (0.1 μ M to 3 μ M) in complete medium for 18 h and then serum-free medium for 24 h. Actin was used as loading control. Data represents the mean protein residue (% of control) from independent experiments.

Taken together, these *in vitro* experiments identify U42 as a promising pharmacological tool against murine ovarian cancer and show that its efficacy strictly depends on the cellular background. These results are in keeping with the current literature that showed structurally similar NAMPT-targeting PROTACs such as PROTAC B3 caused efficient target degradation and high efficacy in A2780 human ovarian cancer cells (51).

Finally, the translational potential of this observation should be considered. ID8 cells are derived from spontaneously transformed murine ovarian surface epithelium and form tumors in immunocompetent syngeneic C57BL/6 mice (65), thus the only model that responded to U42 in this panel is also the one that can be transferred to an *in vivo* setting, where the immune compartment is preserved. This is particularly relevant for a target such as NAMPT, whose extracellular form shapes the tumor microenvironment and maintains immune evasion through the PD-1/PD-L1 axis (34), and it implies that a syngeneic ID8 model is the natural next step to determine whether the concomitant removal of the intracellular and the extracellular form of the enzyme translates into an anti-tumor effect *in vivo* even in OC.

3.2 Evaluation of novel BRD4-targeting compounds in triple-negative breast cancer

The second part of this work focused on targeting BRD4. The 4T1 TNBC murine model was selected because it frequently exhibits elevated c-Myc expression and relies on transcriptional programs sustained by BRD4 at super-enhancers, making the BET machinery a plausible therapeutic vulnerability in this tumor model (43). Furthermore, 4T1 cells are derived from a spontaneous mammary tumor in a BALB/c mouse and have already been extensively characterized in our laboratory within the framework of the eNAMPT axis (34), which makes them a convenient platform for the future *in vivo* evaluation of the most active compounds.

In this part of the work, the compounds were designed and synthesized by the medicinal chemistry group of Prof. Alexander Dömling (Institute of Molecular and Translational Medicine, Palacký University Olomouc, Czech Republic), with whom our laboratory is in collaboration. All the compounds of both series were derived from JQ1, the prototypical BET inhibitor described above, which was modified to explore new molecules. In addition, two molecules of the second series were developed as bona fide degraders, one molecular glue and one PROTAC. Therefore, the collaboration had a double purpose: to search for analogs that could reproduce, and possibly improve, the BRD4 inhibition exerted by JQ1, and to determine whether these chemical modifications translate into an antiproliferative effect in tumor cells.

We first screened a library of 26 novel BRD4-targeting compounds for their ability to affect the viability of 4T1 cells. Cells were exposed to each compound at a single concentration of 30 μ M for 72 hours, and cell viability was determined by MTT assay. JQ1(46) was used as a positive control at 10 μ M and 30 μ M (Fig. 8). JQ1 decreased cell viability to about 87 and 62% of control at 10 and 30 μ M respectively under these conditions and served as a benchmark for

the novel compounds. Screening showed a wide range of activities in the series, with seven compounds, SMA-474, SHM-076, SHM-090, SHM-095, SMA-455, SMA-487 and SHM-094, reducing viability below the level achieved by JQ1 at the same concentration. The most cytotoxic molecule within the library was SMA-474, reducing viability to approximately 44% of control. In contrast, several compounds such as SMA-484, SMA-481 and SMA-432 failed to demonstrate any discernible effect, with viability at or marginally above control levels. This heterogeneity shows that although the synthesized molecules are based on a common design rationale, only a subset of them resulted in effective antiproliferative activity in this cellular model.

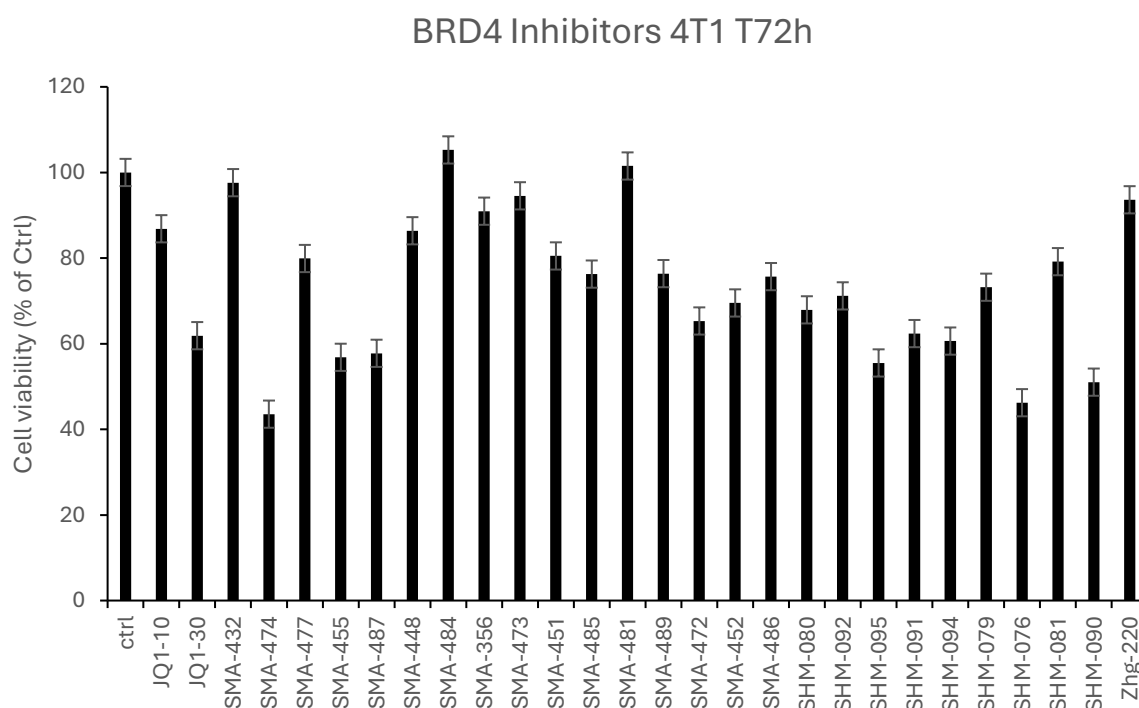


Figure 8. Screening of the first set of BRD4-targeting compounds in 4T1 cells. Cell viability of 4T1 cells measured by MTT assay after 72 h treatment with 30 μ M of each of the 26 compounds of the first series. As reference BET inhibitor, JQ1 was used at 10 μ M and 30 μ M. Data are shown as a percentage of untreated control and represent the mean $\pm$ SEM of 2 independent experiments.

Based on this first screening a smaller second series of compounds was tested in the same experimental conditions (Fig. 9). The series comprised eight inhibitors (SHM-068, SHM-077, SHM-093, SHM-114, SHM-113, SHM-129, SHM-118 and SMA-544), along with two compounds designed as degraders: SMA-545 as a molecular glue and SMA-543 as a PROTAC. The most effective inhibitors were SHM-068, SHM-129 and SHM-077 with viabilities of

approximately 36%, 40% and 45% of control, respectively, and therefore superior to JQ1 at the same concentration which reduced viability to approximately 52% of control. Most strikingly, the molecular glue SMA-545 was the most potent compound of the series, reducing cell viability to about 28% of control. Conversely, PROTAC SMA-543 displayed essentially no antiproliferative activity with viability remaining approximately 90% of control.

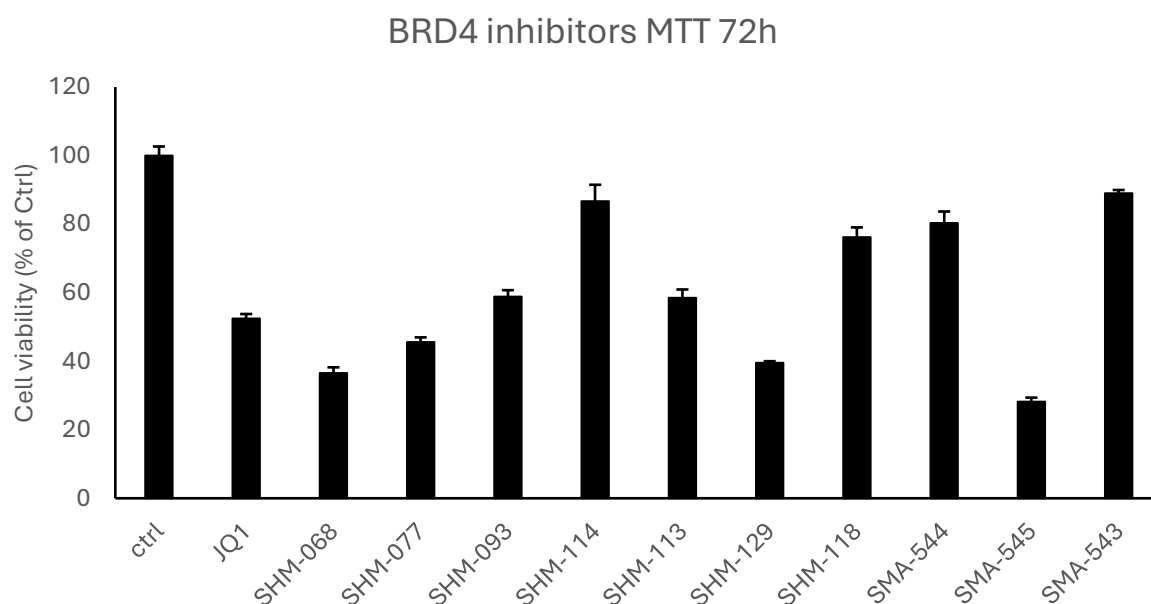


Figure 9. Antiproliferative activity of the second series of compounds targeting BRD4 in 4T1 cells. MTT assay of 4T1 cells after 72 h treatment with 30 μ M of each compound and JQ1 at 30 μ M as reference inhibitor. Data is representative of one independent experiment.

Particularly interesting is the distinct behavior of the two degraders. Indeed, a PROTAC must form a ternary complex of the target protein, the compound, and an E3 ubiquitin ligase to induce degradation (1). Therefore, the lack of activity of SMA-543 could be due to unfavorable linker geometry or incompatible E3 ligase recruitment in this cellular context.

Since two molecules of this second series, SMA-545 as a molecular glue and SMA-543 as a PROTAC, had been specifically designed as degraders, we then tested whether their effect on cell viability was accompanied by a loss of the target protein. 4T1 cells were treated with 30 μ M of either compound for 18 hours and BRD4 levels were analyzed by Western blot including JQ1 at the same concentration as a reference BET inhibitor (Fig. 10). The 18-hour time point was selected to be consistent with conditions previously established in our laboratory for analysis of protein degradation.

In these conditions, no degradation of the target was observed. Treatment with the molecular glue SMA-545 did not appreciably affect BRD4 levels, which were approximately 106% of control, while a modest increase in BRD4 levels was observed upon treatment with the PROTAC SMA-543 (approximately 132% of control). The strongest accumulation of protein was seen with JQ1 which reached around 146% of control and is consistent with the well-documented observation that occupancy-based BET inhibitors displace BRD4 from chromatin and stabilize the protein rather than promote its removal (58).

This result does not exclude a degradative mechanism for these two compounds. The degradation mediated by PROTACs and molecular glues is strictly dose- and time-dependent, as exemplified by the bell-shaped dose-response of U42 discussed in the previous section, and a single dose tested at a single time point is insufficient to define the window in which degradation may occur (1). Therefore, defining the right concentration range and correct kinetics of SMA-545 and SMA-543 is a needed next step before any conclusions can be made about their mechanism of action. Until such conditions are determined, the considerable cytotoxicity of SMA-545 cannot be attributed to the degradation of its intended target.

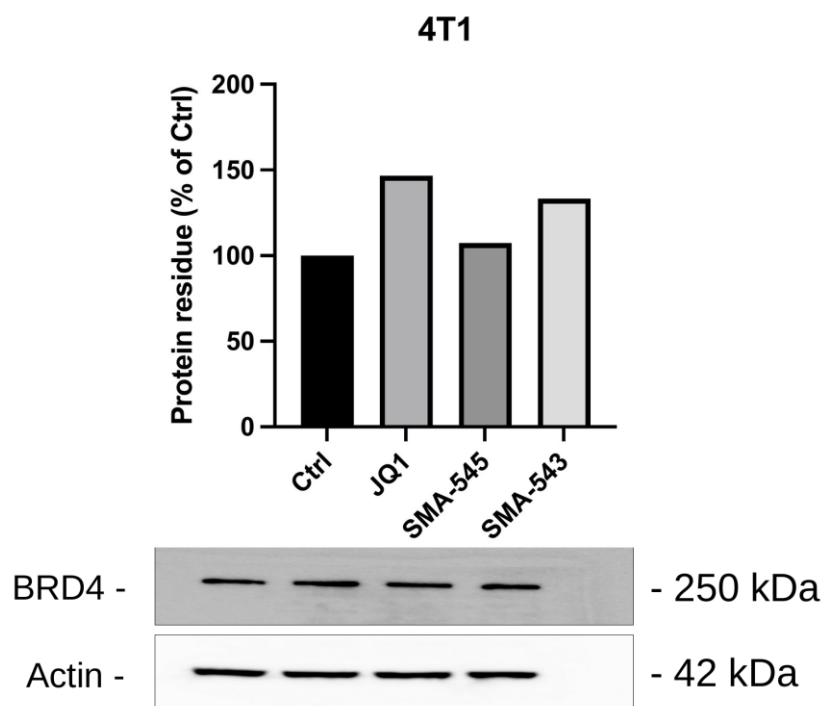


Figure 10. Impact of SMA-545 and SMA-543 on the protein levels of BRD4 in 4T1 cells. Densitometric quantification (top) and representative Western blot image (bottom) of BRD4 protein levels in 4T1 cells treated for 18 h with 30 μ M of the molecular glue SMA-545, the PROTAC SMA-543 or the reference BET inhibitor JQ1. Data are expressed as protein residue (% of control) and are from one independent experiment.

Finally, we tested if the JQ1-based compounds maintain the ability to inhibit BRD4 activity (Fig. 11). Using a BRD4 (BD1+BD2) TR-FRET assay we measured the inhibition of BRD4 binding to the acetylated substrate. Once incubated the test inhibitors, BRD4, the substrate, a sample containing terbium-labelled donor and a dye-labeled acceptor we measure the fluorescence intensity by the emission of the donor and the acceptor. The final data are expressed as a percentage of BRD4 activity. Nonetheless, as shown above, some compounds exhibit cytotoxicity. Therefore, the compounds tested at 10 μ M can no longer be considered BRD4 inhibitors, suggesting that the chemical modifications may alter their binding to the target. JQ1 demonstrated inhibitory activity against BRD4 and, together with the positive and negative controls, confirmed the functional performance of the assay kit.

These results together with the Western blot data suggest that the molecules of these series do not behave as selective BRD4 binders. The series is derived from the JQ1 scaffold, but the chemical modifications introduced in the warhead resulted in compounds that retain, and in some cases improve, antiproliferative activity, but have lost the capability to interact with the bromodomain module, so their cytotoxicity must be ascribed to one or more target(s) still to be identified. This also provides a coherent explanation for the absence of BRD4 depletion described above as neither a PROTAC nor a molecular glue can induce the degradation of a protein that they do not efficiently engage. However, it is worth considering that failure to degrade may be related to factors other than target binding, for example, recruitment of a poorly expressed or poorly compatible E3 ubiquitin ligase in this cell model (15).

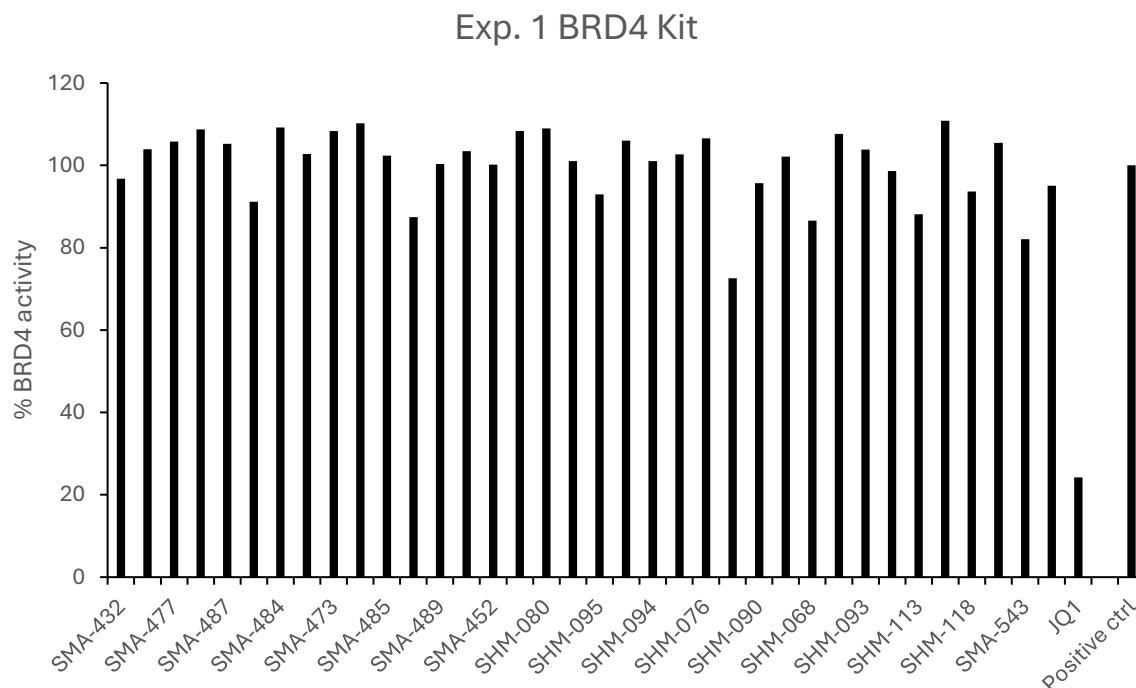


Figure 11. Cell-free TR-FRET binding assay to evaluate BRD4 engagement by the compounds of both series. BRD4 (BD1+BD2) TR-FRET Assay Kit (BPS Bioscience, cat. #32612) was used to determine the displacement of acetylated bromodomain ligand from recombinant BRD4 (BD1+BD2) in presence of each compound expressed as percent binding signal of positive control (corresponding to uninhibited interaction) and negative control (with non-acetylated ligand). Each compound was tested at a final concentration of 10 μ M. A reference BET inhibitor, JQ1, was used. Data are representative of one independent experiment.

4. CONCLUSIONS

This thesis project explored targeted protein degradation as a pharmacological approach for two targets, NAMPT and BRD4, in two aggressive tumor models, ovarian carcinoma and triple-negative breast cancer.

Our work established a baseline characterization of NAMPT in ovarian cancer cell lines, demonstrating that the enzyme is expressed in all the models examined, but that its secretion is strongly cell-line-dependent. In the murine model of OC, PROTAC U42 induced parallel depletion of both iNAMPT and eNAMPT with a $DC_{50} < 0.1 \mu\text{M}$ and the recovery of protein levels at the highest doses reproduced the hook effect that is characteristic of degraders requiring a ternary complex. Degradation and loss of viability were concomitant: the murine ID8 line was sensitive to both, whereas the human lines were resistant to both. These data collectively identify U42 as a promising pharmacological tool against ovarian cancer and, at the same time, show that its efficacy strictly depends on the cellular background, most probably on the availability of a compatible E3 ubiquitin ligase.

The second branch of the project transitioned from a validated degrader to a library of newly synthesized molecules and screened two series of BRD4-targeting compounds in the 4T1 model of TNBC. The screening identified several molecules with higher cytotoxicity than JQ1 at the same dose, with the molecular glue SMA-545 being the most cytotoxic compound in the entire library. In the cell-free binding assay, JQ1 behaved as expected, confirming the functionality of the assay. In contrast, none of the tested compounds was able to displace the acetylated ligand from the bromodomain module, suggesting that the chemical modifications may have impaired their ability to bind to the BRD4 bromodomain. Their cytotoxicity must therefore be ascribed to one or more targets that remain to be identified.

In conclusion, the two parts of this work highlight two distinct outcomes that can arise during the development of targeted protein degraders. In the NAMPT-directed degradation study, effective target engagement resulted in the depletion of both NAMPT isoforms resulting in a decrease of ID8 cell line viability. Conversely, the analysis of the second compound series showed that cytotoxic activity was not associated with engagement of the intended protein target. Importantly, this discrepancy could not be established from phenotypic assays alone and was revealed only through direct target engagement studies.

5. MATERIALS AND METHODS

5.1 Cell culture

Murine ovarian cancer cell line ID8 was cultured in T-75 flasks with DMEM (Dulbecco's Modified Eagle Medium; Sigma-Aldrich) whereas human ovarian cancer cell lines Caov-3 and SKOV3 were cultured in T-25 flasks with IMDM (Iscove's Modified Dulbecco's Medium; Sigma-Aldrich). 4T1 murine triple-negative breast cancer cells were cultured in MEM (Minimum Essential Medium Eagle; Sigma-Aldrich) in culture dishes. The culture media were supplemented with 1% L-glutamine (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). Media were supplemented with fetal bovine serum (FBS; Gibco) at final concentration 10% for ID8, SKOV3 and 4T1 cells and 15% for Caov-3 cells. All cell lines were cultured in a humidified incubator at 37°C, 5% CO₂.

5.2 MTT assay

To assess cell viability, cells were plated in 24-well plates. ID8 cells were seeded at 5.0×10^3 cells/well and Caov-3 and SKOV3 cells were plated at 3.5×10^4 cells/well. These ovarian cancer cell lines were incubated for 24 hours and then treated with increasing concentrations of the PROTAC U42 (0.03 μ M to 1 μ M) for 72 hours. For the screening of the BRD4-targeting compounds, 4T1 cells were seeded at a density of 1.5×10^4 cells/well and, after 24 hours of incubation, treated for 72 hours with 30 μ M of each compound with JQ1 as reference BET inhibitor. All compounds were dissolved in DMSO, and the same final concentration of DMSO was added to untreated controls.

After incubation of treated and untreated cells, culture medium was removed and cells were washed with 1X PBS. The cells were then incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) at a final concentration of 250 μ g/mL in Locke's buffer solution for 1 hour at 37°C with 5% CO₂. The obtained formazan crystals were dissolved in 0.1 M isopropanol-HCl. Optical absorbance was measured at 570 nm in a microplate reader (Victor3 V, PerkinElmer Life Sciences).

5.3 Western blot

To evaluate the degradation profile, ID8, Caov-3 and SKOV3 cells were seeded in 12-well plates at a density of 1.5×10^5 cells/well and treated with increasing concentrations of U42

(0.03 μ M to 1 μ M) for 18 hours after 24 hours of seeding. To evaluate BRD4 degradation, 4T1 cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and treated for 18 hours with 30 μ M of the molecular glue SMA-545, the PROTAC SMA-543 or the reference BET inhibitor JQ1 after 24 hours.

After treatment, cells were lysed with RIPA lysis buffer (20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1% Nonidet P-40) containing protease inhibitors (100 nM PMSF, Sigma-Aldrich; PIC, Merck-Millipore) and phosphatase inhibitors (1 M NaF, 1 M Na_3VO_4 , Sigma-Aldrich). The cell lysates were centrifuged at 13,000 rpm for 15 min at 4°C. Total protein concentration was determined using the Bradford assay (Sigma-Aldrich).

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by the TurboBlot system (Bio-Rad, Hemel Hempstead, UK). The membranes were first stained with Ponceau solution to evaluate the transfer efficiency and protein loading. Membranes were blocked and then incubated with primary antibodies: mouse anti-NAMPT (AdipoGen; #AG-20A-0034, RRID:AB_2490117), rabbit anti-BRD4 (Bethyl Laboratories; #A301-985A-M) and mouse anti-Actin (Sigma-Aldrich; clone 4 #MAB1501) as the housekeeping protein. This was followed by incubation with HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (Bio-Rad Laboratories). Protein bands were visualized by chemiluminescence detection using an ECL solution (Thermo Fisher Scientific). Densitometric analysis was performed using Image Lab software (Bio-Rad Laboratories) to quantify protein expression levels.

5.4 Extracellular release of NAMPT (eNAMPT) in cell culture medium

To examine the extracellular release of NAMPT under starving condition, ID8, Caov-3 and SKOV3 cells were plated in 6-well plates with a density of 6.0×10^5 cells/well in complete culture medium. To measure basal secretion of eNAMPT, after 24 hours the total culture medium was removed, cells were washed with 1X PBS and the medium replaced with serum-free medium. Cells were then incubated under standard conditions (37°C, 5% CO_2) for 4 hours.

In another experiment, ID8 cells were treated with increasing doses of U42 (0.1 μ M to 3 μ M) for 18 hours in complete medium, to evaluate the dose-dependent degradation of both iNAMPT and eNAMPT. The medium was removed and cells were washed with 1X PBS, treated again with the compounds and kept in serum-free medium for another 24 hours.

After the above incubation periods the supernatants of the cell cultures (starved media) were collected. The collected media were transferred to centrifugal filter units and centrifuged at

8,000 rcf for 5 min at 4°C to concentrate secreted proteins. This concentration procedure was repeated stepwise until the desired final volume was obtained, as previously described by Grolla and colleagues (23). At the same time, the cells were washed and lysed in RIPA lysis buffer as mentioned above. For the evaluation of protein expression, the concentrated supernatants and the cell lysates were loaded on SDS-PAGE gels and analyzed by Western blot as described above.

5.5 BRD4 (BD1+BD2) TR-FRET binding assay

Target engagement was measured with the BRD4 (BD1+BD2) TR-FRET Assay Kit (BPS Bioscience, #32612) according to the manufacturer's instructions. All samples and controls were assayed in duplicate in a white, non-binding, low-volume 384-well plate. Each 20 µL reaction contained 5 µL of 100-fold diluted terbium-labeled donor in 1X BRD TR-FRET Assay Buffer 1, 5 µL of 100-fold diluted dye-labeled acceptor in 1X BRD TR-FRET Assay Buffer 1, 2 µL of compound solution, 5 µL of 40-fold diluted BET Bromodomain Ligand in 1X BRD TR-FRET Assay Buffer 1 and 3 µL of recombinant BRD4 (BD1+BD2) diluted to 6 ng/µL (18 ng per reaction). Compounds were screened at a final concentration of 10 µM with JQ1 used as a reference inhibitor. In the positive control the compound solution was exchanged by 2 µL of 10% DMSO in water. In the negative control the BET Bromodomain Ligand was exchanged by Non-acetylated Ligand 1.

The plates were incubated for 2 hours at room temperature and the fluorescence intensity was measured on a plate reader capable of time resolved fluorescence resonance energy transfer, exciting at 340 ± 20 nm and measuring emission at 620 ± 10 nm for the donor and at 665 ± 10 nm for the acceptor, with a lag time of 60 µs and an integration time of 500 µs. The TR-FRET ratio was obtained as the ratio between the emission at 665 nm and the emission at 620 nm and the percentage of the binding signal was obtained as $(FRET_s - FRET_{neg}) / (FRET_p - FRET_{neg}) \times 100$ where $FRET_s$, $FRET_{neg}$ and $FRET_p$ correspond to the values of the sample, of the negative control and of the positive control respectively.

5.6 Statistical analysis

Statistical analysis was conducted using GraphPad Prism software. Values are expressed as mean $\pm$ standard error of the mean (SEM). The experiments performed in the ovarian cancer cell lines were statistically analyzed: a parametric Welch's t-test was used for comparisons between two experimental groups and a one-way Brown-Forsythe and Welch ANOVA for

multiple group comparisons. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant in all analyses.

The first series of BRD4-targeting compounds was screened in 2 independent experiments, whereas the second series of compounds, the Western blot analysis of BRD4 and the TR-FRET binding assay were performed once. Therefore, these datasets were not statistically analyzed and are presented as the percentage of the corresponding untreated control.

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