

Targeting NAD⁺ metabolism in ovarian cancer

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Declaration of Originality

I, Michael James Gruet, declare this thesis and body of work are my own. Any work that was provided through collaboration is appropriately referenced and acknowledged.

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Abstract

Ovarian cancer is the fourth leading cause of female cancer related deaths in the developing world. Epithelial ovarian cancer is the most common type of ovarian cancer, and patients with these tumours are frequently treated with poly(ADP-ribose) polymerase inhibitors. Patients with a homologous recombination deficiency, for example due to mutation in genes such as *BRCA1/2*, benefit most from treatment with PARP inhibitors. However, in recent years it has been shown that HRD-negative populations can also benefit. Mutations that dysregulate RAS/PI3K-signalling frequently occur in epithelial ovarian cancer, and recent clinical trials have shown that this can promote PARP inhibitor resistance. Since dysregulated RAS/PI3K-signalling promotes metabolic reprogramming to support unconstrained proliferation, we investigated whether these mutations could create a targetable “metabolic bottleneck” that could extend the benefit of PARP inhibitors in these RAS/PI3K-mutant tumours. In this thesis, I characterised the role of the NAD⁺ salvage pathway enzyme NAMPT in EOC and found that RAS/PI3K-mutant tumours are exquisitely sensitive to a NAMPT inhibitor. Importantly, RAS/PI3K-mutations increase the sensitivity of EOC cell lines to a synergistic NAMPT and PARP inhibitor combination *in vitro*. This combination significantly improved *in vivo* survival outcomes in ID8-*Trp53*^{-/-}; *Pten*^{-/-} tumours, an alteration that occurs in the most common subtype of EOC, high grade serous ovarian cancer. Using RNA-sequencing we provide novel insights into the role of PARP and NAMPT activity on alternative splicing events in a HGSOC background. Finally, using metabolomics we optimise conditions to successfully utilise “clickable” pre-cursor NAD⁺ probes to detect PARPs post-translational modification, ADP-ribosylation, by chemical proteomics.

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List of Abbreviations

2-D	2-dimensional
2Yn	2-alkyne
3-D	3-dimensional
4EBP-1	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
53BP1	Tumor Protein P53 Binding Protein 1
5-Bn-6-a-NAD ⁺	5-Benzyl-6-alkyne-NAD ⁺
5-Et-6-a-NAD ⁺	5-ethyl-6-alkyne-NAD ⁺
6Yn	6-alkyne
6Yn-Ad	6-alkyne adenosine
6Yn-Ad	6Yn-Adenosine
6Yn-ADP	6Yn-Adenosine diphosphate
6Yn-AMP	6Yn-Adenosine monophosphate
6Yn-ATP	6Yn-Adenosine triphosphate
6Yn-NAD ⁺	6Yn- Nicotinamide adenine dinucleotide
6Yn-ProTide	6-alkyne pronucleotide probe
8Yn	8-alkyne
a.u.	Arbitrary unit
ABC1A/B	ATP Binding Cassette Subfamily B Member 1 and 2
ABCB1	ATP Binding Cassette Subfamily B Member 1
ACMS	2-amino-3-carboxymuconate semialdehyde
ACTG2	Actin gamma 2

Ad	Adenosine
ADPr	ADP-ribose
AF488	Alexa Fluor 488 dye
AK	Adenosine kinase
AKT	Protein kinase B
AKT2	AKT Serine/Threonine Kinase 2
AMPK	AMP-activated protein kinase complex
AMPK α	AMP-activated protein kinase α subunit
AMPylation	Adenylation
ARAF	A-Raf Proto-Oncogene, Serine/Threonine Kinase
ARH(1/3)	(ADP-ribosyl)hydrolase (1/3)
ARID1(A/B)	AT-Rich Interaction Domain 1(A/B)
ART	ADP-ribosyltransferase
AS	Alternative splicing
ASEs	Alternative splicing events
ATM	ATM Serine/Threonine Kinase
ATMIN	ATM Interactor
ATP	Adenosine triphosphate
ATR	ATR Serine/Threonine Kinase
AUC	Area under the curve
AXL	Tyrosine-protein kinase receptor UFO
AzB	Azide-biotin

AzRB	Azido-arginine-biotin
AzT	Azide-TAMRA
AzTB	Azide-TAMRA-biotin
BAD	BCL2 Associated Agonist Of Cell Death
BARD1	BRCA1 Associated RING Domain 1
BIM	BCL2 Like 11
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
BRCA1/2	Breast cancer gene 1/2
BRCT	BRCA1 C Terminus
BRIP1	BRCA1 Interacting Helicase 1
BSA	Bovine serum albumin
C5orf46	Chromosome 5 Open Reading Frame 46
cADPr	Cyclic ADP-ribose
cADPrSs	Cyclic ADP-ribose synthases
CCLE	Cancer cell line encyclopaedia
CCNE1	Cyclin E1
CCOC	Clear cell ovarian cancer
CD38	Cluster of differentiation 38
CD73	5'-Nucleotidase Ecto
CDKN2(A/B)	Cyclin Dependent Kinase Inhibitor 2(A/B)
CE	Collision energy
c-Fos	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit

CHK(1/2)	Checkpoint Kinase (1/2)
c-Jun	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit
c-MYC	MYC Proto-Oncogene, BHLH Transcription Factor
CNVs	Copy number variations
CPA4	Carboxypeptidase A4
CRAF	C-Raf Proto-Oncogene, Serine/Threonine Kinase
CRISPR-Cas9	CRISPR-associated endonuclease caspase 9
CtBP	C terminal-binding protein (CtBP),
CTC1	CST Telomere Replication Complex Component 1
CTNNB1	Catenin Beta 1
CuAAC	Copper-catalysed azide-alkyne cycloaddition
DBD	DNA binding domain
DDR	DNA damage response
DE	Differential expression
DMSO	Dimethyl sulfoxide
DP	Declustering potential
DSBs	Double-stranded DNA breaks
DTAP	dodecyltrimethylammonium bromide
DYNLL1	Dynein Light Chain LC8-Type 1
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor

EIF1AX	Eukaryotic Translation Initiation Factor 1A X-Linked
ELK-1	ETS Transcription Factor ELK1
EMA	European Medicine Agency
EMSY	EMSY Transcriptional Repressor, BRCA2 Interacting
eNAMPT	extracellular NAMPT
EnOC	Endometroid ovarian cancer
EOC	Epithelial ovarian cancer
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERCC1	ERCC Excision Repair 1, Endonuclease Non-Catalytic Subunit
ERK1/2	Mitogen-activated protein kinase
ETC	Electron transport chain
ETS	ETS Proto-Oncogene 1, Transcription Factor
EZH2	Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FFAR1	Free Fatty Acid Receptor 1
FGFR	Fibroblast Growth Factor Receptor
FK	Formylkinurenine
FOSL1	FOS Like 1, AP-1 Transcription Factor Subunit
FOXO	Forkhead box proteins
GDP	Guanosine diphosphate

GI	Gastrointestinal
GLS	Glutaminase
GO	Gene ontology
GOBP	GO biological process
GSK-3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
H2(A/B)	H2(A/B) Clustered Histone
H ₂ O ₂	Hydrogen peroxide
H3	H3 Clustered Histone
H4	H4 Clustered Histone
HCl	Hydrochloric acid
HD	Helical domain (HD),
HDAC	Histone Deacetylase
HEK	Human embryonic kidney
HER2	Human Epidermal Growth Factor Receptor 2
HGSOC	High-grade serous ovarian cancer
HIF-1 α	Hypoxia-inducible factor 1 α
HILIC	Hydrophilic interaction chromatography
hnRNP	Heterogeneous ribonucleoproteins
HPLC	High-performance liquid chromatography
HR	Homologous recombination
HRAS	Harvey Rat Sarcoma Viral Oncogene Homolog

HRD	Homologous recombination deficiency
HRP	Horseradish peroxidase
HRR	Homologous recombination DNA repair
I.P.	Intraperitoneal injection
IC ₅₀	Concentration causing 50% growth inhibition
IDH1	Isocitrate Dehydrogenase (NADP(+)) 1
IDO1	Indoleamine 2,3-Dioxygenase 1
IGF	In-gel fluorescence
JNK	Mitogen-Activated Protein Kinase 8
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase
KMT2(B/D)	Lysine Methyltransferase 2(B/D)
KRAS	Kirsten rat sarcoma virus
LB	Lysogeny broth
LC3-I	Light chain 3
LC3-II	Lipidated LC3
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
LDHA	Lactate Dehydrogenase A
LGSOC	Low-grade serous ovarian cancer
LINC01271	Long Intergenic Non-Protein Coding RNA 1271
LINC01629	Long Intergenic Non-Protein Coding RNA 1629
Lnc-FILIP1L-1	Long non-coding RNA-Filamin A interacting protein 1-like

Lnc-KCNE1B-155	Novel Transcript, Similar To YY1 Associated Myogenesis RNA 1 YAM1
LncRNAs	Long noncoding RNAs
LOH	Loss of heterozygosity
m/z	Mass-to-charge ratio
MacroD(1/2)	Mono-ADP Ribosylhydrolase (1/2)
MAD2L2	Mitotic Arrest Deficient 2 Like 2
MAPK	Mitogen-Activated Protein Kinase
MAR	Mono-ADP-ribose
MARylation	Mono-ADP-ribosylation
MAX	MYC Associated Factor X
MCL1	MCL1 Apoptosis Regulator, BCL2 Family Member
MDM(2/4)	MDM(2/4) Proto-Oncogene
MDR1	Multidrug Resistance Protein 1
MEK	Mitogen-activated protein kinase kinase
MEKi	MEK inhibitor
MET	MET Proto-Oncogene, Receptor Tyrosine Kinase
MIR12136	MicroRNA 12136
MLH1	MutL Homolog 1
MMEJ	Microhomology mediated end joining
MMP	Mitochondrial membrane potential
MMP1	Matrix Metalloproteinase 1
MMR	Mismatch repair

MMRd	MMR deficiency
MMS	Methyl methanesulfonate
MNK	ATPase Copper Transporting Alpha
MOC	Mucinous ovarian cancer
MOPS	3-(N-morpholino)propanesulfonic acid
MRE11	MRE11 Homolog, Double Strand Break Repair Nuclease
MS	Mass spectrometry
MSH(2/6)	MutS Homolog (2/6)
MSK	Mitogen and stress activated protein kinase
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MUS81	MUS81 Structure-Specific Endonuclease Subunit
MYC	MYC Proto-Oncogene, BHLH Transcription Factor
MYCN	MYCN Proto-Oncogene, BHLH Transcription Factor
NaAD	Nicotinic acid adenine dinucleotide
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADK	NAD ⁺ kinase
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADSYN	NAD synthase

NAM	Nicotinamide
NaMN	Nicotinic acid mononucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NAMPTi	NAMPT inhibitor
NAMPTi-R	NAMPT inhibitor resistance
NAMPTis	NAMPT inhibitors
NaOH	Sodium hydroxide
NAPRT	Nicotinic acid phosphoribosyltransferase
NDPK	Nucleotide diphosphokinase
NEB	New England Biolabs
NF1	Neurofibromatosis type 1
NF- κ B	Nuclear Factor Kappa B Subunit 1
NHEJ	Non-homologous recombination repair
NMNAT(1/2/3)	Nicotinamide mononucleotide adenylyltransferase (1/2/3)
NMNT	N-methyltransferase
NR	Nicotinamide riboside
NRAS	NRAS Proto-Oncogene, GTPase
NRH	Reduced nicotinamide riboside
NRK1/2	Nicotinamide Riboside Kinase 1/2
NRKs	Nicotinamide riboside kinases
ns	Not statistically significant
OASL	2'-5'-Oligoadenylate Synthetase Like

OCR	Oxygen consumption rate
OE	Overexpression
Olap	Olaparib
OS	Overall survival
OXPHOS	Oxidative phosphorylation
P38 MAPK	Mitogen-Activated Protein Kinase 14
p70S6K	Ribosomal Protein S6 Kinase B1
PAK-4	P21 (RAC1) Activated Kinase 4
PALB2	Partner And Localizer Of BRCA2
PAR	Poly-ADP-ribose
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) polymerase
PARPi	PARP inhibitor
PARPi-R	PARP inhibitor resistance
PARPis	PARP inhibitors
PARylation	Poly-ADP-ribosylation
PBS	Phosphate buffered saline
PCA	Principal component analysis
PDHK1	Pyruvate dehydrogenase kinase 1
PKD1	3-phosphoinositide-dependent protein kinase-1
PDX	Patient derived xenograft
PFA	Paraformaldehyde

PFS	Progression free survival
PH	Preiss-Handler
PHGDH1	Phosphoglycerate Dehydrogenase
PI3K	Phosphoinositide 3-kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit α
PIK3R1	Phosphoinositide-3-Kinase Regulatory Subunit 1
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PIP ₃	Phosphatidylinositol (3,4,5)-trisphosphate
PMS(1/2)	PMS1 Homolog (1/2), Mismatch Repair System Component
POLE	DNA Polymerase Epsilon, Catalytic Subunit
Pol α	DNA Polymerase Alpha
POL θ	DNA Polymerase Theta
PPM1D1	Protein phosphatase 1D
PPP2R1A	Protein Phosphatase 2 Scaffold Subunit α
pre-mRNA	Precursor-mRNA
PROTACs	Proteolysis-targeting chimeras
PRPPP	Phosphoribosyl pyrophosphate
PTB	Phosphotyrosine-binding
PtdIns	Phosphoinositides
PTEN	Phosphatase and tensin homolog
PTIP	PAX Interacting Protein 1
PTM	Post-translational modification

PTMs	Post-translational modifications
Qa	Quinolinic acid
QC	Quality control
QPRT	Quinolinate phosphoribosyltransferase
RAD51	RAD51 Recombinase
RAF	Raf-1 Proto-Oncogene, Serine/Threonine Kinase
RAS	Rat sarcoma virus
REV7	REV7 Homolog
RIF1	Replication Timing Regulatory Factor 1
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
RP	Reversed-Phase
RPA	Replication protein A
RPMA	Reverse phase protein microarray
RSK	Ribosomal Protein S6 Kinase A1
RT	Room temperature
S100A16	S100 Calcium Binding Protein A16
S6K-1	Ribosomal Protein S6 Kinase B1
SAM	S-Adenosyl methionine

SAPK	Mitogen-Activated Protein Kinase Kinase 4
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SECTM1	Secreted And Transmembrane 1
SEM	Standard error of mean
SERPINE	Serpin Family E Member 1
SF3B1	Splicing factor 3B1
SH2	Src homology-2
SHLD(1/2/3)	Shieldin Complex Subunit (1/2/3)
SIRT	Sirtuins
SLC25A40	Solute Carrier Family 25 Member 40
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent
SNORA57	Small Nucleolar RNA, H/ACA Box 57
snoRNAs	small nuclear RNAs
snRNPs	Small nuclear RNPs
SOS	Son of sevenless
SOX8	SRY-Box Transcription Factor 8
SR	Serine and arginine-rich
SRB	Sulforhodamine
SSB	Single-strand DNA break
SSBR	Single strand break repair
STAT(3-5)	Signal transducer and activator of transcription (3-5)

STK11	Serine/Threonine Kinase 11
SYNE1	Pectrin Repeat Containing Nuclear Envelope Protein 1
TAMRA	Carboxytetramethylrhodamine
TARG1	Terminal ADP-Ribose Protein Glycohydrolase 1
TBS	1X tris-buffered saline
TBS-T	tris-buffered saline-Tween-20
TCA	Trichloroacetic acid
TCGA	The Cancer Genome Atlas
TDO	Tryptophan dioxygenase
TERT	Telomerase Reverse Transcriptase
TFEB	Transcription factor EB
TKR	Tyrosine kinase receptors
TNBC	Triple-negative breast cancer
TP53	Tumor protein P53
Trp	Tryptophan
TSC(1/2)	Tuberous sclerosis (1/2)
ULA	ultra-low attachment
UPLC	Ultra Performance Liquid Chromatography
USP9X	Ubiquitin Specific Peptidase 9 X-Linked
v/v	Volume/volume concentration
w/v	Weight/volume concentration
WB	Western blotting

WEE1	WEE1 G2 Checkpoint Kinase
WGR	Tryptophan-glycine–arginine-rich
WT	Wild-type
WT1	WT1 Transcription Factor
XRCC1	X-ray repair cross-complementing protein 1
ZnF(1/2/3)	Zinc Finger Protein (1/2/3)
ZNF295-AS1	ZNF295 Antisense RNA 1
β -NMN	Beta Nicotinamide Mononucleotide
β -NMNH	Reduced β -NMN (β -NMNH)
γ H2AX	Phospho-histone H2A.X (Ser139)
λ_{abs}	Absorbance wavelength
λ_{em}	Emission wavelength

CHAPTER 1

Introduction

Chapter 1 – Introduction

1.1. Ovarian Cancer

1.1.1. Introduction to Epithelial Ovarian Cancer

Ovarian cancer is the 4th leading cause of female cancer related deaths in the developed world¹ and the 7th most commonly diagnosed cancer in women worldwide². This heterogenous collection of malignancies accounts for 140,200 deaths and over 225,500 new cases each year globally³. The disease has a high death rate because patients are often diagnosed at an advanced stage, ultimately this is one of the main factors causing the diseases high death-to-incidence rate². At early stages (I and II) patients are often asymptomatic², but at later stages (stage III or IV) the majority of women will develop ascites (i.e., excess fluid accumulation in the peritoneal cavity)⁴. As a result, women will present with abdominal bloating, pain and gastrointestinal dysfunction; but these symptoms can initially be mistaken for other conditions, such as irritable bowel syndrome^{1,3}.

Since effective screening strategies do not exist researchers are investigating novel approaches to detect ovarian cancer, such as the Cancer Loyalty Card Study (CLOCS)⁵, which demonstrated that purchases of pain and indigestion medications significantly increase up to 8 months prior to diagnosis. Studies like this could aid the early diagnosis of ovarian cancer, which would be ideal as patients treated at early-stage disease have a 92% 5-year relative survival rate, compared to 29% for those presenting late².

Ovarian cancer is an umbrella term used for distinct histotypes with different molecular features, clinical behaviours and developmental origins⁶. 10% of ovarian cancer cases are non-epithelial and include germ-cell tumours and sex cord stromal tumours³. The remaining 90% of cases are epithelial ovarian cancers (EOCs) and include (**Figure 1**): high-grade serous ovarian cancer (HGSOC, ~70% of EOC cases), low-grade serous ovarian cancer (LGSOC, <5%), endometrioid ovarian cancer (EnOC, 10%), clear cell ovarian cancer (CCOC, 10%) and mucinous ovarian cancer (MOC, <5%). Rarer histologies include small cell carcinoma and carcinosarcoma³.

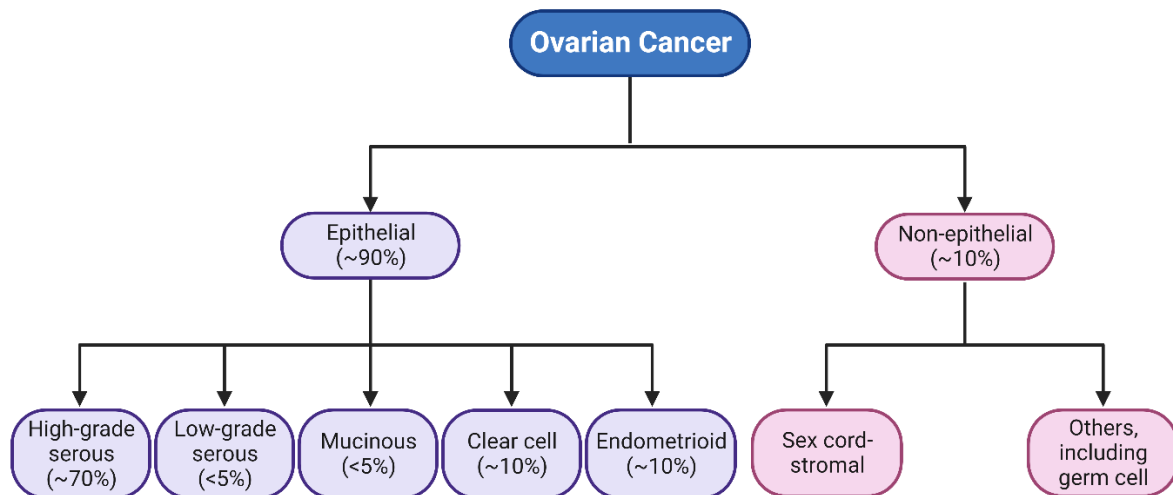


Figure 1. Histologic subtypes of EOC

Histologic subtypes of EOC and their frequency. Figure was adapted from Banerjee and Kaye⁷. Created with BioRender.com

1.1.2. High-grade serous ovarian cancer

HGSOC is the most common subtype of EOC and typically presents in patients at an advanced stage (~70% present at stage III). It is believed these tumours arise from the distal fallopian tube epithelium⁶. Like other histotypes of EOC, first line management typically involves cytoreductive surgery followed by platinum (cisplatin or carboplatin) and taxane (paclitaxel or docetaxel) based chemotherapy. Alternatively, patients will receive neoadjuvant chemotherapy to shrink tumours, followed by cytoreductive surgery and additional chemotherapy³. HGSOC initially responds to chemotherapy (80% objective response rate (ORR)), however, recurrence commonly occurs leading to eventual chemoresistance. As a result, patients only have a 40% 5-year survival rate at advanced stages⁶.

HGSOC tumours have a complex genomic landscape due to genomic instability, extensive copy number aberrations and structural genomic variants (**Figure 2**). *TP53* mutations (e.g., in frame, frameshift insertions and deletions, and missense or nonsense mutations) are nearly universal (>95% of cases) in HGSOC. Cases lacking a *TP53* mutation often have dysfunctional p53 due to copy number gain in genes involved in its regulation and degradation (e.g., MDM2 or MDM4). Roughly 50% of analysed cases have a homologous recombination (HR) deficiency (HRD). For example, *BRCA1* (8% germline and 4% somatic) and *BRCA2* (6% germline and 3%

somatic) play an essential role in homologous recombination (HR), and germline mutations in these genes are the most significant genetic risk factors (40-60% lifetime risk) leading to the development of EOC. Hypermethylation of the *BRCA1* promoter (11%) also occurs. Excluding *BRCA1/2* other Homologous Recombination DNA repair (HRR) pathway genes can be altered. *EMSY* is amplified in 6% of cases and in <5% of cases *PALB2* and *RAD51* family members are mutated. Non-HRR genes are also mutated and include: *RB1* (8% deletion, 2% mutation), *NF1* (8% deletion, 4% mutation), *PTEN* (7% deletion, <1% mutation) and *CCNE1* (14% amplification) ^{1,3,6,8}.

Epithelial Ovarian Cancer and its associated mutations				
HGSOC	LGSOC	MOC	CCOC	EnOC
TP53 BRCA1/2 NF1 PTEN RB1 CDK12 CCNE1 amp High genomic instability Extensive copy number changes Other HRR genes*	BRAF KRAS NRAS ERBB2	KRAS ERBB2 amp	ARID1A PI3KCA PTEN CTNNB1 PPP2R1α	ARID1A PIK3CA PTEN PPP2R1α MMR deficiency

Figure 2. Molecular landscape of EOC

Common genomic features associated with high grade serous ovarian cancer (HGSOC), low grade serous ovarian cancer (LGSOC), mucinous ovarian cancer (MOC), clear cell ovarian cancer (CCOC) and endometrioid ovarian cancer (EnOC). * = CHK2, BARD1, BRIP1, PALB2, RAD50, RAD51C, ATR, EMSY, Fanconi anemia genes. This figure was adapted from Banerjee and Kaye⁷. Created with BioRender.com

Importantly, it has been demonstrated that patients with a HRD (e.g., due to a *BRCA1/2* mutation) have enhanced sensitivity to platinum-based chemotherapy and other DNA damage agents^{6,9}. Moreover, HRD increases cells reliance on poly(ADP-ribose) polymerase (PARP) for survival, and this synthetic lethal interaction has been exploited with PARP inhibitors (PARPis) to treat recurrent disease and for maintenance therapy (see **section 1.2.** for more information)^{6,10,11}.

1.1.3. Low-grade serous ovarian cancer

LGSOC typically presents in patients at an advanced stage (~80% present at stage III) and evidence suggests its common precursor lesion is a serous borderline ovarian tumour (SBOT). LGSOC is considerably less aggressive than HGSOC with a median overall survival (OS) of 99 months (57 months for HGSOC). However, these tumours are intrinsically chemoresistant (<25% ORR) and have a high risk of relapse. As a result, even though survival is typically prolonged post-relapse, treatment of recurrent and persistent LGSOC is a major clinical challenge^{3,6,12,13}.

Unlike HGSOC, LGSOC possesses mutational stability and is considered genomically quiet. These tumours have low genomic complexity and mutational burden. They are typically *TP53* wild type. *KRAS* (33%), *BRAF* (10%), *NRAS* (10%), *USP9X* (13-27%) and *EIF1AX* (6-15%) are commonly mutated. Mutations in other MAPK or AKT-mTOR pathway associated genes (e.g., *ERBB2*, *PIK3CA*, *FFAR1*) can occur but are less common^{1,3,6,13}. Furthermore, *CDKN2A* (10%) mutations are frequently detected^{6,14,15}.

1.1.4. Endometrioid ovarian cancer

EnOC typically presents in patients at an early stage (~50% present at stage I) and its common precursor lesion is endometriosis (up to 40% of cases). Furthermore, a number of cases (5-10%) are associated with the hereditary condition Lynch syndrome, which occurs due to germline mutations in mismatch repair (MMR) genes (e.g., *MLH1*, *MSH2*, *MSH6*, *PMS1*, or *PMS2*). Overall prognosis is favourable, as patients with EnOC have a 60-70% 10-year survival rate. Chemosensitivity is considered intermediate as the ORR is reported to be ~60%^{1,3,6,8,16,17}.

Mutations frequently occur in the following genes: *CTNNB1* (30-50%), *PIK3CA* (30-50%), *KRAS* (25-50%), *ARID1A* (20-40%) and *PTEN* (30-45%). Microsatellite instability can be observed in ~10% of cases. Hypermutation can occur due to MMR deficiency (MMRd) or mutations in *POLE* (5%). A subset of EnOC cases resemble HGSOC due to widespread copy number variations or mutations in *TP53* (20%) and *BRCA1/2* (10%). These mutations are more common in higher grade EnOC and can result in misdiagnosis. However, the *WT1* gene can be used to distinguish between HGSOC (WT1-positive) and EnOC (WT1-negative). *SOX8* mutations (20%) are also detected and frequently co-occur with *TP53* mutations. Unbiased whole exome sequencing suggests other significantly mutated genes could include *KMT2D*,

KMT2B and *PIK3R1*, but considering only 26 samples were sequenced in this study it is unclear if this is representative of all EnOC cases^{3,6,8,16}.

1.1.5. Clear cell ovarian cancer

CCOC typically presents in patients at an early stage (55-70% present at stage I). Like EnOC, its common precursor lesion is endometriosis (up to 50% of cases) and it is associated with Lynch syndrome. At early stages prognosis is favourable, with an 80-90% 10-year survival rate. However, at advanced stages prognosis is extremely poor (10% 10-year survival rate), as these tumours are highly chemoresistant (~30% ORR). As a result, outcomes for late stage CCOC are significantly worse when compared to other EOC subtypes^{6,8 18-20}.

Although copy number variations (CNVs) are less frequent in CCOC when compared to HGSOC, the ratio of chromosomal whole-arm CNVs (associated with mitotic instability) is significantly higher (47% of cases in CCOC vs 21.6% in HGSOC). Amplifications are frequently observed in chromosomes 20q13.2 (~36%), 8q (52%) and 17q (46%). Increased copy numbers of *ERBB2* (chr17q12) (14%) *MET* (chr7q31) (31%) and *AKT2* (chr19q13.2) (14-24%) have been reported. Copy number loss can also be observed at chr13q (28%), 9q (21%) and 18q (21%). This can result in the loss of genes such as *CDKN2A/2B* (9q21.3) (17%). Truncating loss of *ARID1A* (40-62%) and *PIK3CA* mutations (33-51%) commonly occur in CCOC. A range of other genetic aberrations can also be detected in CCOC: *TP53* (<15% have a mutation or aberrant expression), *BRCA1* (6% germline and 5% somatic), *BRCA2* (1% germline and 5% somatic), *PPP2R1A* (7-10%), *SYNE1* (up to 20%), *CTNNB1* (5-10%), *KRAS* (5%), *PTEN* (5%), *TERT* promoter (5-15%), *ARID1B* (10%), *PIK3R1* (7-8%) and *SMARCA4* (5%)^{6,18-20}.

1.1.6. Mucinous ovarian cancer

MOC typically presents in patients at an early stage (75-80% present at stage I). Its common precursor lesion is poorly defined but a few hypotheses exist: (i) metastases from external-adnexal sites, (ii) from mature ovarian teratomas, and (iii) transitional cell (Brenner) neoplasms. Overall, MOC has an intermediate prognosis and intrinsic chemosensitivity is low (~25-40% ORR). At early stages patients have an excellent prognosis, with a >90% 10-year survival rate. However, the prognosis for advanced stage/relapse is extremely poor, with a <20% 10-year survival rate^{3,6,8,21}.

The majority of MOC cases have low genomic complexity but at higher grades/more advanced stages genomic complexity increases. A range of genes are frequently altered: *KRAS* (40-65%), *ERBB2* amplification (20-38%), *c-MYC* amplification (65%), *TP53* (50-75%), deletion or mutation of *CDKN2A* (50%), *PIK3CA* (10-13%) and *BRAF* (10%). *KRAS* mutations and *ERBB2* amplifications are typically mutually exclusive events. In addition, rarer (2-5%) mutations can occur in *PTEN*, *FGFR*, *KIT* and *STK11*^{6,21}.

1.2. PARP biology and PARP inhibitors

1.2.1. ADP-ribosylation and the DNA damage response

To allow for tight temporal control of DNA damage repair, like other cellular functions, it can be regulated through post-translational modifications (PTMs). ADP-ribosylation is an important PTM in the DNA damage response (DDR) pathway²². PARP enzymes (17 exist in humans) bind and cleave nicotinamide adenine dinucleotide (NAD⁺) into nicotinamide (NAM) and ADP-ribose (ADPr), to facilitate the reversible, covalent addition of one (MARylation) or more (PARylation) ADPr units onto acceptor proteins. During PARylation, ADPr units attach through 2',1''-O-glycosidic ribose-ribose bonds (transesterification reaction) to form long (~200 units), linear or branched (every 20-50 units) chains. The majority of ADP-ribosylation carried out under basal conditions and in response to cellular stress is catalysed by PARP-1 (85-90%) and PARP-2 (10-15%)^{23,24}. Negatively charged ADPr moieties can be placed onto a variety of amino acid residues: serine (most common target for DDR), glutamate, aspartate, tyrosine, arginine, lysine or cysteine. However, they can also be added onto DNA or RNA ends^{22,25}. The removal of ADPr is also essential to maintain genome stability and the majority is removed by the hydrolases Poly(ADP-ribose) glycohydrolase (PARG) and (ADP-ribosyl) hydrolase 3 (ARH3). However, other ADPr erasers exist such as (ADP-ribosyl) hydrolase 1 (ARH1), Terminal ADP-Ribose Protein Glycohydrolase 1 (TARG1), MacroD1 and MacroD2 enzymes, and some phosphodiesterases²⁵.

PARP-1, -2 and -3 are the only DNA damage sensors (nuclear localisation) among the family of PARP proteins. PARP-1 and -2 can catalyse PARylation, whilst PARP-3 has MARylation activity²⁶. PARP-1 and -2 do have some functional redundancy, however, PARP-1 is responsible for the majority of ADP-ribosylation in the DDR²². PARP-1 contains a variety of different domains. (i) An amino-terminal DNA binding domain (DBD), that consists of a nuclear

localisation sequence and three zinc fingers (ZnF1-3) for binding to DNA breaks. (ii) An automodification domain, consisting of a BRCA1 C Terminus (BRCT) domain that interacts with DNA, PAR or other proteins. (iii) The carboxy-terminal catalytic domain consists of a tryptophan-glycine–arginine-rich (WGR) domain, which can interact with DNA; a helical domain (HD), which blocks the enzymatic activity of PARP-1 unless bound to DNA breaks; and an ADP-ribosyltransferase (ART) catalytic domain^{22,24,27}.

PARP-1 and PARylation play an important part in regulating various DNA repair processes, including single-stranded break (SSB) and double-stranded break (DSB) repair (**Figure 3**), DNA replication fork stabilisation, and the modification of chromatin structure²⁴. For example, if DNA damage induces the formation of SSBs PARP-1 will bind using its ZnF domains, leading to the formation of a PARP1/DNA nucleoprotein structure. As a result, the HD domains confirmation changes and allosterically activates PARP-1 catalytic activity (i.e., HD domain stops preventing NAD⁺ from binding to the ART binding site). This drives PARylation and activates SSB repair (SSBR) by promoting the accumulation and stabilisation of SSBR components at the site of damaged DNA (i.e., creates a scaffold to recruit SSBR factors). For example, this leads to the PARylation/recruitment of factors such as X-ray repair cross-complementing protein 1 (XRCC1). Finally, PARP-1 PARylates itself (autoPARylation) leading to its dissociation from DNA due to the PTMs negative charge, allowing repair factors to reach the break site^{22,24,27,28}.

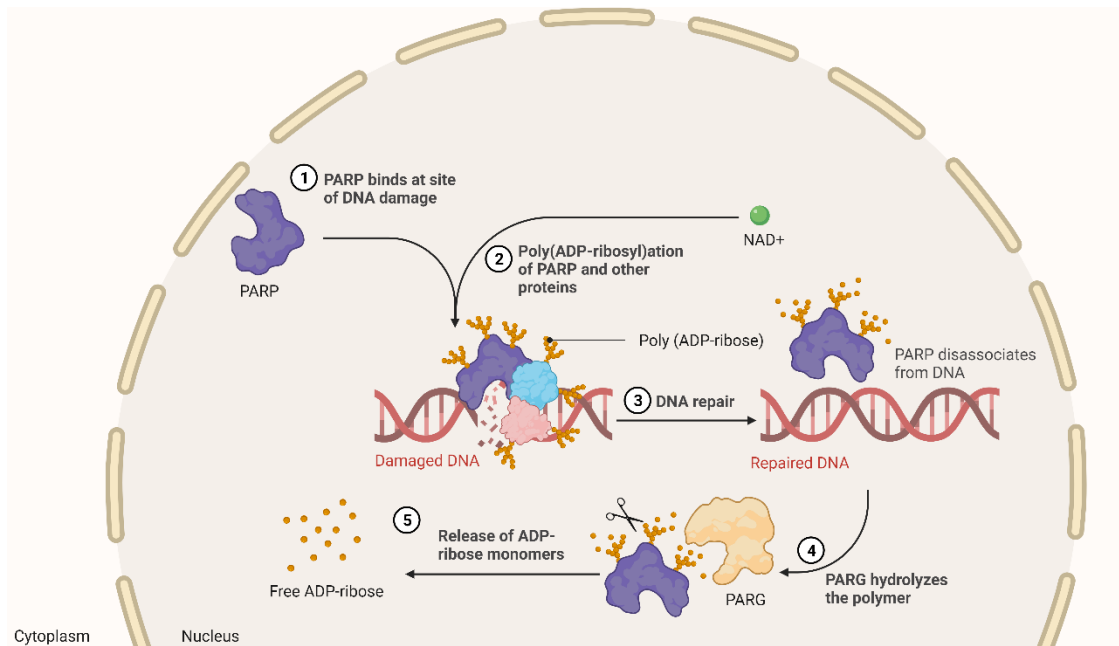


Figure 3. The role of PARP biology in DNA repair

A schematic representation of PARP-1 activity in DNA repair. **(1)** PARP-1 recognises damaged DNA (e.g., single, or double-stranded breaks) and binds at this site using its DNA-binding domain. **(2)** Using NAD^+ as its substrate, PARP-1 synthesises poly(ADP-ribose) chains on itself/target proteins to recruit DNA repair proteins, remodel chromatin and eventually repair DNA. **(3)** PARP-1 autoPARylation leads to PARPs release from DNA due to the PTMs charge. **(4-5)** Enzymes such as PARG break down PAR chains and release free ADP-ribose units. Created with BioRender.com.

1.2.2. PARP inhibitors mechanism of action and synthetic lethality

As mentioned previously, tumours possessing a HRD (e.g., *BRCA1/2*-mutation) are reliant on PARP for survival and treatment with a PARPi leads to a synthetic lethal interaction (i.e., mutation in one of two genes has little effect but defects in both result in cell death)²⁹⁻³¹ (**Figure 4**). The model for PARPi synthetic lethality is as follows: (i) PARPis interact with the catalytic domain of PARP1/2, preventing NAD^+ from binding to PARPs active site; (ii) autoPARylation is blocked, PARP becomes trapped on DNA, and the PARP1/DNA nucleoprotein complex impairs replication fork progression; (iii) replication fork stalling leads to the formation of DSBs, which in a HR-proficient cell could be repaired through the HRR pathway (i.e., high-fidelity, template directed repair restores the original DNA sequence) and cells survive. However, in a HRD cell nonhomologous end-joining (NHEJ) is used instead (low-fidelity, error prone repair), DSB ends are ligated resulting in the deletion or mutation of DNA

sequences. This leads to large scale genomic rearrangements (genomic instability), cell cycle arrest and apoptosis (synthetic lethality)^{27,32}. It should be noted that the ability of PARPi to trap PARP on DNA correlates with cytotoxic potency, and PARPi trapping can be ranked: talazoparib >> niraparib > olaparib = rucaparib >> veliparib³³⁻³⁵. Furthermore, it is likely that other sources of PARP-trapping lesions (e.g., genomic ribonucleotides) which impede DNA replication exist³⁶. Interestingly, there is conflicting evidence in the literature that suggests PARPis could actually increase replication fork speed (rather than causing fork stalling and collapse), as such DNA lesions go unrecognised by the replication machinery, and this results in increased replication stress and DNA damage³⁷.

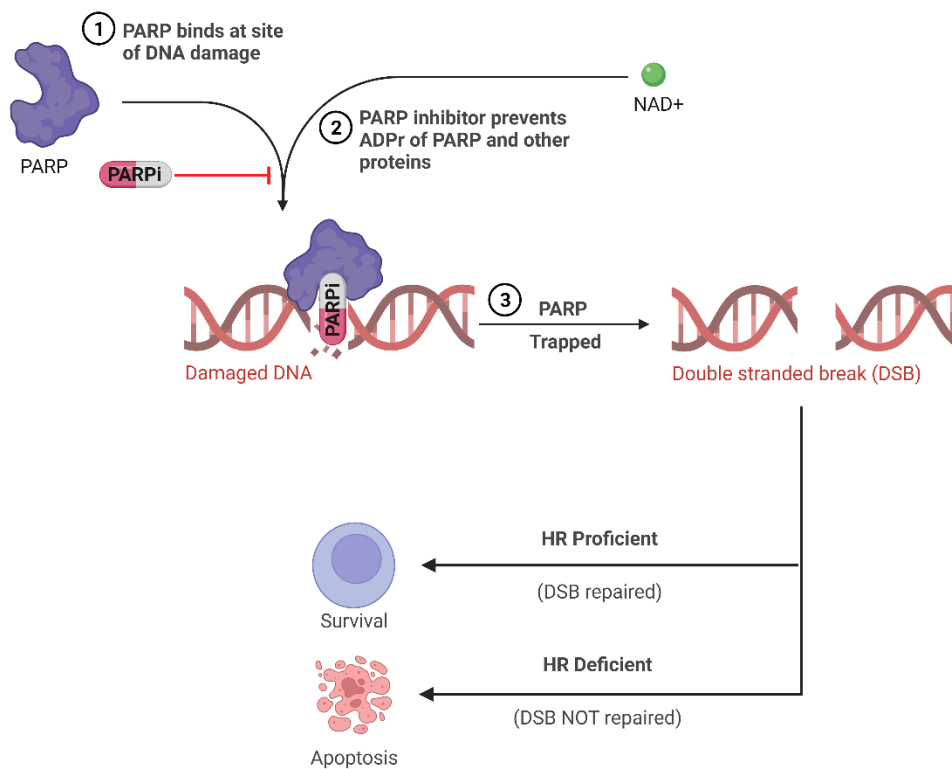


Figure 4. Proposed PARPi mechanism of action

PARPi treatment **(1-3)** inhibits PARylation resulting in PARP-1 being trapped on DNA at the site of DNA damage. The PARP-1/DNA nucleoprotein complex impairs replication fork progression (replication forks stall) and double stranded breaks (DSBs) form. In HR proficient cells DSBs are repaired accurately and cells survive. However, in HR deficient cells DSBs cannot be repaired by HR. Instead, DNA is repaired via the 'error prone' NHEJ pathway, leading to genomic instability, cell cycle arrest and apoptosis²⁷. Created with BioRender.com.

1.2.3. Clinical development of PARP inhibitors

In recent years PARPi including olaparib, rucaparib and niraparib have been approved for the treatment of ovarian cancers by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA). Depending on the PARPi and country of use, each PARPi is approved for administration in different contexts. In the EU, olaparib, rucaparib and niraparib are approved for maintenance monotherapy for patients with platinum sensitive, relapsed HGSOC. Whilst rucaparib can also be used as a third-line treatment in *BRCA1/2* mutated (germline and/or somatic) ovarian cancer. In the US, olaparib, rucaparib and niraparib are also approved for the maintenance treatment of recurrent ovarian cancer. Olaparib can be used for the fourth-line treatment and first-line maintenance of *BRCA1/2*-mutant advanced ovarian cancer. Furthermore, rucaparib is approved for the third-line treatment of *BRCA1/2*-mutant advanced ovarian cancer³⁸.

A number of key clinical trials have been performed in ovarian cancer and have been extensively reviewed^{38,39}. Each of the approved inhibitors has been assessed as a (i) front-line maintenance therapy, (ii) for maintenance in a recurrent setting and (iii) for the treatment of recurrent ovarian cancer (**Table 1**).

For olaparib, its efficacy as a front-line maintenance therapy was tested in the SOLO1 trial (NCT01844986)¹⁰. In this phase 3 trial, women with newly diagnosed advanced *BRCA1/2*-mutated HGSOC or EnOC that responded to platinum-based chemotherapy were selected. The median progression free survival (PFS) was 49.9 months following olaparib treatment, compared to 13.8 months in the placebo group³⁹. Encouragingly, a 7-year follow-up study revealed that 67.0% of olaparib treated patients were alive, compared to 46.5% in the placebo group⁴⁰. Olaparib was assessed as a maintenance therapy in the recurrent setting in Study 19 (NCT00753545)⁴¹ and SOLO2 (NCT01874353)⁴². In study 19 (phase 2 trial) women with platinum-sensitive (received two or more regimens of chemotherapy), relapsed, HGSOC were selected. Patients receiving olaparib had a PFS of 8.4 months compared to 4.8 months in the placebo group⁴¹, but when separating patients by *BRCA*-mutation status PFS was found to be 11.2 months (olaparib) and 4.3 months (placebo)⁴³. By comparison, the SOLO-2 (phase 3) trial assessed olaparib maintenance therapy in women with platinum-sensitive (received two or more regimens of chemotherapy), relapsed, HGSOC or EnOC that harbour *BRCA1/2*-mutations. Patients receiving olaparib had a PFS of 19.1 months, compared to 5.5 months in

the placebo group⁴². Finally, SOLO-3 (NCT02282020) was the confirmatory (phase 3) trial for the treatment of recurrent (platinum sensitive, *BRCA1/2*-mutant) ovarian cancer with olaparib³⁹. Compared to placebo groups olaparib treatment improved PFS to 13.4 months (versus 9.2) and an ORR of 84.6% (versus 61.5%)⁴⁴ was observed.

For niraparib, its efficacy as a front-line maintenance therapy was tested in the PRIMA trial (NCT02655016)⁴⁵. In this phase 3 trial women with newly diagnosed advanced HGSOE/EnOC that had responded to platinum-based chemotherapy were selected. Among patients harbouring tumours with a HRD 21.9- and 10.4-month median PFS was observed in niraparib, and placebo treated groups, respectively. In the overall population PFS was 13.8 months (niraparib) and 8.2 months (placebo)⁴⁵. Niraparib was assessed as a maintenance therapy in the recurrent setting in the NOVA trial (NCT01847274). In this phase 3 trial, patients with platinum-sensitive (received two or more regimens of chemotherapy), recurrent ovarian cancer were selected. Patients were divided into cohorts with/without a germline *BRCA1/2*-mutation. The latter was then further divided into HRD-positive and -negative subgroups. The group harbouring germline *BRCA1/2*-mutations had a PFS of 21.0- (niraparib group) and 5.5-months (placebo group). Overall, the non-germline *BRCA1/2* group had a PFS of 9.3 and 3.9 months following niraparib and placebo treatment, respectively. By comparison, HRD-positive patients (without a germline *BRCA1/2*-mutation) had 12.9- (niraparib group) and 3.8-months (placebo group) PFS⁴⁶. Finally, the QUADRA (NCT02354586) trial investigated the efficacy of niraparib in the treatment of recurrent ovarian cancer (4th/5th line treatment). In this phase 2 trial, patients with platinum-sensitive (received three or more regimens of chemotherapy), recurrent ovarian cancer were selected. Median OS values of 26.0 (*BRCA*-mutant population), 19.0 (HRD-positive) and 15.5 (HRD-negative) months were observed⁴⁷.

For rucaparib, its efficacy as a front-line maintenance therapy was tested in the ATHENA trial (NCT03522246)⁴⁸. In this phase 3 trial women with newly diagnosed advanced HGSOE that responded to chemotherapy were selected and stratified by their HRD status. In the HRD-positive population, median PFS was 28.7- and 11.3-months following rucaparib and placebo treatment, respectively. Whereas in the HRD-negative population it was 12.1 (rucaparib) and 9.1 (placebo) months. Demonstrating patients benefit from rucaparib monotherapy regardless of HRD status⁴⁸. Rucaparib was assessed as a maintenance therapy in the recurrent setting in the ARIEL3 (NCT01968213) and ARIEL4 (NCT02855944) trials. In ARIEL3 (phase 3

trial) patients with platinum sensitive (received two or more regimens of chemotherapy) HGSOC or EnOC were selected. This study demonstrated that *BRCA*-mutant patients have a median PFS of 16.6 months following rucaparib treatment (versus 5.4 months in placebo). Whilst HRD-positive patients treated with rucaparib had a median PFS of 13.6 months (versus 5.4 months in placebo)⁴⁹. ARIEL4 (phase 3) was a confirmatory trial³⁹, in this study women with *BRCA1/2*-mutated ovarian carcinoma that had previously responded to chemotherapy (two or more regimens) were selected. Patients harbouring deleterious *BRCA1/2*-mutations without reversion mutations had a median PFS of 7.4 months in the rucaparib group (versus 5.7 months in the chemotherapy group)⁵⁰. Finally, Study 10 (NCT01482715)⁵¹ and ARIEL2 (NCT01891344)^{52,53} were essential in assessing rucaparib in the treatment of recurrent ovarian cancer. In Study 10 (phase 1/2 trial) platinum sensitive (received two to four regimens of chemotherapy), *BRCA1/2*-mutant HGSOC patients were selected, and the ORR to rucaparib was 59.5%⁵¹. ARIEL2 was a two-part (phase 2) clinical trial. In part 1, patients with recurrent, platinum-sensitive HGSOC were separated into three groups: (i) *BRCA*-mutant (deleterious germline or somatic), (ii) *BRCA*-wild type and chromosomal loss of heterozygosity (LOH) high (LOH high group), (iii) or *BRCA* wild-type and chromosomal LOH low (LOH low group). The greatest benefit in median PFS was observed in the *BRCA*-mutant (12.8 months) population, followed by the LOH high (5.7 months) and LOH low subgroups (5.2 months). This study demonstrated that PARPis could be used to treat patients without *BRCA* mutations⁵². In ARIEL2 part 2 a post hoc exploratory biomarker analysis was performed on ARIEL 2 samples pre- and post-platinum treatment. This study identified genetic, epigenetic, and genomic biomarkers that correlate with rucaparib sensitivity (e.g., *RAD51C/D* mutations and high *BRCA1* promoter methylation) and resistance (e.g., *BRCA* reversion mutations, loss of *BRCA1* methylation, dysregulated RAS signalling, and amplification of AKT genes or cell cycle genes)⁵³.

Table 1. Summary of major PARPi clinical trials in different settings

	Study	Phase	Study arms	Cancer criteria	HR status	Endpoint (PFS and/or ORR)
Front-line maintenance therapy	SOLO-1	III	Olaparib vs Placebo	HGSOC or EnOC	BRCAm	PFS: 49.9 vs 13.8 m
	PRIMA	III	Niraparib vs Placebo	HGSOC or EnOC	All	PFS: 13.8 vs 8.2 m (All), 21.9 vs 8.2 m (HRD)
	ATHENA	III	Rucaparib vs placebo	HGSOC	All	PFS: 28.7 vs 11.3 m (HRD-positive), 12.1 vs 9.1 m (HRD-negative)
Maintenance therapy in the recurrent setting	Study 19	II	Olaparib vs Placebo	HGSOC	All	PFS: 8.4 vs 4.8 m
	SOLO-2	III	Olaparib vs Placebo	HGSOC or EnOC	BRCAm	PFS: 19.1 vs 5.5 m
	NOVA	III	Niraparib vs Placebo	HGSOC	All	PFS: 21.0 vs 5.5 m (gBRCAm), 12.9 vs 3.8 m (HRD-positive, non-gBRCAm), 9.3 vs 3.9 m (non-gBRCAm)
	ARIEL3	III	Rucaparib vs Placebo	HGSOC or EnOC	All	PFS: 10.8 vs 5.4 m (All), 16.6 vs 5.4 m (BRCAm)
	ARIEL4	III	Rucaparib vs Placebo	HGSOC or EnOC	BRCAm	PFS: 7.4 vs 5.7 m
Treatment of recurrent ovarian cancer	SOLO-3	III	Olaparib vs Placebo	HGSOC or EnOC	BRCAm	PFS: 13.4 vs 9.2 m, ORR: 84.6% vs 61.5%
	QUADRA	II	Niraparib	HGSOC	HRD	ORR: 24%
	Study 10	I/II	Rucaparib	HGSOC	gBRCAm	ORR: 59.5%
	ARIEL2	II	Rucaparib	HGSOC	All	PFS: 12.8m (BRCAm), 5.7 m (BRCAwt, LOH high), 5.2 m (BRCAwt, LOH low)

Adapted from Smith and Pothuri³⁹ with additions from other trials^{44,46,48,50-52}. Please note EnOC tumours are high grade. Abbreviations. BRCAm: BRCA mutation; BRCAwt: BRCA wild type; EnOC: endometroid ovarian cancer; gBRCAm: germline BRCA mutation; HGSOC: high grade serous ovarian cancer; HRD: homologous recombination deficiency; LOH: loss of heterozygosity; m: months; ORR: overall response rate; PFS: progression free survival.

1.2.4. PARP inhibitor resistance

PARPi resistance (PARPi-R) is a major problem in the clinic but our understanding of each mode of resistance is expanding. Currently there are four distinct categories of PARPi-R: (i) increased drug efflux, (ii) impact on the activity and abundance of PAR chains, (iii) restoration of HR, and (iv) stabilisation of stalled forks⁵⁴ (**Figure 5**).

The cellular availability of PARPi can be reduced due to the overexpression of ATP Binding Cassette Subfamily B Member 1 and 2 (*ABC1A/B*) genes (encoding P-glycoprotein efflux pumps)^{32,54}. For example, in *Brca1*-deficient murine models of breast cancer that developed resistance to olaparib, *Abcb1a/b* was upregulated, and co-treatment with olaparib and the P-glycoprotein inhibitor tariquidar reversed resistance⁵⁵. *ABCB1* overexpression has also been observed in olaparib-resistant ovarian cancer cell lines (e.g., A2780 cells), and this was also reversible by following combination treatment with olaparib and an *ABCB1* inhibitor (e.g., verapamil or elacridar)⁵⁶. In chemotherapy treated ovarian and breast cancer samples *ABCB1* is upregulated due to transcriptional fusions involving *ABCB1* that do not disrupt its open reading frame but place it under the control of a strong promoter. For example, *SLC25A40-ABCB1* fusion led to the highest level of *ABCB1* expression, but 15 other novel *ABCB1* fusion partners were also identified. It is intriguing that *ABCB1* expression is upregulated following PARPi-therapy, given these multidrug resistance proteins (*ABCB1* also referred to as MDR1) are also involved in the cellular efflux of chemotherapeutic drugs⁵⁷. Furthermore, in the proof-of-concept EVOLVE study (NCT02681237, phase II trial), which assessed an olaparib and cediranib (VEGF inhibitor) combination in HGSOc after progression on a PARPi. *ABCB1* upregulation was observed in 15% of cases after PARPi progression (i.e., compared to matched PARPi-naïve archival tissue). Patients with *ABCB1* upregulation had poorer outcomes, compared to HR mutated and HR wild-type patients⁵⁸.

Alterations in the activity and abundance of PAR chains can be a result of PARP-1 mutations that disrupt protein function⁵⁴. For example, a genome-wide and high-density CRISPR Cas9 screen identified point mutations in PARP-1 that cause PARPi-R (talazoparib)⁵⁹. Mutations in PARP-1 residues M43 and F44 (p.[43delM;44F>I]) were identified, these residues are involved in base stacking interactions formed between PARP-1 ZnFs and DNA. Their mutation impairs PARP-1 trapping by preventing the recruitment and PARylation of PARP-1 at sites of DNA damage. Mutations in residues K119 and S120, which are DNA-contacting residues within the

ZnF2 domain, also abolished PARP-1 recruitment to DNA damage sites. A mutation in ZnF3 (N329Q) also blocked PARP-trapping. However, ZnF3 is not predicted to be part of the DNA-binding interface. Mutations outside of ZnF domains were reported, such as HD742F and 848delY. HD742F alters the HD domain (important for PARP activation) and prevents PARP trapping. By comparison, the 848delY mutant alters a solvent-exposed helix in the catalytic domain and causes a subtle PARP-trapping defect (i.e., slightly reduced trapping and faster dissociation from sites of damage). PARPi-R mutations could also be observed in the WGR and catalytic domains (e.g., at residue A925), but mutations in the BRCT domain were not detected, suggesting this region is dispensable for PARPi trapping and cytotoxicity. It should be noted that PARP-1 mutations could only be tolerated in *BRCA1* mutant cells with some residual *BRCA1* function (e.g., truncating mutations). Importantly, Pettitt and colleagues also observed a PARP-1 mutation (R591C) in an olaparib-resistant patient. This mutation affects the WGR domain, altering inter-domain communication between DNA-binding and catalytic domains. PARP-1^{R591C} mutants can be recruited to sites of DNA damage, but more rapidly dissociated from damaged DNA compared to wild-type PARP-1⁵⁹. Alterations in the activity and abundance of PAR chains can also be a result of the disruption of PARG activity⁵⁴. As discussed in **section 1.2.1**, PARG breaks down PAR chains, preventing PAR accumulation. Loss of PARG can partially restore PARylation following PARPi treatment, reducing trapping and leading to PARPi-R in *Brca2*-deficient mouse mammary tumour models⁶⁰.

Restoration of HR is considered to be the main mechanism of PARPi-R, and can be achieved by secondary mutations that restore the activity of *BRCA*-dependent (e.g., *BRCA1/2*) and -independent (e.g., *RAD51C/D* or *PALB2*) proteins that are involved in DNA repair pathways⁶¹. True reversions (i.e., to wild-type sequence) and second site reversions (i.e., deletions or insertions that restore the reading frame) of *BRCA1/2*⁶²⁻⁶⁴, *RAD51C/D*^{65,66} and *PALB2*⁶⁷ genes have been identified in pre-clinical and clinical studies. For example, in the EVOLVE study (NCT02681237, phase II trial) reversion mutations in *BRCA1*, *BRCA2* and *RAD51B* were reported in 19% of patients that had progressed on a PARPi, and these mutations correlated with poorer outcomes⁵⁸. Partial HR restoration can also be achieved in cells with loss-of-function *BRCA1* mutations (but not *BRCA2*) by downregulating NHEJ^{54,61}. Several genes involved in inhibiting end resection can be lost, upregulating nuclease activity at DSBs and promoting HR. These genes include *53BP1*⁶⁸⁻⁷⁰ and *RIF1*⁷¹⁻⁷⁵, or members of the Shieldin

complex such as *REV7*^{76,77} (also known as *MAD2L2*), *SHLD1*, *SHLD2* and *SHLD3*⁷⁸⁻⁸⁴. Importantly, clinical evidence does exist, as low expression or mutations in these genes has been reported in patient derived xenograft (PDX) models⁵⁴. Furthermore, there is pre-clinical evidence that loss of other factors such as CTC1, Pol α , DYNLL1 or ATMIN could restore HR through similar mechanisms, but this hasn't been observed in patient samples to date^{54,61}.

Stalled replication forks are typically protected by *BRCA1/2* genes. Following their deletion/loss of function, nucleases such as MRE11 (recruited by EZH2) and MUS81 (recruited by PTIP) are recruited to stalled forks, resulting in nascent-strand shortening, fork collapse, chromosomal aberrations, and PARPi-sensitivity. As a result, loss of EZH2 (in *BRCA2*-deficient cells) or PTIP (in *BRCA1/2*-deficient cells) prevents nuclease recruitment, leading to fork stabilisation and PARPi-R⁸⁵⁻⁸⁷. It should be noted loss of these genes hasn't been reported in PARPi-R clinical samples at this moment in time⁵⁴.

Other mechanisms have been reported to cause PARPi-R. The activation of oncogenic pathways such as PI3K-AKT-mTOR and RAS-RAF-MEK-ERK has been associated with PARPi-R in pre-clinical studies⁶¹. In ARIEL2 (NCT01891344) it was reported dysregulated RAS signalling, and amplification of AKT genes could cause resistance to rucaparib⁵³. Interestingly, PI3K/AKT and RAS/RAF pathway alterations frequently occur in each subtype of EOC, so could potentially be targeted⁶. Alterations in cell cycle control (e.g., ATM, ATR, CHK1/2 or WEE1) have also been reported as a mechanism of PARPi-R pre-clinically and in clinical trials^{53,61}.

Modes of resistance to PARPis			
Increased drug efflux	Decreased PARP trapping	Restoration of HR	Stabilisation of replication forks
Upregulation of ABC transporters	Loss or decreased trapping of PARP-1 Loss of PARG	Reactivation of BRCA1/2 Loss of 53BP1 Loss of Shieldin factors	Loss of PTIP Loss of EZH2

Figure 5. The major PARPi resistance mechanisms

Overview of the four categories of PARPi resistance mechanisms. In each category molecular mechanisms that have been identified in preclinical studies, PDX-models and/or primary tumour material are shown. Figure is adapted from Noordermeer and Attikum⁵⁴. Created with BioRender.com.

1.2.5. PARP inhibitor combination therapies

PARPi-R is a major challenge in the treatment of ovarian cancer, so there is significant interest in identifying combination therapies to increase PARPi efficiency, to prevent the development of resistance or overcome acquired resistance⁶¹. PARPis have been combined with DDR inhibitors (e.g., DNA-PK, ATR, ATM, CHK1, CHK2, WEE1 and POL θ) and synergistic effects have been observed in pre-clinical studies and clinical trials^{61,88}. For example, DNA polymerase theta (POL θ) is upregulated in ovarian cancer and compensates for HRD through a form of DSB repair known as microhomology mediated end joining (MMEJ). Inhibitors of POL θ are synthetic lethal in HRD cells, synergistic with PARPis, and can overcome various PARPi-R mechanisms (e.g., 53BP1 and Shieldin complex defects)⁸⁹.

It is possible PARPi combinations could extend their benefit beyond HRD-populations⁸⁸. For example, the combination of PI3K/mTOR inhibition and PARPi has been explored in HR-proficient tumours, and pre-clinical evidence suggests the synergistic interaction is due to the induction of a HRD following PI3K/mTOR inhibition (e.g., can regulate HRR and NHEJ via AKT directly or by regulating HR pathway components gene expression)^{61,88}. Phase I clinical trials suggest the combination of olaparib and capivasertib (AKT inhibitor) is tolerable, and clinical benefit was achieved in ovarian cancer patients with HR-proficient tumours^{90,91}. Furthermore, combination therapy with PARPi and MEK inhibitors (MEKi) leads to synergy in *RAS* mutant cancers (e.g., EOC), as MEKi decreases cells HR repair capacity, leading to increased DNA damage and apoptosis⁹². Phase I (SOLAR, NCT03162627) and II (ComboMATCH, NCT05554328) clinical trials are assessing the combination of olaparib and selumetinib (MEKi) in *RAS*-mutated ovarian cancers, with early reports suggesting the combination is tolerated and has antitumour activity⁹³.

Given PARP-1 is known to play a role in metabolic rewiring in cancer cells (e.g., mitochondrial homeostasis and energy production)^{23,25}, and it uses the metabolite NAD⁺ as the substrate for its catalytic activity²⁷, it is unsurprising PARP and metabolic inhibitor combinations are gaining traction. For example, inhibition of the nucleotide salvage factor DNPH1, which eliminates the cytotoxic nucleotide 5-hydroxymethyl-deoxyuridine monophosphate, potentiates PARPi sensitivity in *BRCA1*-deficient cells⁹⁴.

Interestingly, HRD ovarian tumours have been shown to shift their metabolism from a Warburg phenotype (i.e., tumours show a preference for aerobic glycolysis regardless of oxygen levels) towards oxidative phosphorylation (OXPHOS) to maintain NAD⁺/ATP levels for DNA repair. This reduces their sensitivity to PARPi and can be exploited with an OXPHOS inhibitor such as Metformin⁹⁵.

Genes that are involved in NAD⁺ homeostasis such as nicotinamide nucleotide adenyl transferase 1 (NMNAT-1)⁹⁶ and sirtuins (SIRT)⁹⁷ have been shown to be synthetic lethal with *BRCA1/2*-deficiency. However, the synergistic interaction between nicotinamide phosphoribosyltransferase (NAMPT) inhibitors (NAMPTis) and PARPis has been more extensively investigated⁹⁸⁻¹⁰¹ (**Figure 15**). The synthetic lethal interaction was first observed in 2012 by Ashworth and colleagues, where they observed triple-negative breast cancer (TNBC) models were exquisitely sensitive to the combination of a NAMPT inhibitor (NAMPTi), FK866, with olaparib. They proposed that synergy is due to the NAMPTi reducing NAD⁺ availability, since synergy could be rescued by supplementing media with the NAD⁺ precursor nicotinic acid (NA). Importantly, they observed the combination exacerbated γ H2AX formation (marker of DNA damage), increased apoptosis, and this translated into *in vivo* efficacy. Interestingly, data suggested *BRCA1/2*-defects increased the combinations benefit, so these mutations could potentially be used as biomarkers to direct the combinations use⁹⁸. In 2017 Heske and colleagues observed the synergistic interaction in models of Ewing sarcoma. They provided further insight into the mechanistic drivers of synergy: (i) characterised NAD⁺ salvage pathway metabolism through metabolomics and rescue assays, confirming synergy is due to the reduction of β -nicotinamide mononucleotide (β -NMN) and NAD⁺; (ii) demonstrated that the combination reduces PARylation to a greater extent than either monotherapy treatment; and (iii) used a reverse phase protein microarray (RPMA) to assess the cellular response to the combination, confirming that it upregulates apoptosis (e.g., cleaved PARP, caspase-3, and -7), DNA damage (γ H2AX) and stress activated protein kinases (e.g., SAPK/JNK and p38 MAPK)⁹⁹. In 2023 Sauriol and colleagues demonstrated the combination overcomes acquired and intrinsic PARPi-R in HGSOC *in vitro*, *in vivo* and using patient derived organoid models¹⁰⁰. Finally, a poster presented at the 2023 AACR Annual meeting by Slamon and colleagues, indicates a subset of ovarian cancer patients are extremely sensitive to NAMPTi, and the group further characterised the PARPi/NAMPTi

combination in ovarian cancer *in vitro* and *in vivo*^{101,102}. However, these data have not been published to date.

1.3. NAD⁺ Metabolism

1.3.1. Regeneration of the NAD⁺ pool

To support malignant transformation and rapid proliferation, cancer cells must alter their cellular metabolism (e.g., the Warburg effect shifts ATP generation away from OXPHOS to glycolysis, even under normal oxygen). These metabolic alterations help to maintain energy status, maintain their cellular redox status, and increase the biosynthesis of macromolecules¹⁰³⁻¹⁰⁵. Multiple cellular processes are dependent on the redox cofactor NAD⁺ such as: glycolysis, the tricarboxylic acid cycle, and OXPHOS by the electron transport chain (ETC)¹⁰⁶. In mammals three major NAD⁺ biosynthesis pathways maintain nuclear, cytoplasmic and mitochondrial NAD⁺ pools (**Figure 6**): (i) the *de novo* biosynthesis or kynurenine pathway uses tryptophan (Trp) as a precursor; (ii) the Preiss-Handler (PH) pathway uses nicotinic acid (NA) as a precursor; and (iii) the salvage pathway uses β -NMN, reduced β -NMN (β -NMNH), nicotinamide (NAM), nicotinamide riboside (NR) or reduced NR (NRH) as a precursor¹⁰⁶⁻¹⁰⁹.

The rate limiting step of (i) the *de novo* biosynthesis pathway is catalysed by Tryptophan dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO), which converts Trp into formylkynurenine (FK). FK is converted into nicotinic acid mononucleotide (NaMN) through a series of enzymatic steps. The NMNAT-1 (nucleus), -2 (golgi complex) or -3 (mitochondrial) enzymes adenylate NaMN into nicotinic acid adenine dinucleotide (NaAD), before it is amidated into NAD⁺ by NAD synthase (NADSYN)^{106,107}. Interestingly, the majority of *de novo* biosynthesis is reported to occur in the liver¹¹⁰. The rate limiting step of (ii) the PH pathway is catalysed by nicotinic acid phosphoribosyltransferase (NAPRT), which converts NA into NaMN. Again, this is converted into NaAD and NAD⁺ by NMNAT 1-3 and NADSYN, respectively¹⁰⁴. However, due to rapid excretion and metabolism the *in vivo* concentration of NA is low when compared to precursors such as NAM^{111,112}. Thus, the (iii) salvage pathway is often considered the major route of NAD⁺ biosynthesis¹¹³⁻¹¹⁵, although it is important to note that tissue context can determine the choice of NAD⁺ biosynthetic pathway used in normal tissues and cancer¹¹⁶. The rate limiting step of this pathway is catalysed by the NAMPT enzyme (found in the nucleus, cytoplasm, and mitochondria), which converts NAM and

phosphoribosyl pyrophosphate (PRPP) into β -NMN. NMNAT 1-3 can then convert β -NMN (and ATP) into NAD^{+104} .

Excluding PARPs, three other major classes of NAD^{+} consuming enzymes exist: (i) the cyclic ADP-ribose (cADPr) synthases (cADPrSs) CD38 and CD157, which regulate processes such as Ca^{2+} , glucose and lipid homeostasis; (ii) SIRT, which MARYlate and deacetylase proteins; and (iii) NAM *N*-methyltransferase (NNMT) which can remove NAM from recycling through its methylation^{106,107}.

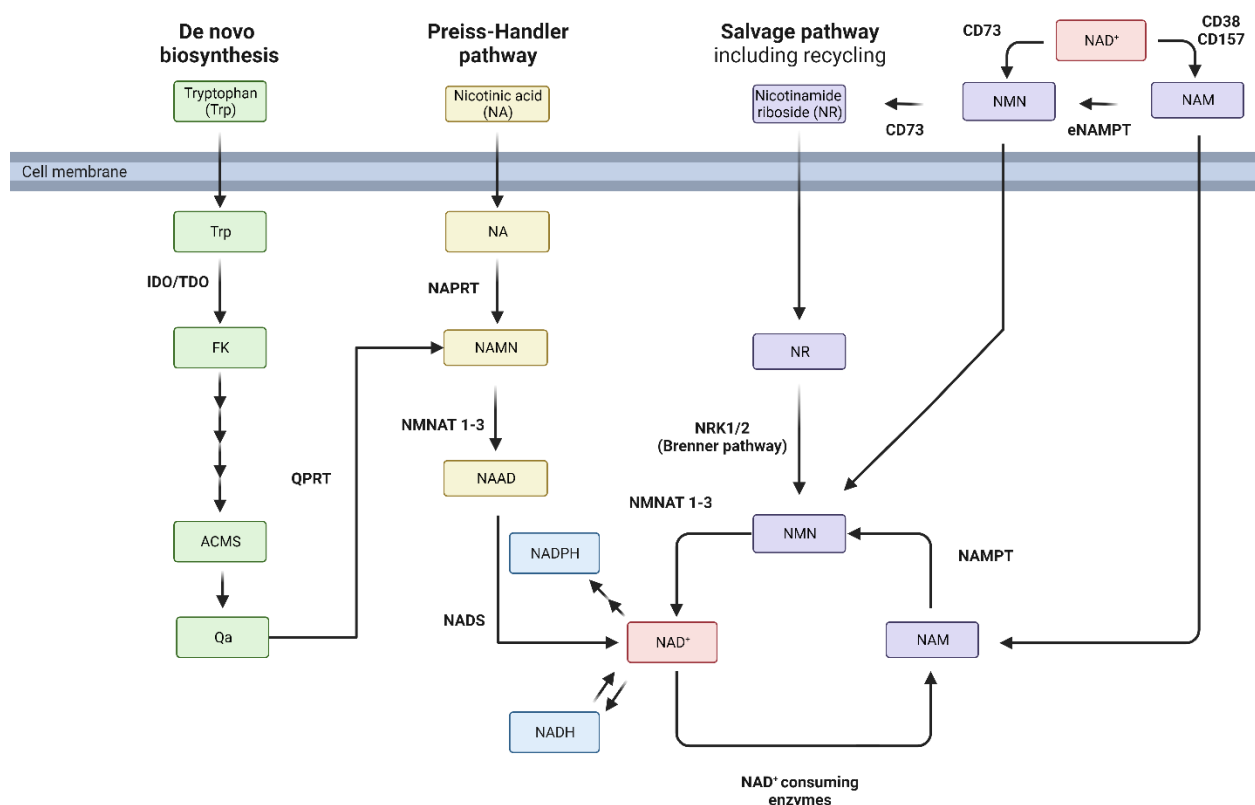


Figure 6. NAD^{+} biosynthesis pathways

NAD^{+} can be synthesised via (i) the *de novo* biosynthesis pathway, (ii) the Preiss-Handler pathway, or (iii) the salvage pathway. In addition to these major routes of NAD^{+} biosynthesis, extracellular NAD^{+} can be hydrolysed into NAM by CD38 and CD157, which can either be transported into the cell or converted into NMN by extracellular NAMPT (eNAMPT). Extracellular NAD^{+} can also be dephosphorylated into NR by CD73. Intracellular NAD^{+} can be consumed by a range of different enzymes (see **section 1.3.1.**), leading to the release of NAM. Figure is adapted from Fang and colleagues¹⁰⁷. Created with BioRender.com. Abbreviations. ACMS: 2-amino-3-carboxymuconate semialdehyde; CD38: cluster of differentiation 38; CD73: 5'-Nucleotidase Ecto; FK: formylkinurenine;

IDO: indoleamine 2,3-dioxygenase; NA: nicotinic acid; NAAD: nicotinic acid adenine dinucleotide; NAD⁺: nicotinamide adenine dinucleotide; NADS: NAD⁺ synthase; NAM: nicotinamide; NAMN: nicotinic acid mononucleotide; NAMPT: nicotinamide phosphoribosyl transferase; eNAMPT: extracellular NAMPT; NAPRT: nicotinic acid phosphoribosyl-transferase; NMN: nicotinamide mononucleotide; NMNATs: nicotinic acid mononucleotide transferases; NR: nicotinamide riboside; NRKs: nicotinamide riboside kinases; Qa: quinolinic acid; QPRT: quinolinate phosphoribosyltransferases; TDO: tryptophan 2,3-dioxygenase; Trp: tryptophan.

1.3.2. Targeting NAD⁺ metabolism in cancer

The NAMPT enzyme is overexpressed in various tumour types (e.g., ovarian cancer) relative to benign tissue^{117,118}. Interestingly, NAMPT overexpression protects cells from genotoxic stress (e.g., methyl methanesulfonate (MMS), etoposide or camptothecin) induced cell death, by maintaining mitochondrial NAD⁺ levels and upregulating the activity of mitochondrial SIRT3 and SIRT4¹¹⁹. As a result, even following the DNA-damage induced hyperactivation of PARP-1 (and cytoplasmic/nuclear NAD⁺ depletion), the release and translocation of apoptosis inducing factor (AIF) from the outer mitochondrial membrane to the nucleus is prevented, suppressing the activation of this caspase-independent mode of programmed cell death¹²⁰.

Interestingly, pre-clinical studies have identified synergistic interactions between NAMPTi and DNA damaging agents such as cisplatin, etoposide and temozolomide^{121,122}. Furthermore, synergy has also been observed with checkpoint immunotherapies (Anti-PD1)¹²³, apoptosis activators^{124,125} and a range of other therapeutic agents¹²².

1.3.3. NAMPT inhibitor mechanism of action

The mechanism of NAMPTi induced cell death is time, cell type and dose dependent. However, generally NAMPTi leads to a substantial reduction (>95%) in intracellular NAD⁺ (24 hours) and ATP levels (48-72 hours) post treatment. Cell death is due to the reduction of NAD⁺ levels below a critical threshold, and this can be rescued with precursors of NAD⁺ biosynthesis. NAMPTi induces mitochondrial dysfunction, as increases in reactive oxygen species (ROS) and loss of mitochondrial membrane potential (MMP) (crucial for maintaining ETC function to generate ATP) are often observed (96 hours). Surprisingly, in some instances NAMPTi-induced cell death appears to require new protein synthesis¹²⁶, but in others it more expectedly

reduces protein synthesis rates to conserve ATP¹²⁷. Cell death can occur through caspase-dependent (e.g., cytochrome c release and caspase 3/7 activation) and -independent mechanisms (e.g., autophagy or oncosis), and interestingly the mode of cell death often correlates with the rate of ATP (i.e., >90% reduction) loss¹²⁶⁻¹³¹. For example, when there is a delay between NAD⁺ and ATP depletion cells typically die by apoptosis (loss of MMP and cell blebbing) or autophagy (cytoplasmic vacuolisation), but when NAD⁺ and ATP depletion occur with little delay cells die by oncosis (cellular swelling and rapid decrease in ATP)^{117,132}. For cells that die by autophagy (characterised by the proteolytic cleavage of LC3-I to LC3-II), NAMPTi induce this through a transcription-dependent pathway, that involves a significant reduction in the gene and protein levels of pERK1/2, pp38MAPK and pSAPK/JNK. Reduced phosphorylation of ERK2 leads to the mobilisation of transcription factor EB (TFEB) from the cytoplasm (inactive) into the nucleus (active), to transcriptionally activate autophagy. As a result, FK866 is synergistic with MAPK inhibitors. NAMPTi also induces autophagy through the PI3K-AKT-mTOR pathway, as FK866 treatment inhibits the phosphorylation of mTOR and its downstream substrate p70S6K (potentially by activating AMPK α). This eventually leads to feedback activation of PI3K and AKT^{104,126,128}. Furthermore, NAMPTi treatment is associated with impaired DNA damage repair, reduced PARylation and G2 cell-cycle arrest¹²⁹.

Interestingly, *BRCA1* levels are correlated with NAD⁺ levels, and this has been observed in multiple primary ovarian cancer cells and in cell lines. For example, *NAMPT* knockdown has been shown to reduce NAD⁺ and *BRCA1* expression levels. This is controlled by the transcriptional co-repressor and metabolic sensor, C terminal-binding protein (CtBP), which leaves the *BRCA1* promoter when NAD⁺/NADH ratios increase, resulting in increased *BRCA1* transcription (and vice versa)^{133,134}. Potentially suggesting NAMPTi could introduce a HRD by regulating *BRCA1* expression.

The metabolic effects of NAMPTi are broad, but have been well characterised in recent years¹¹⁷. For example, the influence of NAMPTi on mitochondrial and glycolytic activity has been assessed using extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) assays. NAMPTi have been shown to significantly decrease both ECAR and OCR, demonstrating mitochondrial and glycolytic activity is diminished^{127,129}. Interestingly, ovarian cancer cells can be divided into subgroups that exhibit NAMPT-dependent and NAMPT-independent glycolysis. Cells lacking *BRCA1/2* mutations are reported to show NAMPT-

dependent glycolysis, and were more susceptible to NAMPTi¹²⁷. Global metabolomics studies have been undertaken following treatment with a NAMPTis. This confirmed a key mechanism of action is the blockade of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase step, leading to ATP depletion and cell death^{112,135,136}. However, NAMPTis also cause significant decreases in carbon flux through the TCA cycle, amino acid metabolism (e.g., aspartate and alanine), purine and pyrimidine metabolism, and guanylate nucleotide synthesis^{112,117,137,138}.

1.3.4. Clinical development of NAMPT inhibitors

Although pre-clinical data for NAMPTis has been encouraging, first generation NAMPTis FK866 (APO866, intravenous infusion or oral administration)¹³⁹ and GMX1778 (CHS-828, intravenous infusion or oral administration)^{140,141} did not achieve objective responses in clinical trials. Some patients treated with GMX1777 (EB1627, oral administration), a pro-drug of GMX1778, were able to achieve stable disease following treatment^{142,143}. However, these agents have ultimately failed as monotherapies in clinical trials because dose limiting toxicity prevents the use of sufficiently high doses/schedules which can achieve clinical efficacy (e.g., insufficient threshold duration and degree of NAD⁺ depletion). The main observed toxicities observed are thrombocytopenia and gastrointestinal (GI) effects (e.g., vomiting, diarrhoea, and esophagitis), the latter is more common when these agents are administered orally. Additional adverse effects include skin rash and lymphopenia. Based on pre-clinical data it is also likely that patients could develop other bone marrow-related toxicities (e.g., anaemia and neutropenia), retinal toxicity, and cardiac toxicity (i.e., due to the lipophilicity of NAMPTis)^{117,122,144}.

To widen their therapeutic window and maximise the therapeutic efficacy of NAMPTis there is a need to identify biomarkers that are predictive of NAMPTi response (e.g., *IDH1*^{145,146}, *BRAF*¹⁴⁷ and *PPM1D*¹⁴⁸ mutations; *PHGDH*¹⁴⁹ or *MYC/MYCN*¹⁵⁰ amplification; *ERCC1*¹⁵¹ or *NAPRT*^{152,153} deficiency) to guide patient stratification^{101,102,144}. Furthermore, there is a need to develop a new generation of NAMPTis with better toxicological profiles. For example, the NAMPTi OT-82^{129,154,155} has shown no cardiac, neurological, or retinal toxicity in mice or non-human primates, unlike other NAMPTis. As a result, OT-82 is being investigated in phase I clinical trials for patients with lymphoma (NCT03921879). However, haematopoietic and lymphoid organs have been reported as primary targets for dose limiting toxicity in pre-clinical studies^{122,144}. Interestingly, dual-inhibitors of NAMPT (and PAK-4^{156,157}, IDO1¹⁵⁸,

EGFR^{159,160}, HER2¹⁶⁰ or HDAC^{161,162}) and NAMPT-degrading proteolysis-targeting chimeras (PROTACs)¹⁶³ are also being developed, and could open new clinical opportunities to target NAMPT in cancer therapy¹⁴⁴.

1.3.5. NAMPT inhibitor resistance

In recent years there has been a growing effort to characterise the mechanisms of intrinsic and acquired resistance to NAMPTi. In some instances, tumours might be intrinsically resistant to NAMPTi because the tissue is completely and irreversibly dependent on the PH biosynthesis pathway for survival, due to the amplification of *NAPRT* and/or *NADSYN1*. Alternatively, if cells possess epigenetically determined dependence on the salvage biosynthesis pathway (i.e., NAMPT) for survival, it is possible resistance can be acquired by enzymatic bypass, as NRK1/2 can synthesise β -NMN(H) from NR(H) in the salvage pathway¹¹⁶. In addition, overexpression of quinolinate phosphoribosyltransferase (QPRT), which produces NAD⁺ from Trp in the *de novo* biosynthesis pathway, can lead to acquired NAMPTi resistance (NAMPTi-R)¹⁶⁴. By comparison, some cells acquire resistance by increasing glycolysis and LDHA (Lactate Dehydrogenase A) activity (i.e., converts NADH and pyruvate into lactate and NAD⁺) or by increasing LDHA activity alone¹⁶⁵. Furthermore, there are also suggestions that upregulation of glutaminase (GLS) is a marker of NAMPTi-R¹²⁷.

Mutations can be introduced in or around NAMPTs nicotinamide/drug binding site to introduce steric hinderance and reduce NAMPTi binding: D93del, S165F, S165Y, H191R, G217R, G217A, G217V, D219S, D219N, K342R, and Q388R. Interestingly, these mutations could not be identified in normal or cancerous tissue, suggesting pre-existing resistance mutations are unlikely to be present in tumours^{115,117,166-168}.

1.4. RAS and PI3K signalling

1.4.1. RAS-RAF-MEK-ERK pathway

The RAS-RAF-MEK-ERK signalling cascade can regulate a range of biological processes, such as proliferation, survival, and metabolism (**Figure 7**). As a result, this pathway is frequently mutated in cancers such as EOC (see **section 1.1** for more detailed information)⁷. Typically, the signalling cascade is activated at tyrosine kinase receptors (TKRs) by growth factors, hormones, or chemokines. However, pathway activation can also occur as a result of somatic

mutation, gene amplification, and through increased paracrine or autocrine signalling¹⁶⁹. For example, *ERBB2* (Erb-B2 Receptor Tyrosine Kinase 2) activating mutations and amplifications (more common) are observed in LGSOC, MOC and CCOC^{6,12,18}.

Following their activation, RTKs dimerise and/or oligomerise to activate their cytoplasmic domains kinase activity, leading to the *trans*-autophosphorylation of their receptor C-terminal tail. These phosphorylated tyrosine residues provide docking sites for adaptor proteins, that contain SH2 (Src homology-2) or PTB (phosphotyrosine-binding) domains, and eventually recruit effectors such as SOS (son of sevenless) to the plasma membrane. SOS is then able to activate KRAS (Kirsten rat sarcoma virus), NRAS (Neuroblastoma RAS viral oncogene homolog) or HRAS (Harvey Rat Sarcoma Viral Oncogene Homolog) at the plasma membrane by increasing the nucleotide exchange rate of RAS-guanosine diphosphate (GDP) (inactive confirmation) into RAS-guanosine triphosphate (GTP) (active confirmation)^{169,170}. Somatic mutations in genes such as *KRAS* and *NRAS* have been observed in EOC subtypes (i.e., LGSOC, MOC, CCOC and EnOC)^{3,6,8,12} and typically involve single-amino-acid substitutions in codons 12, 13 and 61, which impair RAS proteins GTPase activity, leading to the constitutive activation of RAS-GTP^{169,171}. In addition, NF1 (neurofibromin) is a negative regulator of RAS that promotes its conversion from RAS-GTP into inactive RAS-GDP (i.e., stimulates its GTPase activity). However, functional loss of this tumour suppressor gene has been reported in HGSOC and leads to sustained activation of RAS-GTP and RAS-RAF-MEK-ERK signalling^{6,8,172}.

RAS-GTP can activate PI3K (see **section 1.4.2**) and/or the RAF family of proteins: ARAF (A-Raf Proto-Oncogene, Serine/Threonine Kinase), BRAF (B-Raf Proto-Oncogene, Serine/Threonine Kinase) or CRAF (C-Raf Proto-Oncogene, Serine/Threonine Kinase)¹⁶⁹. Mutations in BRAF have been reported in LGSOC and MOC^{3,13,21}, and typically involve alterations in the P-loop (exon 11) or activation segment (exon 15) of the kinase domain. The majority of BRAF mutations (~90%) in cancer involve a valine to glutamic acid substitution at position 600 (*BRAF*^{V600E}). This mutation destabilises an interaction between the proteins P-loop and activation segment which typically locks the kinase in an inactive confirmation. Following the activation of RAFs' catalytic kinase activity (i.e., by RAS or an activating mutation) MEK1 and MEK2 are phosphorylated and activated. Finally, MEK1/2 phosphorylate and activate ERK1/2, which in turn regulates the activity of cytoplasmic proteins and transcription factors (e.g., BIM, MCL1, RSK, MNK, ELK-1, c-Jun, c-Fos, ETS, MYC and MSK)^{169,173}.

1.4.2. PI3K-AKT-mTOR pathway

The PI3K-AKT-mTOR signalling cascade also regulates processes such as proliferation, survival, and metabolism (**Figure 7**). PI3Ks are a family of lipid kinases that phosphorylate phosphoinositides (PtdIns) 3'-hydroxyl group in the inositol ring. There are three classes of PI3Ks (I, II and III) but only class IA PI3Ks are implicated in cancer. These heterodimers are comprised of a regulatory subunit (p85 α , p55 α , p50 α , p85 β , p55 γ) and a catalytic subunit (p110 α , p110 β , p110 δ). PI3Ks can be activated and recruited to the plasma membrane by RTKs when p85 regulatory subunits bind docking sites (phosphorylated tyrosine residues) on RTKs or adaptor molecules. Binding prevents p85 subunits from inhibiting the catalytic activity of p110 subunits, leading to the kinase domains exposure and permits PIP₂ (phosphatidylinositol 4,5-bisphosphate) binding. Alternatively, activated RAS-GTP can bind to the p110 subunit, shifting the active sites position and orientation to favour PIP₂ binding. PI3K then phosphorylates PIP₂ to PIP₃ (phosphatidylinositol 3,4,5-trisphosphate) on the cell membrane^{169,171,174}. *PIK3CA* driver mutations have been detected in LGSOC, CCOC, EnOC and MOC^{6,8,12,21}. Somatic mutations increase PI3Ks lipid binding and/or basal activity. They are typically clustered in two regions of p110 α . First in exon 9, where mutations (e.g., E545K and E542K) alter the helical domain, and interfere with p85 subunits inhibitory activity. Second in exon 20, where mutations (e.g., H1047R) alter the kinase domain and promote constitutive p110 α plasma membrane association^{169,175}. Mutations in p85 subunits (*PIK3R1*) have also been observed in EOC (e.g., CCOC and EnOC)^{16,19} and they are generally truncations or in-frame deletions that disrupt the inhibitory contact between p85 and p110 subunits, leading to constitutive PI3K activation¹⁶⁹.

The tumour suppressor PTEN (phosphatase and tensin homolog) negatively regulates the PI3K signalling cascade by dephosphorylating PIP₃ into PIP₂. As a result, loss of PTEN expression/function (e.g., observed in HGSOC, EnOC and CCOC)^{3,6,8,16} leads to PIP₃ accumulation^{169,176}. PIP₃ localises proteins with a PH (pleckstrin homology) domain, such as AKT, to the cell membrane to promote its phosphorylation at threonine 308 by PDK1 (3-phosphoinositide-dependent protein kinase-1) and serine 473 by mTORC2 (Mammalian target of rapamycin complex 2). Phospho-AKT (active) can then directly phosphorylate and activate mTORC1 (Mammalian target of rapamycin complex 1), or indirectly activate it by phosphorylating TSC2 (Tuberous sclerosis 2) and disrupting the TSC1/2 complex (negative

regulator of mTORC1). As a result, mTORC1 can phosphorylate downstream factors such as S6K-1 (Ribosomal Protein S6 Kinase B1) and/or 4EBP-1 (Eukaryotic Translation Initiation Factor 4E Binding Protein 1). Alternatively, AKT can directly regulate biological processes by altering the activity of proteins such as forkhead box proteins (FOXO), caspase 9, BAD (BCL2 Associated Agonist Of Cell Death) or GSK-3 (Glycogen synthase kinase 3)^{169,176,177}. It should be noted that feedback and cross-talk mechanisms exist between RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways, that can allow tumour cells to escape the activity of inhibitors directed against these cascades through compensatory mechanisms¹⁶⁹.

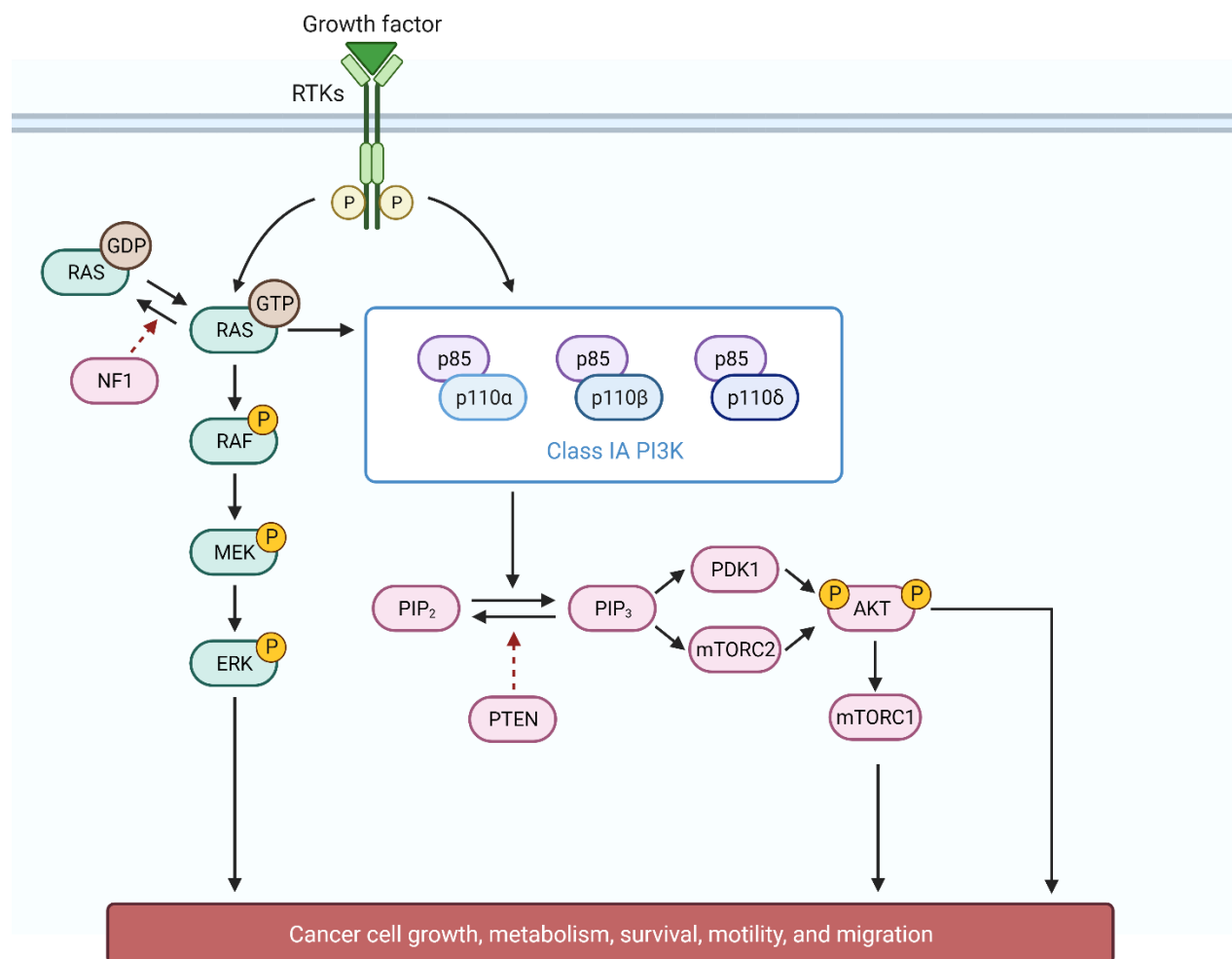


Figure 7. RAS and PI3K-signalling pathways

A schematic representation of the RAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways. Created with BioRender.com

1.4.3. RAS and PI3K signalling: PARP, NAMPT and metabolism

It is well established that cancer cells must reprogramme their metabolism to sustain their increased rate of growth and division. For example, cancer cells preferentially catabolise glucose through aerobic glycolysis (the Warburg effect), rather than oxidative phosphorylation, to maintain ATP levels and accumulate anabolic intermediates for macromolecule biosynthesis. Metabolic reprogramming (e.g., the shift towards aerobic glycolysis) is driven by the activation of oncogenes (e.g., *KRAS*, *NRAS*, *BRAF*, *PIK3CA* and *PIK3R1*) and/or the loss of tumour suppressors (e.g., *NF1* and *PTEN*), which upregulate KRAS-RAF-MEK-ERK and PI3K-AKT-mTOR pathways to influence a plethora of metabolic processes. Importantly, these alterations can create “metabolic bottlenecks” which tumours rely on for growth, and these dependencies can be exploited with metabolic inhibitors¹⁷⁸⁻¹⁸⁰.

KRAS-mutant tumours can become reliant on the activity of NADK (NAD⁺ kinase), which phosphorylates NAD⁺ to synthesise NADP⁺. NADP⁺ can be subsequently converted into NADPH, which plays an essential role in reductive biosynthesis and redox control. Pharmacologic inhibition or genetic deletion of NADK leads to potent growth inhibitory effects in *KRAS*-mutant tumours, in part by decreasing cells ability to cope with oxidative stress^{180,181}. Interestingly, *KRAS*-mutant tumours have been shown to have lower intracellular NAD⁺ concentrations^{182,183}, presumably due to increased demand for the metabolite. Indeed, a relationship between the NAD⁺ salvage pathway enzyme NAMPT, and RAS/PI3K signalling is emerging. For example, *NAMPT* gene expression (and in some instances its dependency for NAD⁺ biosynthesis) can be controlled at the transcriptional level by MYC (MYC Proto-Oncogene, BHLH Transcription Factor), MAX (MYC Associated Factor X), STAT3-5 (signal transducer and activator of transcription 3-5), HIF-1 α (Hypoxia-inducible factor 1-alpha) and NF- κ B (Nuclear Factor Kappa B Subunit 1)^{116,147,184-186}. Given these factors are downstream of RAS and PI3K signalling cascades it is perhaps unsurprising that oncogenic BRAF^{V600E} mutations have been shown to lead to NAMPT overexpression, and this “metabolic bottleneck” can lead to profound NAMPTi sensitivity^{147,185-187}. Tumours with a MYC amplification have robust NAMPT expression and become exquisitely sensitive to NAMPTis¹⁸⁸. Furthermore, the expression of NAMPT has been shown to inversely correlate with PTEN expression¹⁸⁹. By comparison, overexpression of *KRAS* (i.e., without an oncogenic mutation) is not sufficient to upregulate *NAMPT* expression¹⁹⁰.

It is also worth noting that PARP enzymes can regulate metabolism (e.g., mitochondrial function, carbohydrate, amino acid, and lipid metabolism)¹⁹¹⁻¹⁹⁷, as well as the RAS-RAF-MEK-ERK^{198,199} and PI3K-AKT-mTOR²⁰⁰ signalling cascades. This highlights that oncogenic signalling (e.g., by RAS and PI3K), PARP activity, and NAD⁺ availability is highly intertwined.

1.5. Chemical and Biological Tools for Studying Protein ADP-ribosylation

1.5.1. Challenges of detecting ADP-ribosylation events

To better understand the role MARYlation and PARylation (collectively referred to as ADP-ribosylation) in diseases such as cancer, there is a need to identify novel ADP-ribosylation events and the substrate specificities of different PARPs, so that we can elucidate the functional and therapeutic relevance of individual ADP-ribosylation events. Mass spectrometry (MS)-based methods are continually advancing to further our understanding of the ADP-ribosylated proteome (or PARylome). However, ADP-ribosylation is challenging to study due to the physiochemical properties of ADPr itself. (i) ADPr is a highly charged PTM, a characteristic that is typically incompatible with most MS-based methods. (ii) It is a highly heterogenous modification, ADPr can be detected as a monomer (MARYlation) or as a polymer (PARylation); the latter can be between 2-200 units and either branched or linear. (iii) ADPr is labile (i.e., easily broken down or displaced) during collision-induced dissociation, due to its pyrophosphate bond, adenine moiety and amino acid side-chain linkage. As a result, MS-proteomics can yield neutral-loss fragments rather than sequence-specific ions, increasing the complexity of data analysis and preventing the identification of modification sites. (iv) ADPr can be found on a range of amino acid residues, including those with positively charged side chains (e.g., arginine, histidine, and lysine), negatively charged side chains (e.g., aspartic acid and glutamic acid), polar uncharged side chains (e.g., serine, threonine and tyrosine), or amino acids that are polar and charged (e.g., cysteine). To add further complexity, it is unclear which residue the majority of ADP-ribosylation occurs on, with some indicating it is predominantly on glutamate and aspartate residues, with others suggesting it predominantly occurs on serine residues. (v) Finally, ADP-ribosylation is typically found at very low levels in cells and is quickly degraded by enzymes such as PARG²⁰¹⁻²⁰⁶.

1.5.2. Approaches for studying ADP-ribosylation

A variety of approaches have been employed in MS-based proteomics workflows to study the PARylome. (i-iii) Antibody-, recombinant macrodomain- and biotinylated ADPr probe-mediated affinity purification methods^{203,207-214}, (iv) borate-bead (chromatography) based enrichment of a 1,2-*cis*-diol moiety within ADPr (followed by NH_2OH elution)²⁰⁵, and (v) adapted phosphoproteome enrichment strategies²¹⁵ have all been applied successfully to study the PARylome. However, each approach suffers from one or more of the aspects that make ADP-ribosylation difficult to study (e.g., limited affinity and specificity). New technologies are being developed to overcome some of these issues, such as the development of “clickable” (i.e., bio-orthogonal chemistry) NAD^+ analogues.

1.5.3. Introduction to click chemistry

Bio-orthogonal reactions (e.g., azide-alkyne click chemistry) involve the incorporation of chemical groups with high selective reactivity into small molecules (e.g., NAD^+) and/or protein modifications (e.g., ADP-ribosylation) that enable their selective enrichment for downstream applications (e.g., in-gel fluorescence (IGF), pull-down or MS-based proteomics). Functional groups must not be found endogenously, should be chemically inactive (inert) in living systems, and must be small so they can be incorporated into small molecules (e.g., drugs or metabolites) without blocking/disrupting their ability to modify proteins (e.g., ADP-ribosylation of PARP itself or its substrate proteins). As highlighted in **Figure 8** proteins of interest must first be selectively tagged with a bio-orthogonal probe, and then a copper(I) azide-alkyne cycloaddition (CuAAC) reaction can attach (“click”) a bio-orthogonal modification. For example, probe labelled proteins can be modified with an azido-TAMRA-biotin (AzTB) ‘capture’ reagent to enable the fluorescence visualisation of probe incorporation in-gel after SDS-PAGE separation. AzTB also possesses a biotin moiety which enables the selective enrichment of labelled proteins after stringent detergent washing on avidin beads. Alternatively, enzyme-cleavable capture reagents, such as azido-arginine-biotin (AzRB) can be attached, which possess an internal arginine that remains after tryptic digestion and can be incorporated into MS-based proteomic workflows to selectively enrich probe-modified proteins. It should be noted, the CuAAC reaction is cytotoxic, so can only be performed after cell lysis (e.g., could not be used to perform imaging in live cells).

Furthermore, the kinetics of the CuAAC reaction are concentration dependent so it is unlikely proteins with low abundance will be captured²¹⁶⁻²¹⁸.

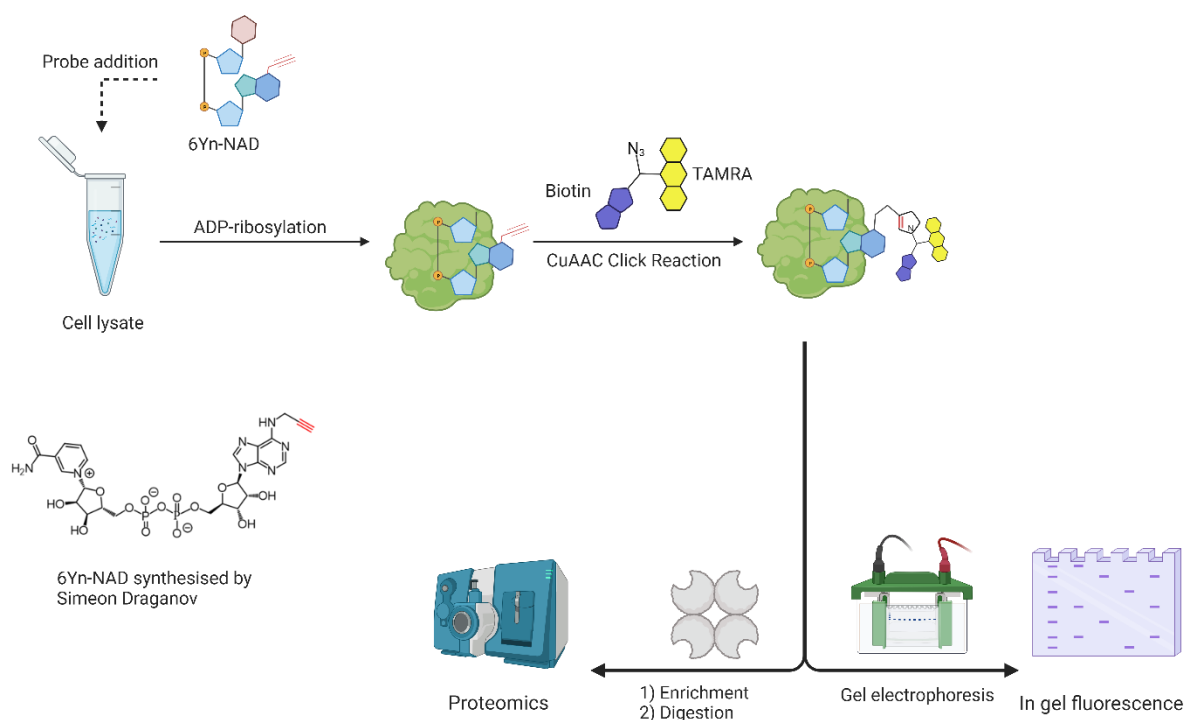


Figure 8. An overview of the click chemistry workflow

Due to the charge of 'clickable' NAD⁺ analogues (e.g., 6Yn-NAD⁺) studies are typically performed in cell lysates (**section 1.5.4**). Labelled (ADP-ribosylated) proteins can be captured using a reagent such as AzTB (shown above) or AzRB. Afterwards, ADP-ribosylation can be visualised by in gel-fluorescence or proteins can be enriched on avidin beads for downstream processes such as proteomics. Created with BioRender.com.

1.5.4. Clickable-NAD⁺ analogues

A range of 'clickable' NAD⁺ analogues have been developed (**Figure 9**). An alkyne group has been added to position 6 of the adenine ring of NAD⁺ to create 6-alkyne NAD⁺ (6Yn-NAD⁺)^{219,220}. It has been demonstrated that PARP-1, PARP-2, Tankyrase-1 (PARP5a), Tankyrase-2 (PARP5b) and PARP-10 can all utilise the clickable analogue for ADP-ribosylation²²⁰⁻²²². Importantly, the substrate activity of endogenous NAD⁺ and 6Yn-NAD⁺ for PARP-1 has been measured in $k_{cat}/K_m(\text{min}^{-1} \text{M}^{-1})$. Depending on the study, the PARP-1 catalysed automodification (PARP-1 activity) of 6Yn-NAD⁺ has been reported to be 10-10.8-fold lower than endogenous NAD⁺. Whilst, hydrolysis (NADase activity) has been reported to

be anywhere between 0.86-9.82 fold lower^{220,221}. Furthermore, the level of PARG-mediated degradation observed with NAD⁺ and 6Yn-NAD⁺ derived PAR polymers is comparable (i.e., both rapidly removed by PARG)²²¹.

An alkyne group has also been added to position 2 of the adenine ring of NAD⁺ to create 2-alkyne NAD⁺ (2Yn-NAD⁺)^{223,224}. Again, it can be utilised by PARP-1, PARP-2, Tankyrase-1, Tankyrase-2, and PARP-10²²²⁻²²⁴. PARP-1 activity is reported to be 9.14-fold lower using 2Yn-NAD compared to endogenous NAD⁺, and the NADase activity has been reported to be 0.97-fold lower. Interestingly, 2Yn-NAD⁺ is significantly more resistant to PARG-mediated degradation, which could increase the baseline level of 2Yn-derived PAR for MS-detection, but this may not be ideal as this can induce physiological changes in cells^{221,225}. Interestingly, 6Yn-NAD⁺ has been reported to catalyse slightly more ADP-ribosylation than 2Yn-NAD⁺ with the PARP-1, PARP-2 and PARP-10 enzymes, but not PARP-5a²²¹.

An NAD⁺ analogue with an azido substitution at 3'-OH of nicotinamide riboside has also been developed (referred to as analogue 6 by Zhang et al)²²¹. Interestingly, analogue 6 appears to be extremely selective for PARP-1 and PARP-2, with no activity reported for Tankyrase-1, Tankyrase-2, or PARP-10. Zhang and colleagues rank the substrate activities of analogue 6, 6Yn-NAD⁺ and 6Yn-NAD⁺ for human PARPs (i.e., PARP-1, -2, -5a, and -10). For PARP-1 and PARP-2, analogue 6 catalysed significantly more auto-PARylation compared to 6Yn-NAD⁺ and 2Yn-NAD⁺. Unlike 6Yn-NAD⁺ and 2Yn-NAD⁺, analogue 6 could not catalyse ADP-ribosylation by PARP-5a (PARylation) or PARP-10 (MARylation). Presumably due to variations in the length and sequence of the enzymes extended loop, preventing the accommodation of the 3'OH modification into the active site. Importantly, PARP-1 activity with analogue 6 is only 2.90-fold lower than endogenous NAD⁺, and the NADase activity is only 0.60-fold lower. Demonstrating this analogue could outperform 6Yn-NAD⁺ and 2Yn-NAD⁺ to study PARP-1 or PARP-2 mediated ADP-ribosylation. However, like the 2Yn-NAD⁺ analogue, analogue 6 derived PAR polymers are highly resistant to PARG mediated degradation²²¹.

NAD⁺ analogues have also been designed to profile the target specificities of individual PARP enzymes using a chemical genetic ("bump-hole") strategy. In brief, this strategy involves incorporating a substituent (e.g., alkyl or benzyl groups can act as a "bump") at position C-5 or C-8 on the nicotinamide moiety of NAD⁺, so that the NAD⁺ variant cannot be incorporated

into the active site of wild-type PARPs (i.e., sterically blocked). However, individual PARP enzymes can be engineered to introduce a unique hydrophobic pocket into their nicotinamide-binding site (“hole”), to accommodate NAD⁺ variants with a substituent (i.e., “gatekeeper” mutation). By changing lysine (K) residue 903 in PARP-1 to a smaller amino acid, such as alanine (A), the PARP-1^{K903A} mutant can accommodate an NAD⁺ analogue that contains an alkyne at position N-6 on the adenine ring (for click conjugation) and an ethyl group at C-5 of the nicotinamide ring (“bump”), this probe is known as 5-ethyl-6-alkyne-NAD⁺ (5-Et-6-a-NAD⁺). Another analogue was synthesised that contained a benzyl group at C-5 of the nicotinamide ring instead, known as, 5-Benzyl-6-alkyne-NAD⁺ (5-Bn-6-a-NAD⁺). In addition to PARP-1^{K903A}, the conserved lysine (K) could also be mutated into an alanine (A) in PARP-2^{K903A} and PARP-6^{K903A} to accommodate these analogues. Furthermore, by mutating leucine (L) 659 or isoleucine (I) 631 in PARP-7, I987 in PARP-10, I987 in PARP-11, or L1782 in PARP-14 into a glycine (G) (i.e., PARP-7^{L659G}, PARP-7^{I631G}, PARP-10^{I987G}, PARP-11^{I987G} and PARP-14^{L1782G}), these bumped analogues can be utilised by a range of PARPs. Unfortunately, these bumped NAD⁺ analogues only preserve PARylation activity, so cannot be used to study PARylation²²⁶⁻²²⁹.

Considering this, Gibson and colleagues developed an analogue-sensitive approach that preserves PARylation activity and synthesised an NAD⁺ analogue with a bifunctional alkyne group at position 8 of the adenine ring, which facilitates click chemistry and prevents utilisation by wild-type PARPs. Importantly, 8-Bu(3-yne)T-NAD⁺ can be utilised by PARPs by again, mutating large “gatekeeper” amino acids in the active site to a glycine (G) or alanine (A): PARP-1^{L877A}, PARP-1^{I895A}, PARP-2^{L443A}, PARP-3^{L394A} ^{219,230}.

Unfortunately, NAD⁺ analogues (like endogenous NAD⁺) are not cell membrane permeable, so studies have often been limited to cell lysates, or cell membranes have been permeabilised with agents such as Triton X-100²²¹ or the DOTAP (1,2-Dioleoyl-3-trimethylammonium propane)²³¹. To overcome this issue, and study ADP-ribosylation in live cells without use of permeabilization agents, clickable NAD⁺ precursor probes have been developed.

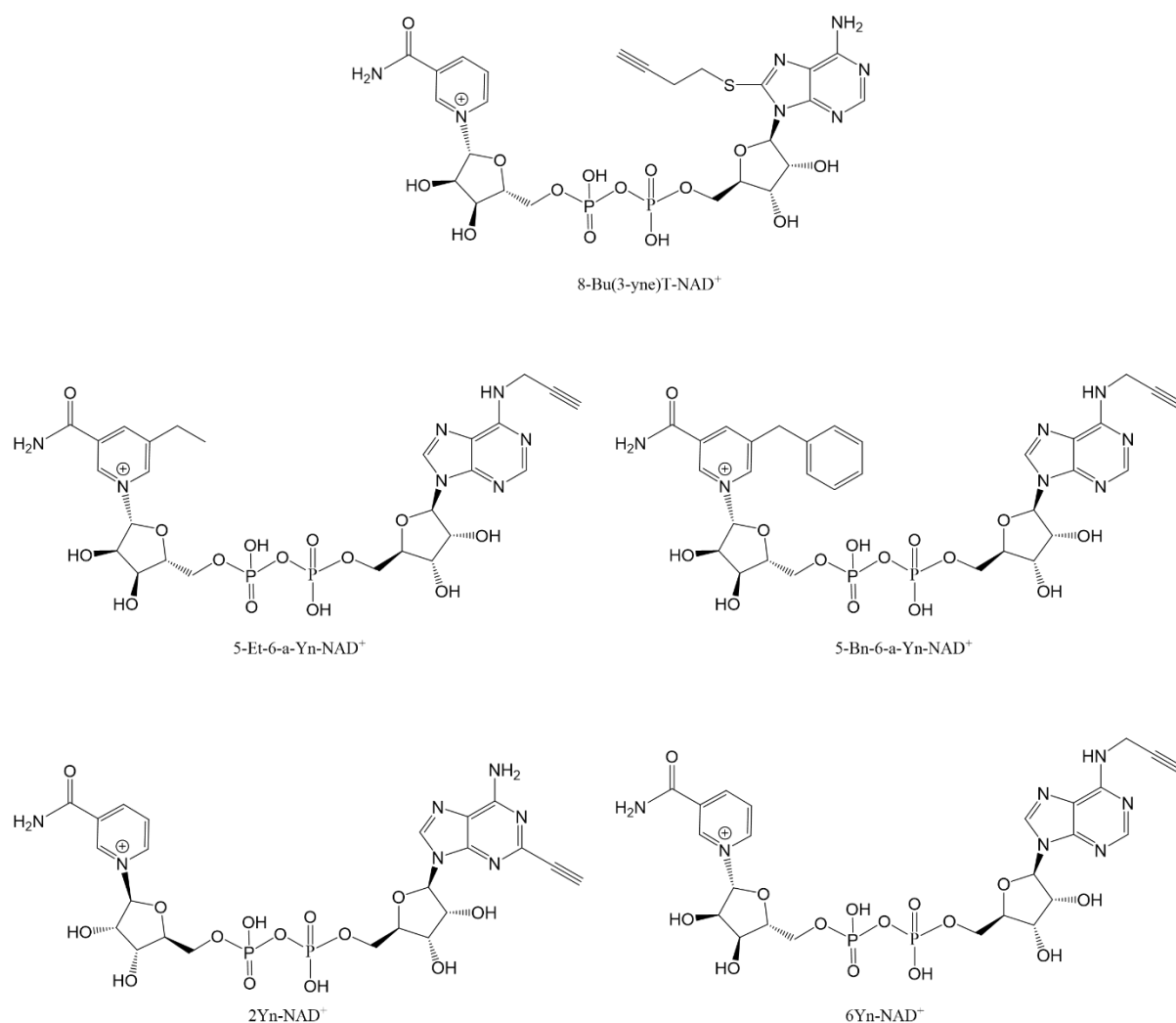


Figure 9. Structure of different NAD⁺ analogues

Chemical structures of the 8-Bu(3-yne)T-NAD^{+219,230}, 5-Et-6-a-Yn-NAD⁺, 5-Bn-6-a-Yn-NAD⁺²²⁶⁻²²⁹, 2Yn-NAD^{+223,224} and 6Yn-NAD^{+219,220}. Created using ChemDraw 19.0.

1.5.5. Clickable-NAD⁺ precursor analogues and their biosynthesis

The first clickable precursor NAD⁺ analogue, was the 6-alkyne adenosine (6Yn-Ad) analogue²³². In theory, this analogue should be converted into 6-alkyne AMP (6Yn-AMP) by adenosine kinase (AK). It should then be converted into 6-alkyne ADP (6Yn-ADP) by AMP kinase (AMPK). ATP synthase or nucleotide diphosphokinase (NDPK) should then convert 6Yn-ADP into 6-alkyne ATP (6Yn-ATP). Which can be utilised by NMNAT 1-3 in the final step of the NAD⁺ biosynthesis pathway, which should convert 6Yn-ATP and β -NMN into 6Yn-NAD^{+192,233} (**Figures 10 and 11**). Encouragingly, previous studies^{232,234} suggested the probe is readily converted into 6Yn-NAD⁺ and used to ADP-ribosylation. Through proteomics Westcott and

colleagues identified 'ADP-ribosylated' proteins in live HEK293T cells following the induction of oxidative stress with hydrogen peroxide (H_2O_2)²³². However, PARP-1 enrichment was not detected in any condition in their proteomics dataset, which is a strange result considering PARP-1 readily ADP-ribosylates itself. This approach was developed further by Kalesh and colleagues who provided evidence suggesting co-treating cells with 6Yn-Ad and 2-alkyne adenosine (2Yn-Ad) could improve coverage of the ADP-ribosylome in live MDA-MB-231 cells²³⁴.

Interestingly, there is evidence demonstrating that an NR analogue, with a 3'-OH modification (NR6), can be converted into β -NMN6 (i.e., β -NMN with a 3'-OH modification) by NRK1. Which is then be converted into analogue 6 (ribose functionalised NAD^+ analogue) by NMNAT1. This has been demonstrated using human NRK1 and NMNAT1 enzymes, and if feasible in live cells would reduce the number of steps necessary for NAD^+ precursor probes to be incorporated into the NAD^+ pool (presumably increasing their utilisation by PARPs for ADP-ribosylation). However, conversion of NR6 into analogue 6 was not tested in live cells by Zhang and colleagues, suggesting either this experiment was out of the studies scope or since analogue 6 derived PAR polymers are PARG resistant, the probe may be poorly tolerated^{60,221,225}.

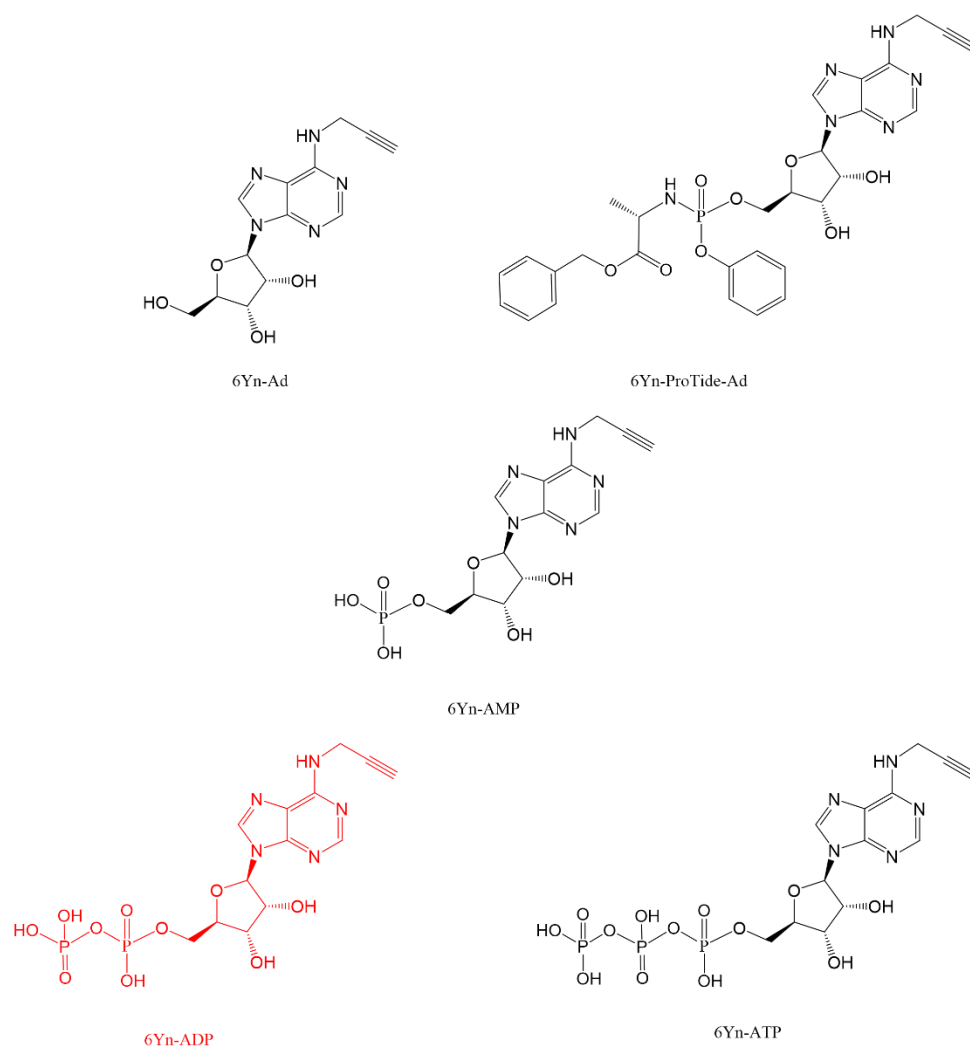


Figure 10. Chemical structures of 6Yn-Ad, 6Yn-ProTide, 6Yn-AMP, 6Yn-ADP and 6Yn-ATP

Chemical structures of 6Yn-Ad²³² and 6Yn-ProTide²³⁵, along with intermediates in the 6Yn-NAD⁺ biosynthesis pathway (see Figure 11). Structures in black were synthesised by Dr Simeon Draganov. 6Yn-ADP (red) was not synthesised. Created using ChemDraw 19.0.

A prodrug approach has also been developed to deliver nucleoside monophosphates into cells (phosphoramidate ProTide method). For example, the charge of a propargyl adenosine pronucleotide probes (6Yn-ProTide) phosphate group can be masked to aid its passive cell-membrane permeabilization. Once inside the cell 6Yn-ProTides' masking groups are cleaved in a four-step process (two enzyme-mediated and two spontaneous). The cleavage involves enzymatic hydrolysis (e.g., by cathepsin A or other esterases), spontaneous cyclisation and ring opening, followed by the activation of aryloxy phosphoramidate. This releases 6Yn-AMP, which has the advantage of bypassing the first phosphorylation step that 6Yn-Ad must pass through (i.e., conversion of 6Yn-Ad into 6Yn-AMP by adenosine kinase). Although Kielkowski

and colleagues demonstrate that 6Yn-ProTide is readily converted into 6Yn-ATP in HeLa cells (by LC-MS/MS metabolomics), they did not assess its conversion into 6Yn-NAD⁺. Furthermore, they did not observe the enrichment of PARP-1 in the absence of oxidative stress (although known ADP-ribosylated proteins were detected) by proteomics. Instead, they demonstrate that 6Yn-ATP can be used to mediate another PTM, known as AMPylation²³⁵⁻²⁴⁰. Grammel and colleagues demonstrate 6Yn-Ad (and likely 6Yn-ProTide) can also be incorporated into RNA or poly(A) tails²⁴¹, highlighting the need to incorporate appropriate controls when using these probes to study ADP-ribosylation (e.g., PARPis and Benzonase® Nuclease). Ultimately, there is a need to improve our understanding of the intracellular conversion of NAD⁺ precursor probes, to identify conditions that maximise their incorporation into the NAD⁺ pool and utilisation by PARPs for ADP-ribosylation^{242,243}.

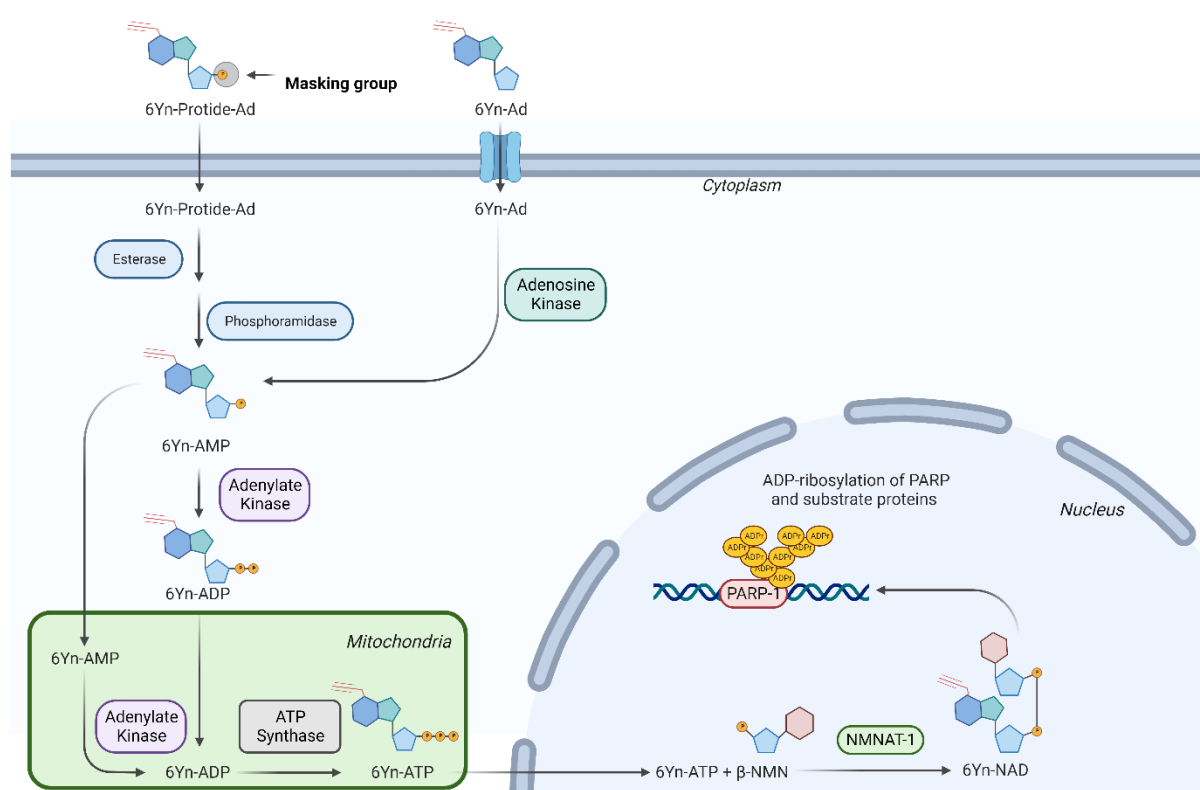


Figure 11. 6Yn-Ad and 6Yn-ProTide biosynthetic pathway

Schematic representation of the intracellular conversion of 6Yn-Ad or 6Yn-ProTide into the full length 6Yn-NAD⁺ analogue. General scheme of the adenosine pathway is adapted from Boison and Yegutkin²³³. Created with BioRender.com.

1.6. PARPs, NAD⁺ and alternative splicing

1.6.1. Alternative splicing and cancer

The majority (>95%) of human transcribed genes undergo alternative RNA splicing to increase the diversity of the proteome. Following the transcription of intron-containing genes, precursor messenger RNA (pre-mRNA) undergoes maturation by a large ribonucleoprotein (RNP) complex called the spliceosome (also directs constitutive splicing), which catalyses the excision of introns and ligation of exons (requires the hydrolysis of large amounts of ATP). The spliceosome is composed of five different RNP subunits, termed small nuclear RNPs (snRNPs) (i.e., U1, U2, U4/U6, and U5 snRNPs), and associated protein cofactors. Splicing decisions (e.g., splice site choice, recruitment of the spliceosome and spliceosome-associated factors) are controlled by (i) primary splicing signals at/near exon-intron boundaries. These signals include 5' or 3' splice sites, the polypyrimidine tract, and the branch site (i.e., loosely conserved short sequence 15-50 nucleotides upstream of the 3' splice site, most include an adenine nucleotide). (ii) *Cis*-acting regulatory RNA elements throughout pre-mRNA, these splicing regulatory elements can act as splicing enhancers or silencers, and are location dependent (i.e., either intronic or exonic). (iii) *Trans*-acting splicing factors (proteins) are generally recruited by *cis*-acting elements to bind at their target site on pre-mRNA to promote/reduce their usage. There are five major mechanistic modes of alternative splicing (AS) in human cells (**Figure 12**): (i) intron retention, an intron is retained in a mature mRNA transcript; (ii) exon skipping, an exon is spliced out of mature mRNA; (iii-iv) alternative 5'- or 3'-splice sites, a smaller exon is retained in mature mRNA; and (v) mutually exclusive exons, different exon combinations are selected in mature mRNA²⁴⁴⁻²⁴⁶.

Given AS can generate alternative mRNA variants and protein isoforms with distinct structural and biological activities, it is perhaps unsurprising that alterations in this pathway are associated with cancer progression and altered treatment responses^{244,247}. For example, a *BRCA1*^{Δ11q} splice variant, which skips the majority of exon 11 to bypass inactivating mutations in this region, promotes PARPi-R and cisplatin resistance⁶³. Whilst a *BRCA2*^{ΔE5+7} splice variant, which results in exons 5 and 7 being skipped, is also associated with chemo-resistance²⁴⁸.

In recent years, it has been suggested that splicing is not an isolated process that is solely regulated by splicing signals, or *cis*- and *trans*-acting factors. This has led to the 'co-

transcriptional splicing hypothesis', which states that splicing and transcription (e.g., by RNA polymerase II) occur at the same time, and local chromatin structure is responsible for the cross-talk between transcription and splicing²⁴⁹⁻²⁵². The basic repeating unit of chromatin is a histone-DNA complex known as a nucleosome (protein octamers), which consist of ~146 bp of DNA around dimers of four histone proteins (i.e., H2A, H2B, H3 and H4). Its primary function is to package DNA into a more compact structure to protect organisms' genetic information from damage. However, its compaction can be alleviated for nuclear processes which require access to DNA (e.g., transcription, replication, and repair). The structure of chromatin is regulated by nucleosome positioning (e.g., at promoters) and histone modifications^{24,249}. There are two non-mutually exclusive models that have been proposed to explain how chromatin structure regulates AS: (i) the kinetic model states that chromatin structure influences splicing outcomes by regulating the speed of RNA polymerase II (RNAPII) elongation; (ii) the adapter/recruitment model states that splicing choices are directed/orchestrated by chromatin or its associated splicing factors bridging chromatin to nascent mRNAs. Importantly, nucleosome and histone modifications have been shown to affect the association of splicing factors with chromatin and the efficiency of splicing²⁵⁰⁻²⁵³.

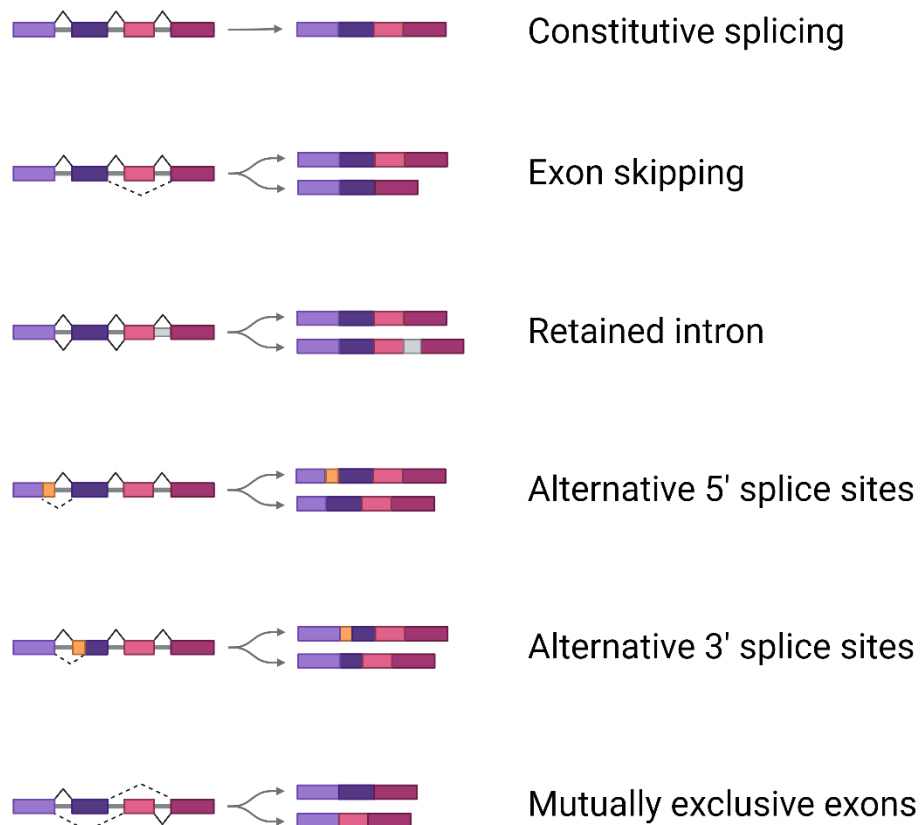


Figure 12. Alternative splicing events

A schematic representation of constitutive splicing and the five major types of alternative splicing events, compared to constitutive splicing. Adapted from Wang and Aifantis²⁴⁴. Created with BioRender.com.

1.6.2. PARP, NAD⁺ and alternative splicing

A large focus of PARP research has been on its role in regulating DNA damage responses. However, in recent years PARP/ADP-ribosylation has been shown to regulate a wide array of processes such as chromatin structure²⁵⁴, and nearly every step in RNA biogenesis, including splicing. With evidence suggesting PARP regulates AS patterns (i) directly through its physical association with chromatin structures, and (ii) indirectly through the PARylation of splicing factors²⁴. Interestingly, studies suggest that PARP-1-chromatin association (i.e., PARP-1 binds nucleosomes at target exon/intron boundaries) can influence AS patterns according to both models of chromatin structure-dependent regulation of AS^{253,255}. For example, RNAPII has been shown to pause at PARP-1-chromatin structures within the gene body, in support of the kinetic model^{256,257}. By comparison, in the adapter/recruitment model PARP-1 appears to bridge the spliceosome to RNA, by binding pre-mRNA and recruiting a member of the U2

snRNP complex known as splicing factor 3B1 (SF3B1), which is involved in pre-mRNA branch site recognition^{253,258,259}. To further support its role in the adapter/recruitment model, PARP-1 can regulate AS by modulating the activity of all three major types of RNA binding proteins involved in splicing. These include (i) snRNPs, (ii) serine and arginine-rich (SR) proteins, and (iii) heterogeneous ribonucleoproteins (hnRNP). This can be mediated through protein-protein interactions, ADP-ribosylation or their non-covalent interaction with PAR^{209,230,253,258,260-262}. Given PARP plays an integral role in regulating splicing it is perhaps unsurprising that inhibiting PARP and the genetic deletion³⁶/pharmacological inhibition^{263,264} of splicing factors can lead to synergistic interactions in diseases such as HGSOC.

Although no one has investigated how inhibiting NAD⁺ biosynthesis (e.g., NAMPT) alters splicing, given NAD⁺ consuming enzymes such as SIRT6 (e.g., SIRT1-7) also regulate chromatin structure through histone deacetylation²⁶⁵⁻²⁶⁷, it is likely targeting NAMPT can also regulate AS patterns in a PARP/SIRT-dependent and -independent manner. Furthermore, it should be noted that major studies investigating the role of PARP-1/ADP-ribosylation in regulating AS have been carried out in S2 *Drosophila*²⁵⁸ and HeLa²⁵⁹ cells. With the exception of work undertaken by Xu and colleagues (manuscript to be submitted)²⁶⁴, who carried out RNA-sequencing and splicing analysis in olaparib treated KURAMOCHI cells, very little is known about the influence of PARPi on splicing in HGSOC.

1.7. Aims and objectives

1.7.1. Chapter 2

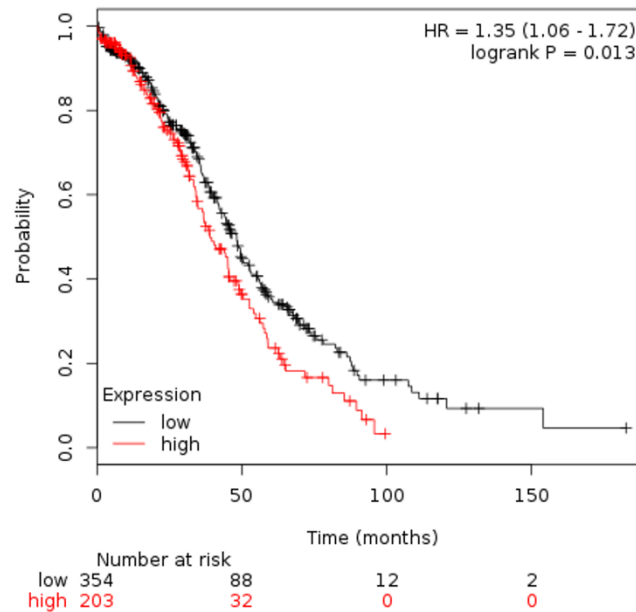
FK866 is a potent first-generation inhibitor of the NAMPT enzyme, which catalyses the conversion of NAM into NMN (rate limiting step) in the NAD⁺ salvage pathway. Unfortunately, dose limiting toxicity has hampered the efficacy of NAMPTis in the clinic. Considering this there is a need to identify biomarkers that can be used to select appropriate patients for treatment and improve the therapeutic window of these metabolic inhibitors, and/or identify suitable drug combination strategies to enhance therapeutic opportunities¹¹⁷.

Preliminary bioinformatic analysis carried out by Dr Adrian Benito-Mauricio demonstrated that high levels of *NAMPT* expression correlate with poor patient outcomes in HGSOC patients (**Figure 13A**). In agreement with a previous study assessing *NAMPT* expression in benign ovarian tissue and HGSOC samples¹¹⁸, suggesting that NAMPT may play an important role in

EOC. Using the Genomics of Drug Sensitivity in Cancer (GDSC) resource Dr Benito-Mauricio demonstrated that EOC cell lines are not intrinsically sensitive to FK866 when compared to other cancer types (**Figure 13B**). However, when segregating EOC cell lines based on their KRAS-mutation status he found that activation of KRAS reduced FK866 IC₅₀ values, to levels comparable with FK866-sensitive blood cancers, suggesting that KRAS mutation status could be used as a biomarker to guide treatment (**Figure 14**). Given there are suggestions that NAMPT expression (and in some instances its dependency for NAD⁺ biosynthesis) can be regulated by RAS-RAF-MEK-ERK (e.g., oncogenic BRAF^{V600E} upregulates NAMPT) and/or PI3K-AKT-mTOR (e.g., PTEN expression inversely correlates with NAMPT expression) signalling cascades via transcriptional regulation (e.g., by MYC, MAX, STAT3-5, HIF-1 α and NF- κ B)^{116,147,184-186,189,190} we set out to investigate whether the presence of RAS/PI3K-activating mutations could lead to a “metabolic bottleneck” that could be exploited with a NAMPTi.

The role of NAMPT in epithelial ovarian cancer

A NAMPT expression and patient outcomes in HGSOC (TCGA dataset)



B NAMPT inhibitor (FK866) sensitivity in different types of cancer (GDSC database)

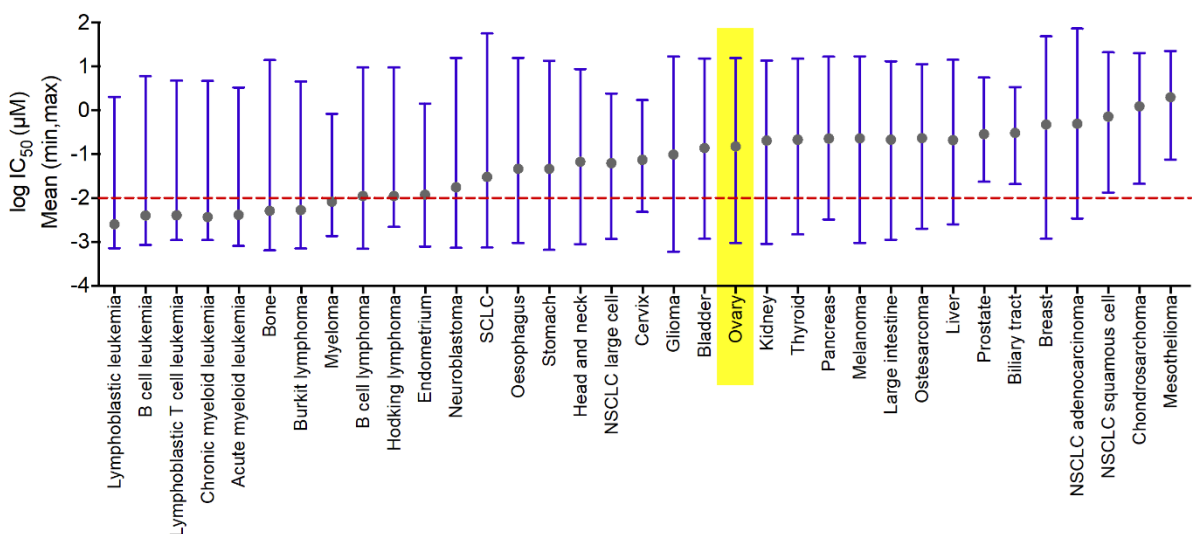


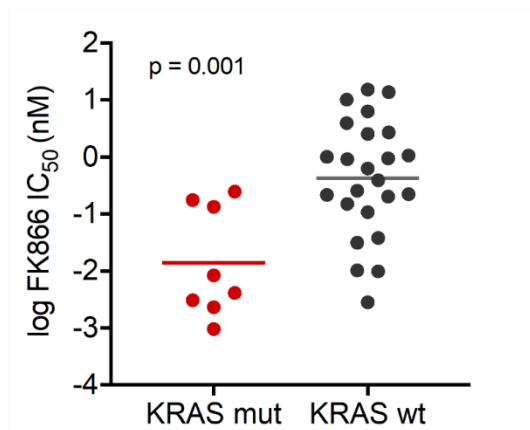
Figure 13. The role of NAMPT in EOC

(A) Using The Cancer Genome Atlas (TCGA) database and the Kaplan-Meier plotter tool (<https://kmplot.com/analysis/>) Dr Adrian Benito-Mauricio investigated the relationship between NAMPT expression (high vs low) and survival outcomes in HGSOC (unpublished data). **(B)** Using the Genomics of Drug Sensitivity in Cancer (GDSC) resource (<https://www.cancerrxgene.org/>) Dr Adrian Benito-Mauricio characterised FK866 (NAMPTi) sensitivity in different types of cancer, ovarian cancer is highlighted in yellow (unpublished data).

Given ovarian tumours with dysregulated RAS/PI3K signalling have been shown to have poorer PARPi responses in clinical trials⁵³, and PARP uses NAD⁺ as a substrate²⁷, we also set out to investigate whether combining a PARPi (olaparib) and NAMPTi (FK866) could improve the efficacy of PARPis in RAS/PI3K-mutant EOC. The combination of a PARPi and NAMPTi has been shown to be synergistic in TNBC⁹⁸, Ewing sarcoma⁹⁹, and more recently in HGSOC¹⁰⁰. However, excluding *BRCA1/2* status⁹⁸ no other biomarkers have been identified to guide the use of this combination.

Activating KRAS-mutations sensitise epithelial ovarian cancer cell lines to FK866

A FK866 sensitivity in KRAS-mutant (mut) and -wild-type (wt) EOC cell lines (GDSC)



B Comparing KRAS-mut and -wt IC₅₀ values with NAMPTi-sensitive blood cancers (GDSC)

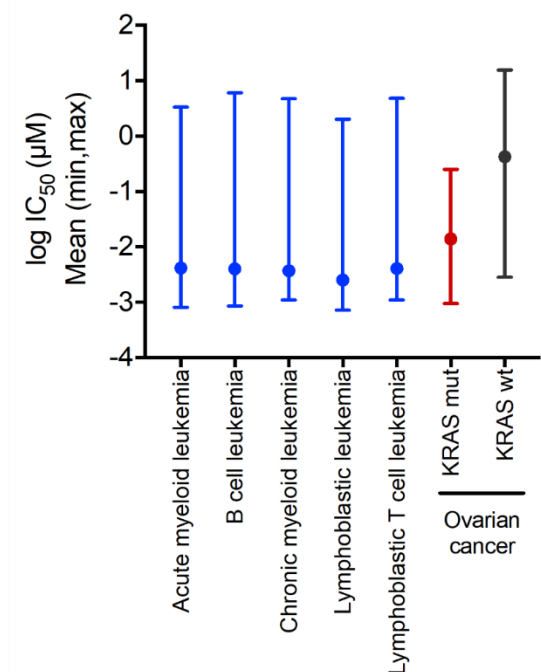


Figure 14. Activating KRAS-mutations sensitise EOC cell lines to FK866

Using the Genomics of Drug Sensitivity in Cancer (GDSC) resource (<https://www.cancerrxgene.org/>) Dr Adrian Benito-Mauricio **(A)** characterised FK866 sensitivity in KRAS-mutant (mut) and -wildtype (wt) EOC cell lines, and **(B)** compared this with IC₅₀ values observed in NAMPTi-sensitive blood cancer cell lines (unpublished data).

Hypothesis:

“KRAS/PI3K-activating mutations increase NAD⁺ demand and sensitise cells to the olaparib/FK866 combination”.

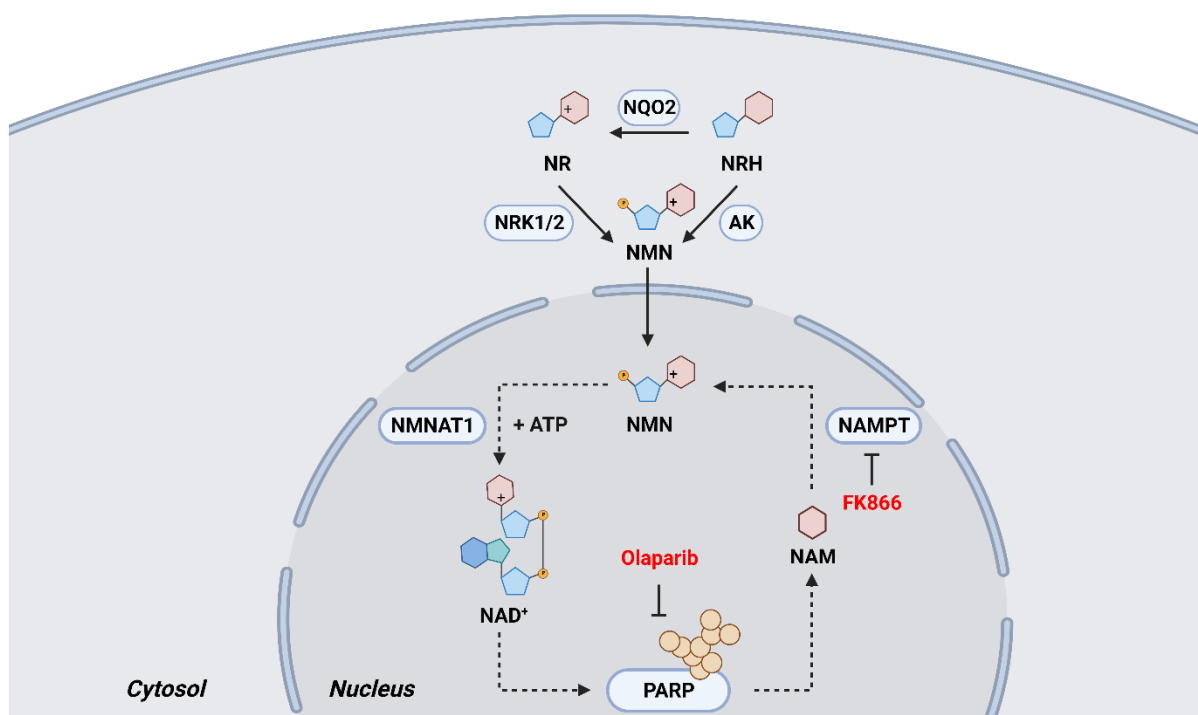


Figure 15. Combined inhibition of PARP and NAMPT

Schematic representation of the nuclear NAD⁺ salvage pathway and how it can be targeted through the combined inhibition of NAMPT (FK866) and PARP (olaparib)^{98,109}. Created with BioRender.com. Abbreviations: NRH: Reduced NR; AK: Adenosine Kinase; NQO2: NAD(P)H dehydrogenase, quinone 2.

Objectives:

- Investigate whether KRAS/PI3K-activating mutations increase metabolic NAD⁺ demand and affect NAMPTi (FK866) sensitivity in a panel of EOC cell lines.
- Investigate the influence of NAD⁺ availability on PARPi (olaparib) sensitivity in 2D and 3D.
- Determine whether KRAS/PI3K-activating mutations could be used as a treatment selection biomarker for NAMPTi/PARPi combination therapy.
- Probe the mechanism of action of the combination in EOC cell lines.

1.7.2. Chapter 3

HGSOC is the most common type of EOC, accounting for ~70% of all cases. It is characterised by mutations in *TP53* (>95% of cases), *BRCA1* (12% of cases), *BRCA2* (9% of cases), or the loss

of tumour suppressors such as *PTEN* (7% of cases) or *NF1* (8% of cases)^{6,8}. Loss of *PTEN* prevents the dephosphorylation of PIP₃ into PIP₂, leading to PIP₃ accumulation and the constitutive activation of PI3K-AKT-mTORC signalling¹⁷⁶. By comparison, *NF1* stimulates RAS GTPase activity promoting its conversion from RAS-GTP (active confirmation) into RAS-GDP (inactive confirmation). Its functional loss leads to sustained activation of RAS-RAF-MEK-ERK signalling¹⁷², and can also activate the PI3K cascade¹⁶⁹. Walton and colleagues^{268,269} previously developed transplantable murine models (ID8) of HGSOC, using CRISPR/Cas9 gene editing. ID8 derivatives were developed that harboured single (*Trp53*^{-/-}) or double (*Trp53*^{-/-};*Nf1*^{-/-}, *Trp53*^{-/-};*Pten*^{-/-}, *Trp53*^{-/-};*Brca1*^{-/-} and *Trp53*^{-/-};*Brca2*^{-/-}) knockouts. Importantly, Walton and colleagues showed that loss of *Nf1* led to increased RAS-RAF-MEK-ERK signalling, but did not upregulate PI3K signalling, whereas *Pten* loss only upregulated PI3K signalling²⁶⁸. Therefore, we selected these models to investigate whether the RAS-RAF-MEK-ERK or PI3K-AKT-mTORC1 axis plays a more important role in sensitising cells to either monotherapy or the combination *in vitro*. Furthermore, these models can be injected into immunocompetent mice (i.e., functioning immune microenvironment), increasing the physiological relevance of endpoint and survival *in vivo* studies.

Hypothesis:

“Loss of *NF1*/*PTEN* increases NAD⁺ demand and sensitivity to the olaparib/FK866 combination”.

Objectives:

- Characterise FK866 sensitivity in a panel of isogenic HGSOC models (ID8 cells) with genetic deletion of genes such as *Trp53*, *Nf1*, *Pten*, *Brca1* or *Brca2*.
- Investigate whether loss of *Nf1* or *Pten* sensitises ID8 cells to olaparib and/or FK866.
- Probe the mechanism of action of the combination in ID8-*Trp53*^{-/-};*Pten*^{-/-} cells.
- Investigate whether the combination can reduce ID8-*Trp53*^{-/-};*Pten*^{-/-} tumour growth and improve survival outcomes.

1.7.3. Chapter 4

Previous studies using “clickable” 6Yn-NAD⁺ precursor probes provide conflicting evidence for their utility to study ADP-ribosylation. For example, there is IGF/proteomics evidence to

suggest that the adenosine analogue 6Yn-Ad is converted into 6Yn-NAD⁺ and utilised by PARPs in the presence of H₂O₂-induced oxidative stress (using HEK293T cells)²³² and in the absence of oxidative stress (using MDA-MB-231 cells)²³⁴. Whereas a study using the 6Yn-ProTide probe suggests that in the absence of oxidative stress the probe is converted into 6Yn-ATP and used for AMPylation, but not ADP-ribosylation (using HeLa cells)²⁷⁰.

Importantly, preliminary work carried out by Dr Simeon Draganov indicated that clickable adenosine-based probes can non-specifically label proteins in cell lysates if suitable adjustments to the sample preparation workflow aren't made^{242,243} for analysis by IGF, western blotting, or proteomics (i.e., an additional protein precipitation step in methanol: chloroform must be included after cell lysis but prior to click chemistry). To add further complexity, at the 6Yn-ATP step of the biosynthetic pathway instead of being converted into 6Yn-NAD⁺, the ATP analogue can be used for AMPylation, preventing its incorporation into the NAD⁺ pool and utilisation by PARPs for ADP-ribosylation. Furthermore, due to the number of steps involved in this metabolic pathway if inefficient labelling is observed, it can be challenging to identify where metabolic bottlenecks are occurring without robust metabolomics^{242,243}.

Westcott and colleagues did detect 6Yn-NAD⁺ in cells following 6Yn-Ad treatment (showed a single MS and MS/MS spectra). However, they did not measure any other steps in the metabolic pathway, the influence of co-treating cells with H₂O₂ and 6Yn-Ad on 6Yn-NAD⁺ synthesis, or measure the concentration of 6Yn-NAD⁺ relative to endogenous NAD⁺²³². By comparison, Kielkowski and colleagues do measure conversion of the 6Yn-ProTide probe into 6Yn-ATP (show the chromatographic peak area), but did not measure the level of intracellular 6Yn-NAD⁺, or investigate the influence of oxidative stress (activates PARP and drives ADP-ribosylation) on 6Yn-ATP levels²⁷⁰. Finally, Kalesh and colleagues did not assess the conversion of 6Yn-Ad into 6Yn-NAD⁺ by metabolomics²³⁴.

Ultimately, there is a need to develop a targeted metabolomics method to characterise the conversion of 6Yn-Ad/6Yn-ProTide probes into 6Yn-AMP, 6Yn-ATP, and 6Yn-NAD⁺ analogues. Whilst measuring their incorporation into endogenous ATP and NAD⁺ pools. This approach could be used to identify conditions which maximise the synthesis of 6Yn-NAD⁺, its utilisation

by PARP for ADP-ribosylation, and ultimately identify novel ADP-ribosylation events in diseases such as EOC.

Hypothesis:

“Targeted metabolomic analysis can be used to optimise the conversion of 6Yn-Ad or 6Yn-ProTide probes into 6Yn-NAD⁺ for the chemical proteomic detection of ADP-ribosylated proteins”.

Objectives:

- Develop in-house targeted RP and HILIC UPLC-MS/MS methods for intracellular metabolic analysis of 6Yn-NAD⁺ biosynthesis intermediates.
- Use the in-house RP and HILIC UPLC-MS/MS methods to investigate whether NAD⁺ precursor availability, NAMPT expression and/or PARP activation can influence the conversion of 6Yn-Ad or 6Yn-ProTide probes into 6Yn-NAD⁺.
- Use the in-house RP UPLC-MS/MS method to assess whether DOTAP membrane permeabilization leads to optimal internalisation of full length 6Yn-NAD⁺.

1.7.4. Chapter 5

PARP is known to regulate AS through the ADP-ribosylation of splicing factors, and by regulating the structure of chromatin^{253,255}. Studies in *S2 Drosophila*²⁵⁸ and HeLa²⁵⁹ cells have identified a number of AS events that occur following the genetic deletion of PARP-1 or the inhibition of PARylation. To date, excluding a study carried out by Dr Yuewei Xu (manuscript to be submitted)²⁶⁴, where the influence of olaparib on AS was assessed in HGSOc KURAMOCHI cells, very little is known about the influence of PARPis on AS in cancer. Furthermore, given PARP-1 uses NAD⁺ to carry out its catalytic activity (e.g., ADP-ribosylation of splicing factors) and other NAD⁺-dependent enzymes can regulate the structure of chromatin (e.g., SIRT6)²⁶⁵⁻²⁶⁷, it is likely that NAD⁺ availability can alter AS through PARP-dependent and -independent mechanisms.

Hypothesis:

“NAD⁺ metabolism can regulate splicing via PARP-dependent and -independent activity”.

Objectives:

- Characterise the role of NAD⁺ availability and/or PARP activity on alternative splicing in the HGSOC COV318 cell line.
- Characterise the role of NAD⁺ availability and/or PARP activity on gene expression in the HGSOC COV318 cell line.

CHAPTER 2

Evaluating the combined effect of FK866 and olaparib in a panel of EOC cells lines

Chapter 2 – Evaluating the combined effect of FK866 and olaparib in a panel of EOC cell lines

2.1. Background

As discussed in **section 1.7.1**, preliminary bioinformatics experiments performed by Dr Adrian Benito-Mauricio suggest EOC tumours with oncogenic *KRAS* mutations are exquisitely sensitive to the NAMPTi FK866. Given there are suggestions that NAMPT expression (and in some instances its dependency for NAD⁺ biosynthesis) can be regulated by RAS-RAF-MEK-ERK (e.g., oncogenic BRAF^{V600E} upregulates NAMPT) and/or PI3K-AKT-mTOR (e.g., PTEN expression inversely correlates with NAMPT expression) signalling cascades via transcriptional regulation (e.g., by MYC, MAX, STAT3-5, HIF-1 α and NF- κ B)^{116,147,184-186,189,190} we set out to investigate whether the presence of RAS/PI3K-activating mutations could lead to a “metabolic bottleneck” that could be exploited with a NAMPTi. We also investigated whether combining a PARPi (olaparib) and NAMPTi (FK866) could improve the efficacy of PARPis in RAS/PI3K-mutant EOC.

To investigate whether KRAS/PI3K-activating mutations increase NAD⁺ demand and sensitise cells to the olaparib/FK866 combination, we assembled a panel of 10 EOC cell lines with mutations that reflect different EOC subtypes (i.e., HGSOC, EnOC, CCOC, MOC and LGSOC) (**Figure 16**). COV318, COV504 and 59M cells were included as they possess *TP53* mutations but lack *BRCA1/2* mutations and KRAS/PI3K-activating mutations. KURAMOCHI and OVSAHO cells were included as they possess *TP53* and *BRCA1/2* mutations. OVCAR-8 cells were included because they possess a *TP53* mutation and a *KRAS* activating mutation. It should also be noted that the *BRCA1* promoter is reported to be methylated in this cell line²⁷¹, suggesting OVCAR-8 cells may have a HRD. COV644 cells were included because they lack *TP53*, *BRCA1/2* and KRAS/PI3K-activating mutations. According to previous reports these 7 cell lines are likely to model HGSOC²⁷², which is the most common subtype of EOC. A2780, HEY-A8 and TOV21G cells lack *TP53* mutations but possess RAS (e.g., *KRAS* or *BRAF*) and/or PI3K (e.g., *PTEN* or *PI3K*) pathway alterations. Since these 3 cell lines lack *TP53* mutations it is likely they model EOC subtypes such as LGSOC, MOC, EnOC or CCOC²⁷².

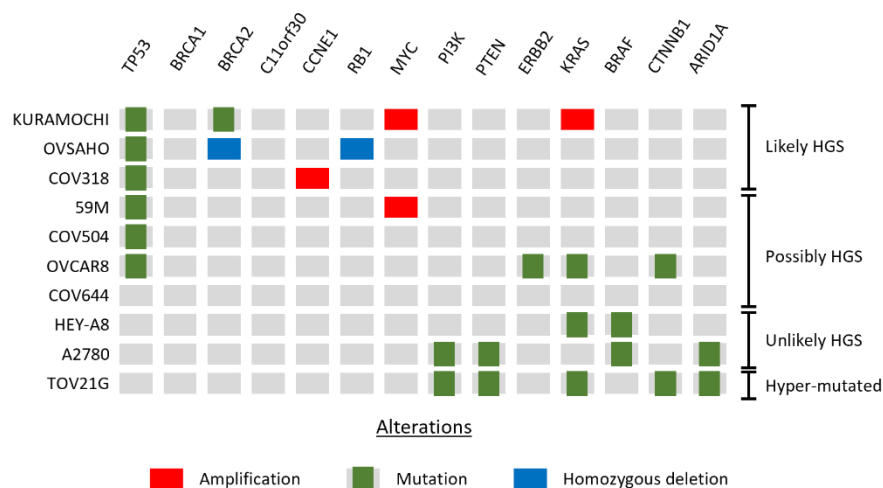


Figure 16. Mutational profile of selected EOC cell lines

Selected genetic events specific to ovarian cancer are shown, alongside their ranking as models of HGSOC (right). This figure has been adapted from work by Domcke and colleagues (2013)²⁷².

2.2. Materials & Methods

2.2.1. Cell culture and drug preparation

A panel of 10 EOC cell lines (**Figure 16**) were used in subsequent assays: COV318, COV504, COV644, 59M, OVSAHO, KURAMOCHI, A2780, HEY-A8, OVCAR-8 and TOV21G cells. All cells were cultured in phenol red-free DMEM (ThermoFisher, A1443001) supplemented with 5.6mM glucose (ThermoFisher, A2494001), 10% foetal bovine serum (FBS; ThermoFisher, 10270106), 2mM L-glutamine (ThermoFisher, 25030-081), 100 units/ mL penicillin, 100µg/mL streptomycin (ThermoFisher, 15070-063). Cells were maintained at 37°C in a humidified atmosphere containing 10% CO₂. Cell lines were authenticated using Short Tandem Repeat (STR) profiling by Public Health England. To avoid genetic drift human cell lines were cultured for no more than ten passages. Cell lines were regularly tested for mycoplasma contamination.

Stocks of FK866 (APExBIO, A4381) and olaparib (Selleckchem, S1060) were prepared in DMSO (Sigma-Aldrich, D2650). In the short term, re-suspended aliquots were stored at -20°C.

2.2.2. Sulforhodamine B (SRB) cell growth assay

Human cell lines were seeded on 96-well plates at a pre-determined density, as shown in **Table 2**. Cell counts were performed using a Vi-CELL XR Cell Viability Analyzer (Beckman

coulter). The following day, wells were aspirated and replaced with media containing vehicle (0.1% DMSO), the NAMPTi FK866 (50pM-50nM) or the PARPi olaparib (1nM-200μM). Where indicated, drugs were added in combination and/or media was supplemented with 250μM of β-NMN (Sigma-Aldrich, N3501). After 6-days of treatment, cells were fixed for 1 hour at 4°C with 10% (w/v) trichloroacetic acid (TCA) solution (Sigma-Aldrich, T6399). Wells were washed with water and left to dry at room temperature (RT) overnight. Cells were stained for 30 minutes at RT with 0.4% SRB (Sigma-Aldrich, 230162) solution (w/v) in 1% (v/v) acetic acid (ThermoFisher, 10005920). Wells were washed four times with 1% acetic acid (v/v) to remove excess dye and plates were left to dry overnight at RT. 200μL of 10mM Tris Base solution (Sigma-Aldrich, T1699) was added to wells and plates were placed on a shaker to solubilise the protein bound SRB dye. Absorbance was then measured on a SpectraMax Gemini XPS Microplate Reader (Molecular Devices) at 565nm. Unless otherwise specified, a minimum of three technical replicates were used for each condition. Cell free wells were used as blanks to remove background. Data was normalised to vehicle control (VC) wells and dose response curves were generated using nonlinear regression function [inhibitor] vs. response (three parameters) in the GraphPad Prism 10.0.0 software.

Table 2. EOC cell line panel seeding densities for the SRB assay

Cell line	Seeding density
COV318	2,500
COV504	4,000
COV644	4,000
KURAMOCHI	4,000
OVSCHO	4,000
59M	4,000
A2780	1,000
HEY-A8	1,000
OVCAR-8	2,000
TOV21G	1,500

2.2.3. ATP assays

The cell line panel was seeded onto two parallel plates at a density of 10,000 cells per well. A clear plastic plate was used for an SRB assay and white walled 96 well plates were used for

the CellTiter-Glo® assay (Promega, G7570). 24-hours after seeding cells were treated with FK866. After 48-hours treatment, SRB plates were processed using the SRB assay protocol, and white walled plates were processed using the CellTiter-Glo® assay, following manufacturers guidelines. Luminescence was measured on a FLUOstar Omega Microplate reader (BMG LABTECH). Cell free wells were used as blanks to remove background. Data was normalised to VC wells and graphs were generated using the GraphPad Prism 10.0.0 software. Absolute levels of ATP were determined by normalising to cell biomass (SRB assay), and concentrations were subsequently calculated using an ATP (Sigma-Aldrich, FLAAS-1VL) standard curve.

2.2.4. NAD/NADH-Glo™ Assay

The cell line panel was seeded onto two parallel plates at a density of 10,000 cells per well. A clear plastic plate was used for an SRB assay and white walled 96 well plates were used for the NAD/NADH-Glo™ assay (Promega, G9072). 24-hours after seeding, cells were treated with FK866. After 24-hours treatment, SRB plates were processed using the SRB assay protocol, and white walled plates were processed using the NAD/NADH-Glo™ assay. In brief, media was aspirated from wells and replaced with 50µL of PBS or 50µL of PBS with diluted NAD⁺ standards (50-2000nM). 50µL of 1% dodecyltrimethylammonium bromide (DTAB) with 0.2N NaOH was added to each well and plates were briefly mixed to ensure homogeneity and cell lysis. For each well, 50µL was transferred into an empty well and 25µL of 0.4N HCl was added. Plates were covered and incubated for 15 minutes on a ThermoMixer® C (Eppendorf) heat block at 60°C. Plates were allowed to equilibrate for 10 minutes at RT before adding 25µL of 0.5M Trizma® Base (Sigma-Aldrich, 93352) solution. 50µL of this mixture was then transferred into a white walled 96 plate along with 50µL of the NAD/NADH-Glo detection reagent, which contained: 1000µL Luciferin Detection Reagent, 5µL Reductase enzyme, 5µL Reductase substrate, 5µL NAD Cycling Enzyme and 25µL NAD Cycling Substrate. Plates were mixed for 30 minutes at RT before luminescence was measured on a FLUOstar Omega Microplate reader (BMG LABTECH). Cell free wells were used as blanks to remove background. Data was normalised to VC wells and graphs were generated using the GraphPad Prism 10.0.0 software. Absolute levels of NAD⁺ were determined by normalising to cell biomass (SRB assay), and concentrations were subsequently calculated using an NAD standard curve.

2.2.5. 3-D spheroid assays

COV318, KURAMOCHI, OVSAHO, A2780, HEY-A8 and OVCAR-8 cells were used for 3-D spheroid assays. Cell suspensions were passed through a 0.45µm filter before seeding 100µL of cells onto ultra-low attachment (ULA) 96-well black plates with a clear round bottom, at a density of 5,000 cells per well. Cells were left for 24-hours to allow spheroids to form, and 100µL of media containing either VC or drug (2X concentration) was added to each well. After 6-days of treatment, spheroids were visually assessed on an Eclipse TE2000-U Inverted microscope (Nikon) at 10X magnification, and images were captured using the QCapture software. Alternatively, images were captured on an Incucyte® S3 Live-Cell Analysis Instrument (Sartorius) and the accompanying Incucyte® Software (v2020C). After imaging, 50µL of media was removed from each well and 50µL of the CellTiter-Glo 3D cell viability assay (Promega, G9682) added. Cell viability was measured following manufacturers guidelines. Luminescence was measured on a FLUOstar Omega Microplate reader (BMG LABTECH). Cell free wells were used as blanks to remove background. A similar approach was used for the CellTox™ Green Cytotoxicity Assay. Data was normalised to VC wells and graphs were generated using the GraphPad Prism 10.0.0 software.

2.2.6. Caspase-Glo® 3/7 Assay System

COV318, KURAMOCHI and OVCAR-8 were selected for apoptosis assays, using seeding densities shown in **Table 2**. A clear plastic plate was used for an SRB assay and white walled 96 well plates were used for the Caspase-Glo® 3/7 Assay (Promega, G8092). 24 hours after seeding, cells were treated with vehicle, olaparib and/or FK866. After 6-days treatment, SRB plates were processed using the SRB assay protocol, and white walled plates were processed using the Caspase-Glo® 3/7 Assay, following manufacturers guidelines. Luminescence was measured on a FLUOstar CLARIOstar Plus Microplate Reader (BMG LABTECH). Cell free wells were used as blanks to remove background. Caspase data was normalised to cell biomass. Data was then normalised to VC wells and graphs were generated using the GraphPad Prism 10.0.0 software.

2.2.7. Drug combination and synergy analysis

COV318, KURAMOCHI, OVSAHO, A2780, HEY-A8 and OVCAR-8 cells were selected for synergy assays, using seeding densities shown in **Table 2**. Cells were seeded in clear plastic 96 well plates and treated with vehicle, olaparib (100nM, 1000nM, 10,000nM, 25,000nM and

50,000nM) and/or FK866 (0.1nM, 0.5nM, 1nM, 5nM and 10nM) the following day in a 6 x 6 matrix format. A single technical replicate was used for each condition and data was averaged from three independent experiments. Cells were treated for 6-days and data was acquired using the SRB assay. Data analysis was performed using the SynergyFinder+ web application²⁷³ and the Bliss Independence model of combination analysis. Synergy scores >10 were considered synergistic, scores between -10 to 10 were considered additive and scores < -10 were considered antagonistic. Cell free wells were used as blanks to remove background. Data was then normalised to VC wells and graphs were generated using the GraphPad Prism 10.0.0 software.

2.2.8. Targeted LC-MS/MS analysis for β -NMN and NAD⁺

COV318 and OVCAR-8 cells were seeded on 6 well plates at a density of 200,000 cells per well. The following day, media was aspirated and replaced with fresh media containing vehicle, FK866 and/or olaparib. Separate plates were included for media blanks, extraction blanks and for cell counts at the time of extraction to normalise data by cell number. 24-hours after treatment commenced, cell counts were performed on a Vi-CELL XR Cell Viability Analyzer (Beckman coulter). The remaining plates were transferred onto a ThermalTrayTM (Biocision) that had been pre-cooled with dry-ice and 2mL Eppendorf tubes were pre-cooled in a CoolRack[®]. Wells were aspirated and cells were washed with ice-cold Ringers solution (2 mL, one tablet per 500 mL of MilliQ H₂O, Sigma-Aldrich, 96724). Wells were aspirated and cells were quenched with 1mL of ice cold LC-MS grade 80:20 methanol:water (v/v) (ThermoFisher, 34966) for 20 minutes. Wells were scraped and the extracts were transferred to the 2mL tubes. The previous step was repeated with 500 μ L of 100% methanol. Next, extracts were centrifuged for 20 minutes at 18,000 x g (4°C) using a EppendorfTM Centrifuge 5430 R (Eppendorf), to pellet out cellular debris. Supernatants were collected in high recovery LCMS vials, dried under nitrogen flow, and stored at -80°C. Before LC-MS/MS analysis samples were reconstituted in 50 μ L of LC-MS grade H₂O (ThermoFisher, 10505904), vortexed for 1 minute and stored in an autosampler at 10°C.

LC-MS/MS assays were performed using an Agilent 1290 Infinity LC system (Agilent Technologies) and a 4000 QTRAP triple quadrupole instrument (AB SCIEX) coupled to an ESI source (Turbo V). An ACQUITY UPLC[®] HSS (High Strength Silica) T3 Column (Waters) was used for Reversed-Phase (RP) Liquid Chromatography, with a column temperature of 40°C. To

increase the columns lifetime a ACQUITY UPLC HSS T3 VanGuard Pre-column (Waters Corp, 186003976) was used. Elution was performed at a flow rate of 600 μ L/min using the gradient described in **Table 3** and 5 μ L of sample was used per injection. Needle wash steps were performed with Acetonitrile:Isopropanol:H₂O (40:40:20, v/v/v) (ThermoFisher, 10055454) (ThermoFisher, 10684355). Mobile phase A was comprised of H₂O and 0.2% formic acid (v/v) (Scientific Laboratory Supplies, 56302). Mobile phase B contained acetonitrile (ACN) and 0.2% formic acid (v/v).

Table 3. RP UPLC gradient elution in positive mode

Time (min)	Flow rate (μ L/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	600	99.5	0.5
2.00	600	99.5	0.5
5.00	600	85.0	15.0
10.00	600	0.5	99.5
13.00	600	0.5	99.5
13.10	600	99.5	0.5
15.00	600	99.5	0.5

To optimise conditions for targeted metabolomics in Multiple Reaction Monitoring (MRM) mode (QqQ) standards for each compound were directly infused into the MS/MS analyser. From this MRM transitions, declustering potential (DP) and collision energy (CE) values were identified for each target analyte as shown in **Table 4**.

Table 4. MRM parameters for the RP UPLC-MS/MS NAD⁺ assay

Metabolite	Q1	Q3	RT (min)	DP	CE	Detected in cells?
<u>NAD_1</u>	664.0	136.0	0.88	70	70	✓
<u>NAD_2</u>	664.0	97.0	0.88	70	70	✓
<u>β-NMN_1</u>	335.0	123.0	0.68	100	20	✓
<u>β-NMN_2</u>	357.0	235.0	0.68	50	14	X

Main ions are underlined

The Analyst (v1.6.2, SCIEX) software was used to perform peak integration. Purified stock compounds were used to produce a calibration curve (0.025-10 μ g/mL) for each run. Using this and cell counts, molar concentrations per million cells were calculated for each metabolite.

2.2.9. Protein extraction and protein assay

On the day of extraction media was removed from 6-well plates. Plates were placed onto a pre-cooled ThermalTray™ and washed with ice-cold PBS. Cells were lysed with

radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich, R0278) supplemented with a Halt™ Protease and Phosphatase Inhibitor Cocktail (ThermoFisher, 78440). Lysates were transferred into 1.5mL Eppendorfs, vortexed for 30 seconds and then placed on ice for 10 minutes. This was repeated two more times and then lysates were centrifuged at 17,400 x g for 10 minutes (4°C) to remove cellular debris. Supernatants were transferred into fresh Eppendorf tubes. Protein supernatants and BSA standards (ThermoFisher, 10531674) were quantified using a Pierce™ BCA Protein Assay Kit (ThermoFisher, 23225) on a FLUOstar CLARIOstar Plus Microplate Reader (BMG LABTECH). Cell free wells were used as blanks to remove background. Data was analysed using Microsoft Excel for Windows (Version 2306). A standard curve was used to calculate each samples protein concentration.

2.2.10. Western blotting

Prior to loading, 10-30µg of samples were mixed with ddH₂O and 1x (v/v) Laemmli buffer (BIO-RAD, 1610747) and 10% (v/v) β-mercaptoethanol (BIO-RAD, 1610710). Samples were boiled for 10 minutes at 95°C on a ThermoMixer® C (Eppendorf) heat block. Unless stated otherwise, samples were loaded onto 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (BIO-RAD, 4561094 or 4561096) along with the Amersham™ ECL™ Rainbow™ Marker (Merck, GERPN800E). Proteins were separated by electrophoresis using a PowerPac HC Power Supply (BIO-RAD, 1645052EDU) at 200V for 30-50 minutes. The Tris-Glycine running buffer (90 % nanopure water, 25 mM Tris, 192 mM Glycine, 0.1 % sodium dodecyl sulfate) was prepared in house. Proteins were transferred onto nitrocellulose membranes (BIO-RAD, 1620115) by wet transfer (100V for 60 minutes). Transfer buffer (25 mM Tris, 190 mM Glycine, 20% Methanol, pH 8.3) was prepared in house.

To confirm proteins had been efficiently transferred membranes were briefly stained with Ponceau S solution (Sigma-Aldrich, P7170). Membranes were then cut into sections, washed with 1 x tris-buffered saline with 0.1% tween-20 (TBST; prepared in-house) to remove excess Ponceau, and blocked for 1 hour at RT, in 5% milk (Mercy, 70166) or 5% BSA (Sigma-Aldrich, SRE0096) in 1 x TBST. Phospho-antibodies were blocked with BSA and total antibodies were blocked with milk. After blocking, membranes were incubated overnight at 4°C with primary antibodies (**Table 5**) in milk (total) or BSA (phospho), excluding the phospho-Histone H2A.X antibody, which was in milk. The following day, membranes were washed three times (for 5-10 minutes) in TBST. Afterwards, membranes were incubated for 1 hour (RT) with horseradish

peroxidase (HRP) conjugated secondary antibodies in milk (total) or BSA (phospho), excluding phospho-Histone H2A.X, which was in milk. Membranes were then washed three times with TBST and incubated for 5 minutes at RT with SupersignalTM West Pico plus chemiluminescent substrates (ThermoFisher, 34580). Blots were developed using Amersham Hyperfilm ECL film (GE Healthcare, 28-9068). In some instances, the Optimax X-ray film processor (Protec) was unavailable, when this occurred the Immobilon Forte Western HRP substrate (Merck, WBLUF0100) was used, and membranes were imaged on an ImageQuant LAS 4000 (Fujifilm) instrument. Densitometry analysis was performed using Fiji V2.9.0, and the values were normalised to β -actin or α -tubulin. Graphs were generated using the GraphPad Prism 10.0.0 software.

Table 5. Antibodies used for western blotting

Antibodies	Species	Source	Identifier	Dilutions
β -actin	Mouse	Abcam	Ab6276	1:10,000
phospho-Histone H2A.X (S139)	Mouse	Sigma-Aldrich	05-636	1:1000
PAR/MAR	Rabbit	Cell Signalling	83732	1:1000
PARP-1	Rabbit	Cell Signalling	9542S	1:1000
Akt (pan) (C67E7)	Rabbit	Cell Signalling	4691	1:2500
p44/42 MAPK (Erk1/2)	Rabbit	Cell Signalling	4695S	1:2000
Phospho-Akt (Ser473)	Rabbit	Cell Signalling	4060	1:2000
Phospho-ERK1/2 (Thr202/Tyr204)	Rabbit	Cell Signalling	4370S	1:2000
Chk1 (2G1D5)	Mouse	Cell Signalling	2360	1:1000
RPA32 (4E4)	Rabbit	Cell Signalling	2208S	1:1000
pChk1 (Ser345)	Rabbit	Cell Signalling	133D3	1:1000
pRPA32 (S4/S8)	Rabbit	Fortis Life Sciences	A300-254A	1:1000
NMNAT-1 (B-7)	Mouse	Santa Cruz Biotechnology	sc-271557	1:500
α -Tubulin (B-7)	Mouse	Santa Cruz Biotechnology	T9026	1:1000
Histone H3, CT, pan	Rabbit	Merck	07-690	1:5000
DNA Topoisomerase I	Mouse	BD Biosciences	556597	1:1000
HRP-conjugated goat anti-rabbit	Goat	Invitrogen	31460	1:2500
HRP-conjugated goat anti-mouse	Goat	Invitrogen	31340	1:2500

2.2.11. Statistical analysis

Unless otherwise stated, statistical tests were performed using GraphPad Prism (10.0.0), Microsoft Excel for Windows (Version 2306) or R for windows (4.3.1.).

2.3. Results

2.3.1. RAS- and PI3K-mutant EOC cell lines are sensitive to the NAMPT inhibitor FK866

In the following experiments, a panel of EOC cell lines were used (**Figure 16**) to investigate whether alterations in RAS/PI3K signalling sensitised cells to the NAMPTi FK866. From this, it

was revealed that EOC cells harbouring mutations in KRAS or PI3K signalling pathways were sensitive to FK866 compared to EOC cell lines lacking these alterations (**Figure 17A and Appendix Figure 2**). Of these cells OVCAR-8, TOV21G and A2780 were the most sensitive, with IC_{50} values ≤ 0.62 nM. Although HEY-A8 cells were ranked 4th in terms of FK866 sensitivity (IC_{50} of ~ 2.17 nM), they were not much more sensitive when compared to KRAS/PI3K-wild type cell lines (IC_{50} values of ~ 2.33 - 4.72 nM).

To investigate whether FK866 sensitivity could be explained by the level of NAD⁺ depletion, NAD⁺ assays were performed (**Figure 17B-C**, majority of NAD⁺ assays were performed by Dr Adrian Benito-Mauricio). From this, it was confirmed that after 24 hours 5nM FK866 could efficiently deplete the NAD⁺ pool ($>75\%$ depletion), but minor differences emerged between cell lines at this dose. When cells were treated with a lower dose (0.5nM) clear differences emerged (**Figure 17B**) and the level of NAD⁺ depletion was found to correlate with FK866 sensitivity ($r = 0.8206$, $p = 0.0036$) (**Figure 17C**). For example, the most sensitive cells (OVCAR-8, TOV21G and A2780) were the only ones to achieve an NAD⁺ depletion $> 80\%$. By comparison, HEY-A8 cells achieved an NAD⁺ depletion of $\sim 60\%$. Whereas cells that are more resistant to FK866 (e.g., KURAMOCHI, COV504, 59M and COV318) were able to achieve greater or similar levels of NAD⁺ depletion in comparison to HEY-A8 cells. Suggesting that depletion of NAD⁺ alone isn't sufficient to reduce the survival of cells.

NAMPT inhibition with FK866 is known to inhibit ATP synthesis, as NADH is a substrate for the mitochondrial electron transport chain and NAD⁺ is a co-factor required for glycolysis^{126,131}. To confirm this mechanism of action in EOC, cellular ATP levels were measured. From this it was revealed 48 hours treatment with 5nM of FK866 could significantly reduce ATP levels in the majority of the EOC cells tested (**Figure 17D-E**). However, the greatest level of ATP depletion ($>50\%$) was observed in the KRAS/PI3K-mutant cells. Importantly, lower levels of ATP correlated with lower FK866 IC_{50} values ($r = 0.9119$, $p = 0.0002$) (**Figure 17E**). It should be noted, when correlating FK866 IC_{50} values with each cell lines estimated doubling time a similar trend was observed, those with the shortest doubling time (i.e., KRAS/PI3K-mutant cells) had lower IC_{50} values ($r = 0.8895$, $p = 0.0006$) (**Appendix Figure 3**).

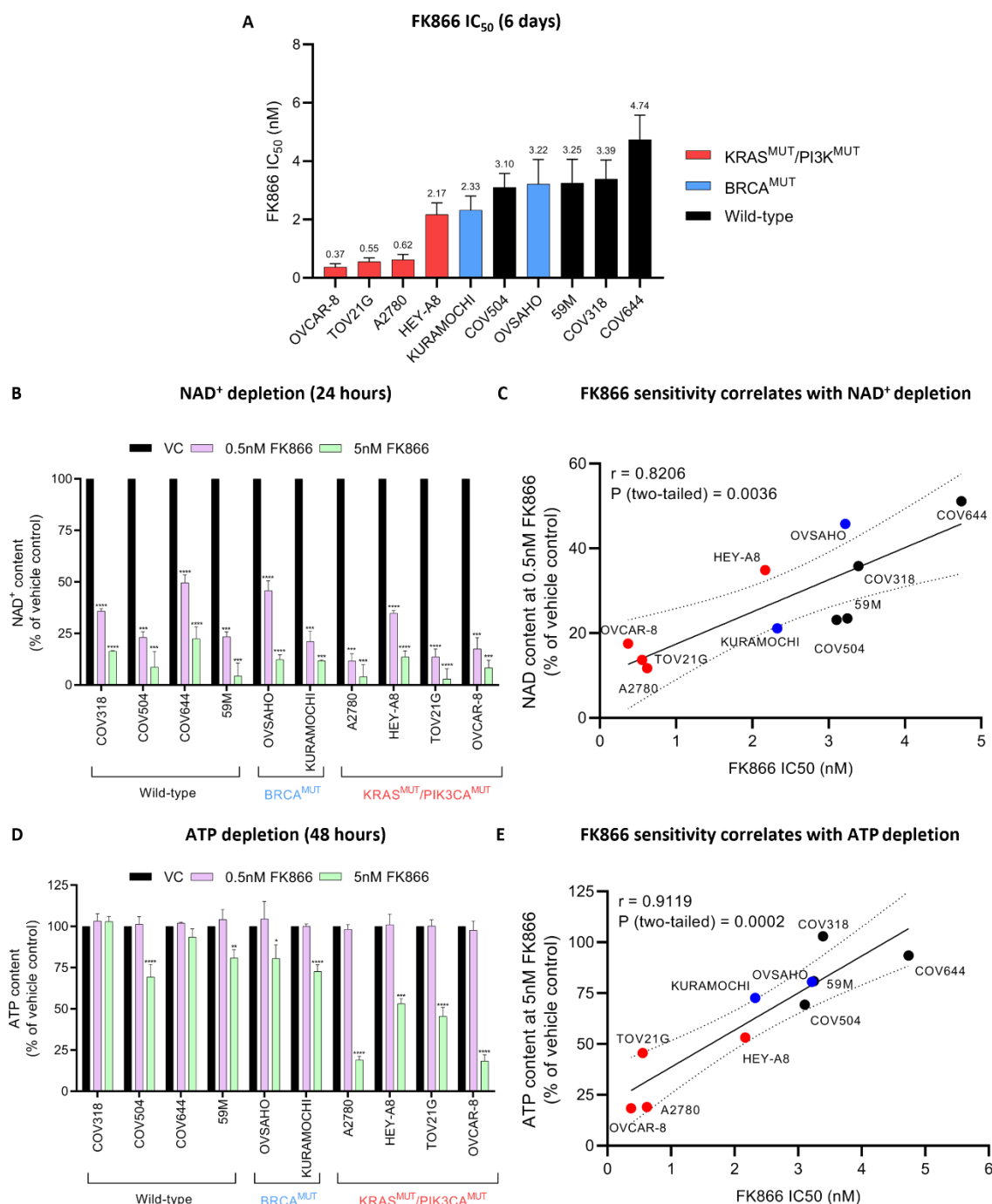


Figure 17. EOC cell lines are sensitive to the NAMPT inhibitor FK866

(A) EOC cell lines FK866 sensitivity after 6-days treatment. Cell proliferation was measured using the SRB assay. For dose response curves see **Appendix Figure 2**. **(B)** EOC cells were treated (24 hours) with vehicle (0.1% DMSO), 0.5nM FK866 (pink) or 5nM FK866 (green) for 24 hours. NAD⁺ content was measured using the NAD/NADH-GloTM assay. Data was normalised to cell biomass (SRB assay). The majority of NAD⁺ data was generated by Dr Adrian Benito-Mauricio. **(C)** Pearson correlation coefficient (*R*) and linear regression were used to analyse the relationship between NAD⁺ content following treatment (24 hours) with FK866 and FK866 sensitivity (IC₅₀). *r* and *p* values are shown. **(D)** EOC cells

were treated (48 hours) with vehicle (0.1% DMSO), 0.5nM FK866 (pink) or 5nM FK866 (green) for 48 hours. ATP content was measured using the CellTiter-Glo® 2.0 assay. Data was normalised to cell biomass (SRB assay). **(E)** Pearson correlation coefficient (R) and linear regression were used to analyse the relationship between ATP content following treatment (48 hours) with FK866 and FK866 sensitivity (IC_{50}). r and p values are shown. For figures (a-b and d) error bars = SD. All figures are presented as the mean of three independent experiments. In figures (b and d) statistical significance between vehicle and monotherapy groups was determined using one-way ANOVA followed by Dunnett's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Since 3-D models better recapitulate the *in vivo* tumour microenvironment (e.g., differences in metabolism, pH and oxygen levels emerge throughout a spheroid²⁷⁴), FK866 efficacy was also investigated in 3-D spheroid models of EOC. Due to their ability to readily form spheroids (**Appendix Figure 4**), COV504 (KRAS wild-type) and OVCAR-8 (KRAS mutant) cells were selected for a CellTox™ Green Cytotoxicity Assay (**Figure 18**). From this, we observed that 72 hours treatment with >1nM FK866 could upregulate cell death (cytotoxicity) in OVCAR-8 (KRAS mutant) but not COV504 (KRAS wild-type) spheroids. Furthermore, OVCAR-8 spheroids became darker in appearance following treatment, though there was not a significant change in spheroid size (data not shown).

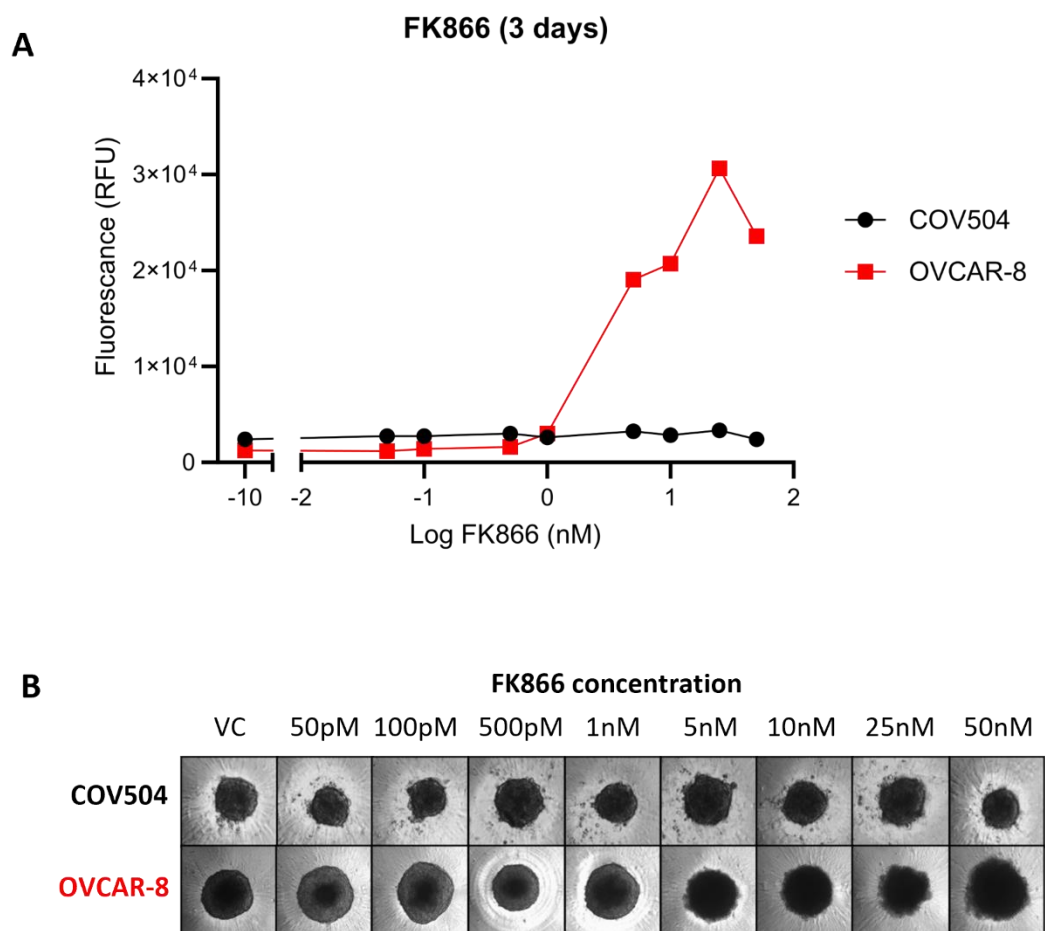


Figure 18. FK866 upregulates cell death in KRAS-mutant OVCAR-8 cells

(A-B) COV504 and OVCAR-8 cells were seeded on ULA attachment 96 well plates for 72 hours. After large spheroids had formed cells were treated with vehicle (0.1% DMSO) or FK866 (50pM – 50nM) for 72 hours. At endpoint, (b) images were taken of spheroids on a Eclipse TE2000-U Inverted microscope (Nikon) at 10X magnification. Then (a) cytotoxicity was measured using the CellTox™ Green Cytotoxicity Assay. Data are from three technical replicates in one independent experiment. (b) Red titles indicate cells have a mutation in KRAS/PI3K signalling and black titles indicate cells don't possess mutations in either pathway.

2.3.2. FK866 synergises with olaparib in EOC cells

To test the sensitivity of the EOC cell line panel to PARPi treatment cells were treated for 6-days with olaparib (**Appendix Figure 1**). From this we observed that NAMPTi sensitive cells (OVCAR-8, TOV21G and A2780) were also sensitive to PARPi. Surprisingly, these cells were more sensitive than selected BRCA-mutant models (KURAMOCHI and OVSAHO).

To investigate whether FK866 enhances PARPi responses the EOC cell line panel was treated with olaparib and low doses of FK866 (100pM or 500pM) (**Figure 19 and Appendix Figure 5**). Apart from A2780, OVCAR-8 and TOV21G cells (where 500pM FK866 was cytotoxic) the selected doses of FK866 did not have an impact on cell proliferation as a monotherapy. In all cell lines that were co-treated with 500pM of FK866, olaparib IC₅₀ values could be reduced by >2-fold. Furthermore, excluding COV318, COV644, 59M and KURAMOCHI cells, co-treatment with 100pM of FK866 could also reduce olaparib IC₅₀ values by ~2-fold or more (**Figure 19**).

To determine whether the combination of FK866 and olaparib was synergistic, 6 cell lines from the EOC panel were selected for a 6 x 6 matrix experiment (**Figure 20**). This included three KRAS/PI3K-mutant lines (A2780, HEY-A8 and OVCAR-8), two BRCA-mutant lines (KURAMOCHI and OVSAHO) and one cell line that lacked mutations in these pathways (COV318) (**Figure 16**). Although treating COV318 cells with the combination reduced cell proliferation to a greater extent than monotherapy treatment at selected doses, the combination could only exert an additive effect (ΔBliss -10 to 10). By comparison, the BRCA-mutant cell lines (KURAMOCHI and OVSAHO) had at least two sections of the matrix where strong synergy (ΔBliss >10) between olaparib and FK866 could be observed. Interestingly, in the KRAS/PI3K-mutant cell lines (A2780, HEY-A8 and OVCAR-8) there were 4-5 doses where synergy could be observed, suggesting that cells possessing KRAS/PI3K pathway mutations may benefit from treatment with the combination to a greater extent than other cell types.

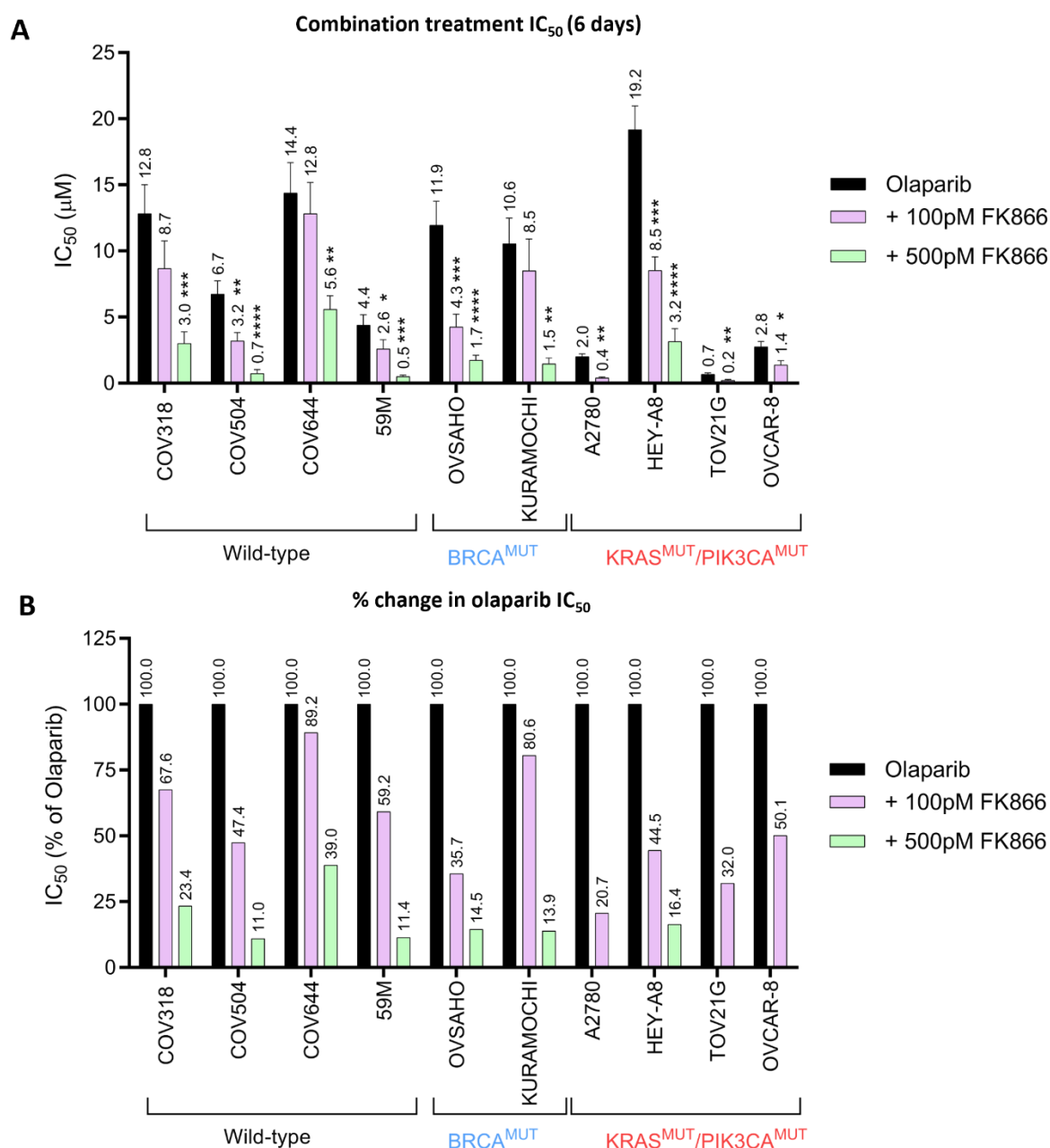


Figure 19. FK866 potentiates the cytotoxic effects of olaparib in EOC cell lines

(A-B) EOC cell lines were co-treated with olaparib and FK866 to assess their sensitivity to the combination after 6-days treatment. Cell proliferation was measured using the SRB assay. (a) IC_{50} values and (b) the % change in IC_{50} values relative to olaparib are shown for each cell line. For (a) error bars represent the 95% confidence interval. All figures are presented as the mean of three independent experiments. In (a) statistical significance between olaparib and combination groups was generally determined using one-way ANOVA followed by Dunnett's multiple comparisons test. For A2780, TOV21G and OVCAR-8 data, where the +500pM group was excluded; olaparib and the

combination group were compared by unpaired t test with Welch's correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). For dose response curves see **Appendix Figure 5**.

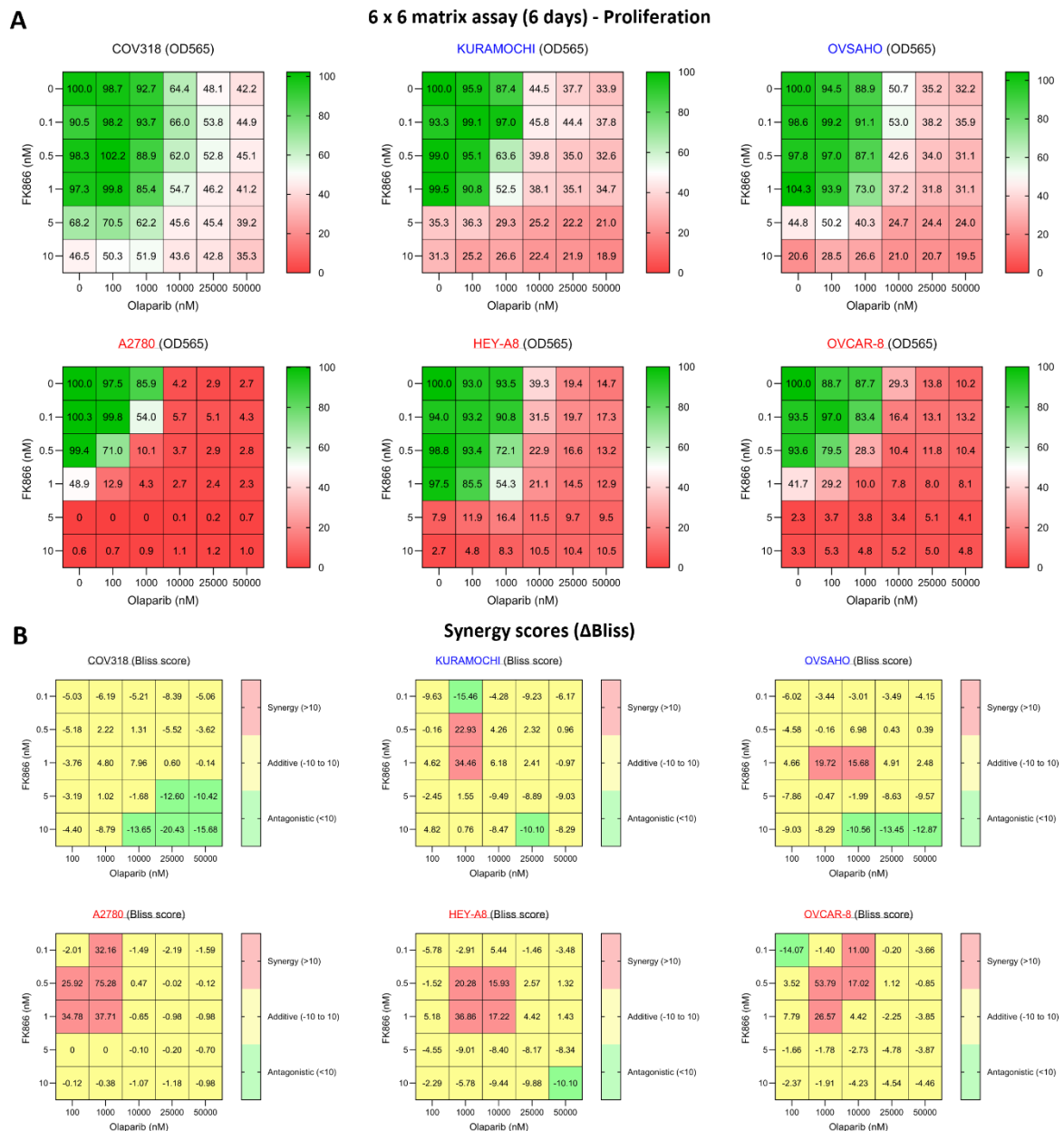


Figure 20. Bliss synergy analysis in EOC cell lines

(A-B) EOC cell lines were co-treated with olaparib (100 to 50,000nM) and FK866 (0.1 to 10nM) to assess their sensitivity to the combination after 6-days treatment. Cell proliferation was measured using the SRB assay. (a) Proliferation and (b) synergy (Δ Bliss format) data are shown. (a) Proliferation data is the average of three independent experiments, with one technical replicate per experiment. (b) Δ Bliss scores <10 are considered antagonistic (green), -10 to 10 is considered additive (yellow) and >10 is considered synergistic (red). Red titles indicate cells have a KRAS/PI3K pathway mutation. Blue

titles indicate cells have a mutation in BRCA1/2. Black titles indicate cells don't possess mutations in either pathway.

2.3.3. FK866 and olaparib upregulates apoptosis in KRAS/PI3K and BRCA-mutant cells

Next, we assessed the influence of olaparib, FK866 and the combination on apoptosis using the Caspase 3/7 assay (**Figure 21**). At selected doses, olaparib and/or FK866 could not upregulate caspase 3/7 activity in COV318 cells. By comparison, in KURAMOCHI (BRCA-mutant) and OVCAR-8 (KRAS-mutant) cells a significant increase in caspase 3/7 activation could be observed following combination treatment, but not following treatment with olaparib or FK866 alone. The largest increase in caspase activation was observed in OVCAR-8 cells.

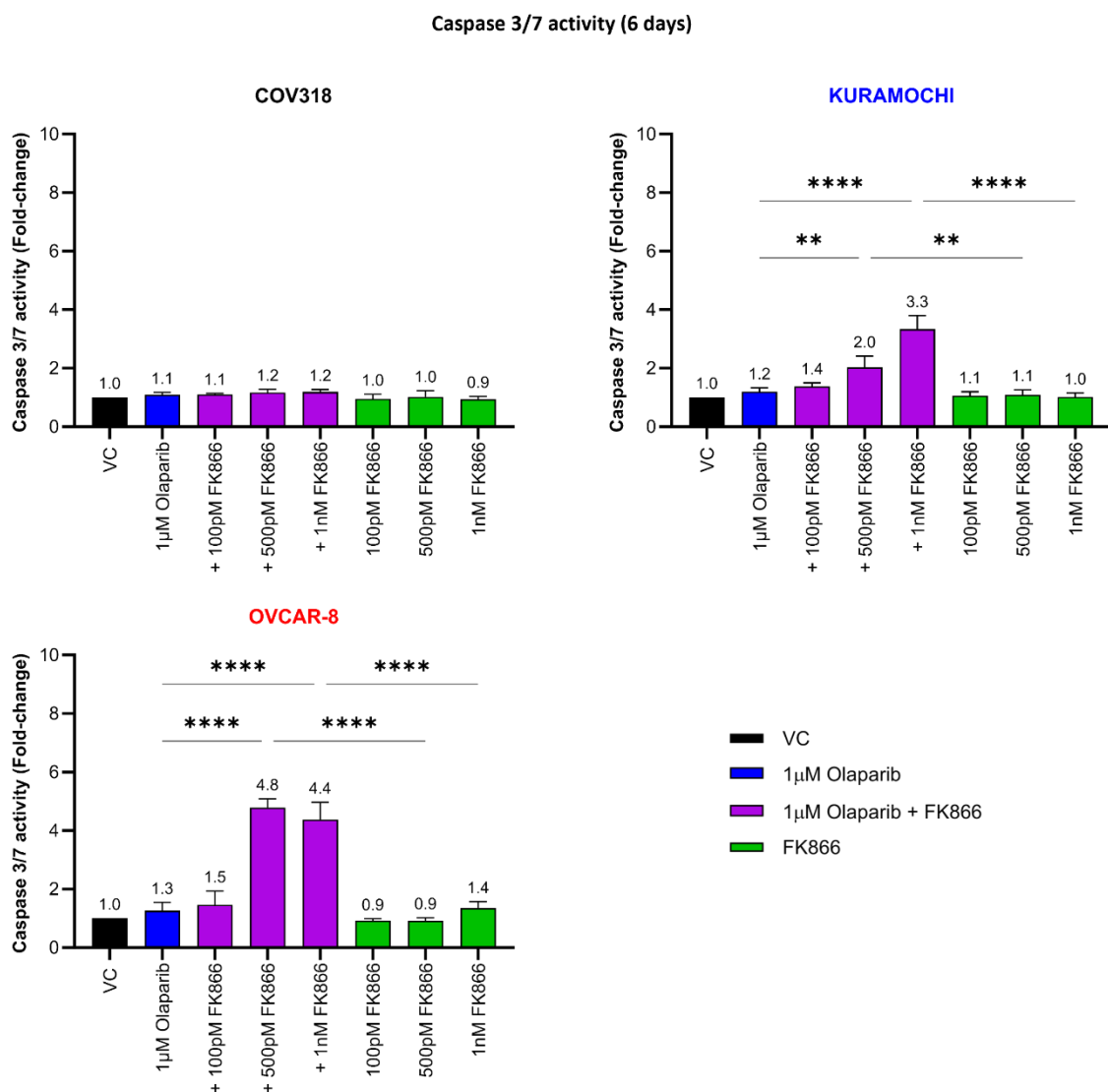


Figure 21. Combination treatment upregulates caspase 3/7 activity in KRAS/PI3K- and BRCA-mutant EOC cells

COV318, KURAMOCHI and OVCAR-8 cells were co-treated with olaparib (1µM) and FK866 (0.1 to 1nM) to assess their influence on caspase 3/7 activity (apoptosis). This was measured using a Caspase-Glo® 3/7 assay. Data is the average $\pm$ SD of three independent experiments. Statistical significance was determined using one-way ANOVA followed by Šídák's multiple comparisons test ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Red titles indicate cells have a KRAS/PI3K pathway mutation. Blue titles indicate cells have a mutation in BRCA1/2. Black titles indicate cells don't possess mutations in either pathway.

2.3.4. FK866 and olaparib reduces the viability of KRAS/PI3K-mutant 3-D spheroids

The efficacy of the combination was also tested in 3-D spheroid models of EOC (**Figure 22 and Appendix Figure 6**). This included three KRAS/PI3K-mutant lines (A2780, HEY-A8 and OVCAR-8), two BRCA-mutant lines (KURAMOCHI and OVSAHO) and one cell line that lacked mutations in these pathways (COV318) (**Figure 16 and Appendix Figure 4**). Surprisingly, COV318 and KURAMOCHI cells were resistant to olaparib and the combination in 3-D. The OVSAHO cell line did respond to olaparib, but co-treatment with 0.5nM FK866 could not significantly reduce cell viability. The A2780, HEY-A8 and OVCAR-8 spheroids all responded to olaparib. Furthermore, co-treatment with olaparib and 0.5nM FK866 could significantly reduce cell viability in all three of the selected KRAS/PI3K-mutant spheroids. These results highlight the importance of testing compounds in 3-D culture, as spheroids better model the tumour microenvironment (e.g., development of metabolic gradients and superior cell-cell interactions) compared to 2-D culture. Overall, these data support the use of a NAMPTi and PARPi combination in KRAS/PI3K-mutant EOC cell lines.

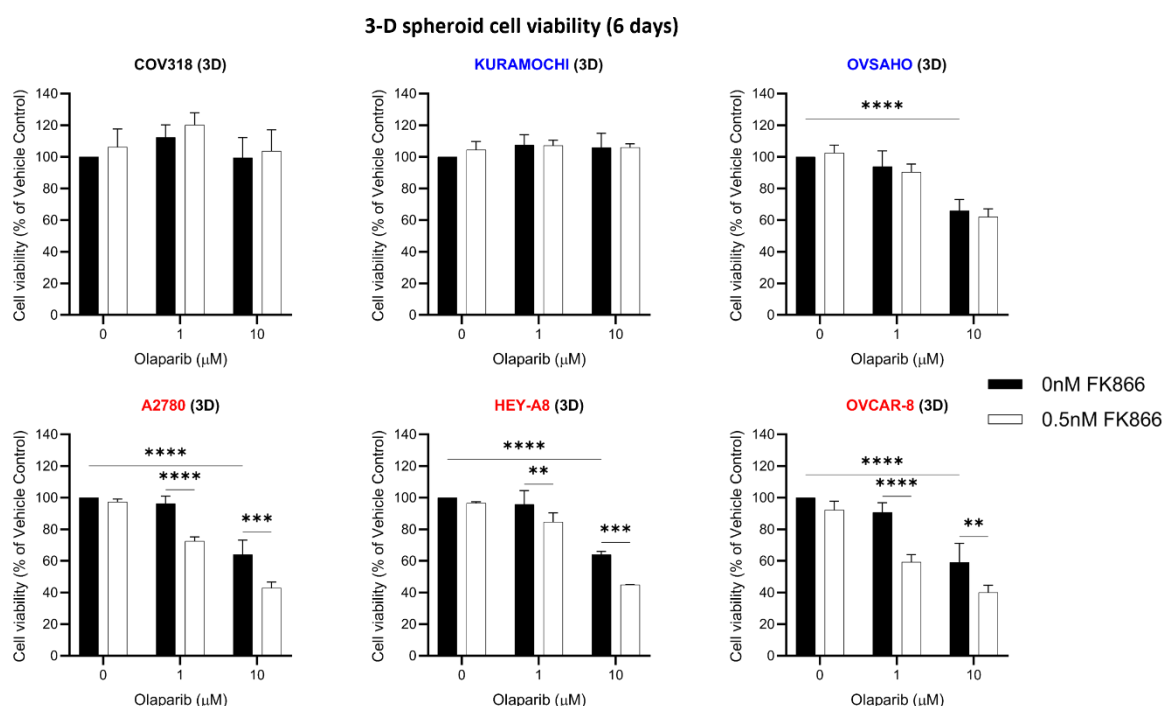


Figure 22. The combination reduces the viability of KRAS/PI3K-mutant EOC spheroids

3-D EOC spheroids were co-treated with olaparib (1 or 10μM) and 0.5nM FK866 to assess their sensitivity to the combination after 6-days treatment. Cell viability was measured using the CellTiter-Glo® 3D assay. Data is the average ± SD of three independent experiments. Statistical significance was

determined using 2-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$, $**p < 0.01$, $*** p < 0.001$, $**** p < 0.0001$). Red titles indicate cells have a mutation in KRAS/PI3K signalling. Blue titles indicate cells have a mutation in BRCA1/2. Black titles indicate cells don't possess mutations in either pathway. For 3-D dose response curves see **Appendix Figure 6**.

2.3.5. β -NMN supplementation is sufficient to rescue the survival of EOC cells treated with FK866 and olaparib

To investigate whether the synergistic interaction between olaparib and FK866 is due to the depletion of β -NMN/ NAD^+ , as has been shown previously⁹⁸⁻¹⁰⁰ (**Chapter 1**), media was supplemented with β -NMN at the point of drug treatment (**Figure 23**). At the selected doses, excluding A2780 and OVCAR-8 cells, FK866 monotherapy did not impact cell proliferation. In A2780 and OVCAR-8 cells, β -NMN supplementation did protect them from FK866-induced toxicity. $1\mu\text{M}$ olaparib treatment slightly reduced cell proliferation in OVSAHO, A2780 and OVCAR-8 cells, but β -NMN supplementation did not have a protective effect. As expected, the combination reduced proliferation to a greater extent than monotherapy treatments in all cell lines tested. However, only a minor reduction in proliferation was observed in COV318 cells and the response in OVSAHO cells was highly variable. As a result, a significant rescue could only be observed following combination treatment in KURAMOCHI, A2780, HEY-A8 and OVCAR-8 cells. In these cell lines, with the exception of A2780 cells, a complete rescue could be achieved following β -NMN media supplementation. It was interesting to observe β -NMN supplementation could only partially rescue A2780 cells following combination treatment, given it completely protected cells in the FK866 monotherapy group. Overall, these data indicate that the depletion of β -NMN is necessary for the combination to reduce cell survival.

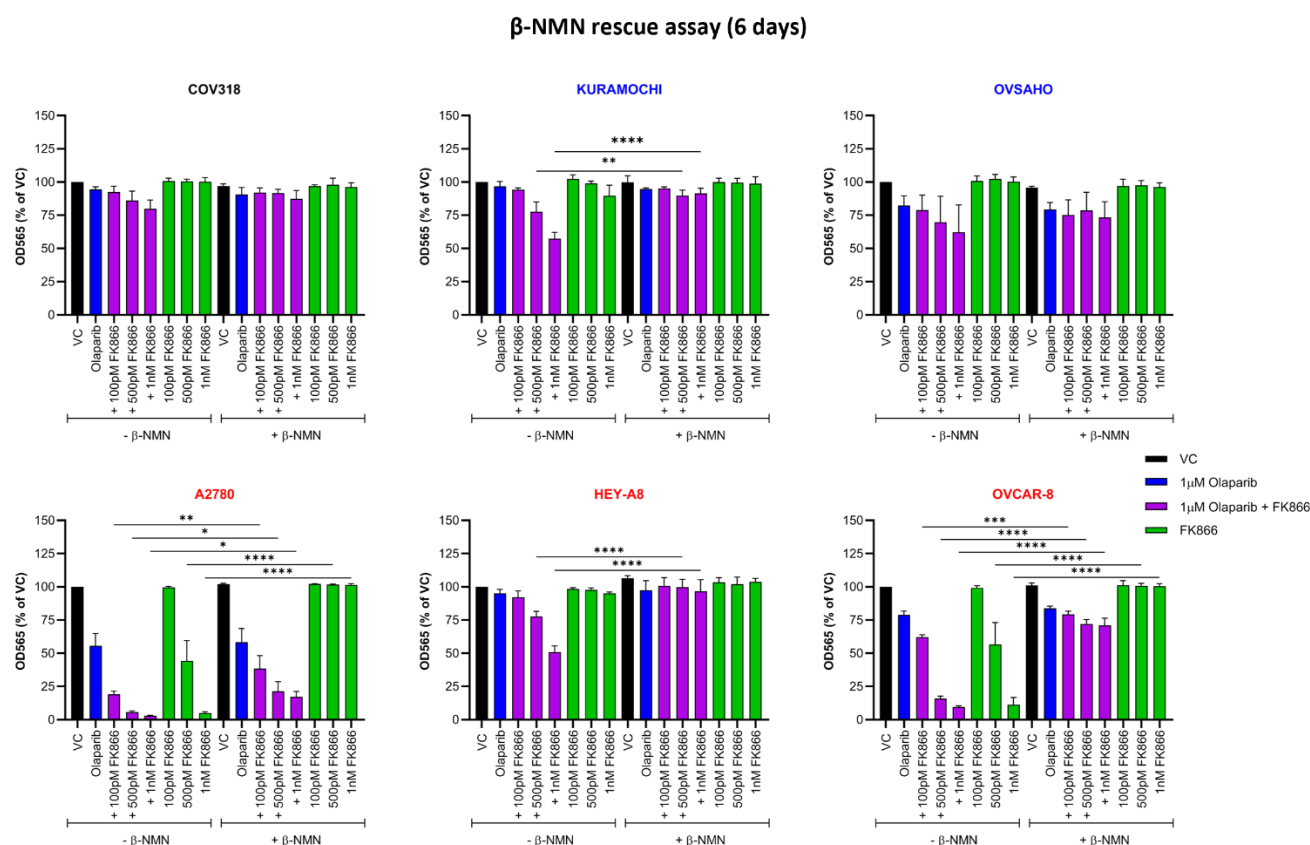


Figure 23. β-NMN media supplementation protects EOC cell lines from olaparib and FK866

EOC cell lines were co-treated with 1μM olaparib and FK866 (0.1 to 1nM) ± 250μM of β-NMN to investigate whether the NAD⁺ salvage pathway intermediate could rescue cells. Cell proliferation was measured after 6 days using the SRB assay. Data is the average of three independent experiments ± SD. Statistical significance was determined using one-way ANOVA followed by Šídák's multiple comparisons test (p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001). Red titles indicate cells have a mutation in KRAS/PI3K signalling. Blue titles indicate cells have a mutation in BRCA1/2. Black titles indicate cells don't possess mutations in either pathway.

2.3.6. KRAS-mutant EOC cells have a smaller β-NMN and NAD⁺ pool

To gain further insight into the impact of the combination on β-NMN and NAD⁺ pools a UPLC-MS/MS metabolomics assay was performed using COV318 (KRAS-wild type) and OVCAR-8 (KRAS-mutant) cells (**Figure 24** and **Appendix Figure 7**). From this, it was revealed that COV318 cells have larger β-NMN/NAD⁺ pools compared to OVCAR-8 cells. Although FK866 and the combination significantly depleted β-NMN/NAD⁺ pools in both cell lines, OVCAR-8 cells were more reliant on NAMPT activity to maintain β-NMN/NAD⁺ levels. For example, in COV318 cells treatment with FK866 and the combination reduced NAD⁺ levels by ~65% and

63%, respectively. Whereas, in OVCAR-8 cells FK866 and the combination reduced NAD⁺ levels by ~93% and 88%, respectively. A similar trend could be observed with β -NMN. Perhaps unsurprisingly, given PARP consumes NAD⁺ to ADP-ribosylate proteins (**Chapter 1**), olaparib treatment increased β -NMN/NAD⁺ levels. Despite inhibiting NAD⁺ consumption, olaparib did not have a greater impact on β -NMN/NAD⁺ levels than FK866, as pools remained depleted in combination groups. Overall, these data provide further evidence that KRAS-mutant cell lines are more reliant on NAMPT to maintain their β -NMN/NAD⁺ pools (see **section 2.4.1.**) and confirm that β -NMN/NAD⁺ are depleted following treatment with the combination.

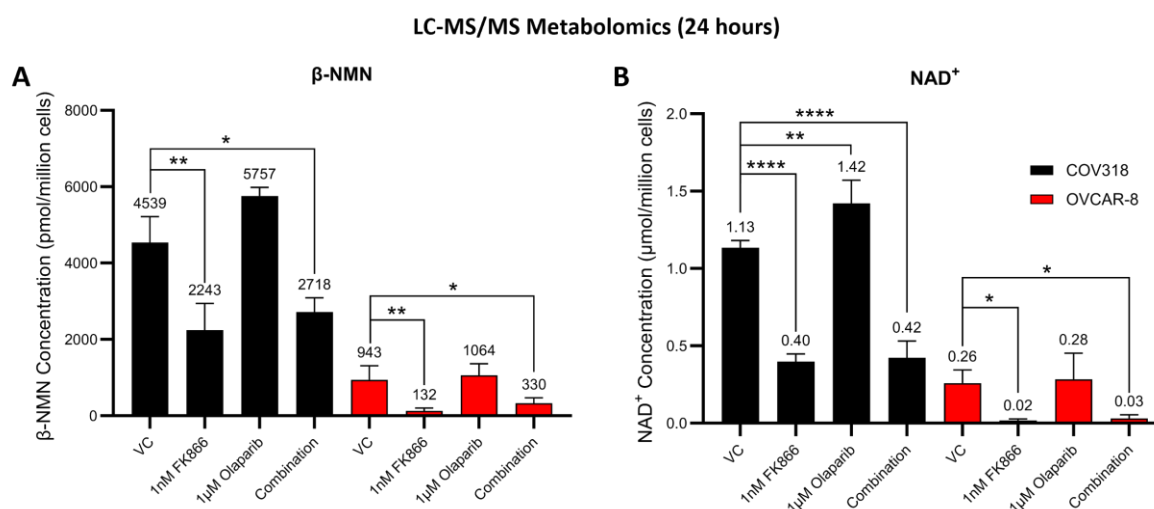


Figure 24. KRAS-mutant OVCAR-8 cells have a smaller β -NMN/NAD⁺ pool compared to KRAS-wild type COV318 cells and they are more reliant on NAMPT activity to maintain β -NMN/NAD⁺ levels

COV318 (KRAS-wild type, black) and OVCAR-8 (KRAS-mutant, red) cells were treated for 24 hours with vehicle, 1 μ M olaparib, 1nM FK866 or the combination and then extracted for RP UPLC-MS/MS analysis. **(A)** β -NMN and **(B)** NAD⁺ levels were calculated (molar concentration/per million cells) using a standard curve. Data is the mean of three independent experiments $\pm$ SD. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). For % change in β -NMN and NAD⁺ levels see **Appendix Figure 7**.

2.3.7. PARPi and NAMPTi block PARylation but not ERK1/2 or AKT signalling in EOC

In multiple myeloma it has previously been shown that NAMPTi can downregulate p-ERK1/2 and p-AKT¹²⁸. Furthermore, in Ewing Sarcoma FK866 and the combination were shown to inhibit PARylation⁹⁹ (**Chapter 1**). To investigate whether treatment with the combination has a similar impact on ERK1/2, AKT or PARylation in EOC western blotting analysis was performed

(Figure 25). From this experiment we found that olaparib, FK866 and the combination had no influence on AKT or ERK1/2 signalling after 24 hours treatment at selected doses. To our surprise, similar levels of AKT and ERK1/2 phosphorylation were observed in COV318 and OVCAR-8 cells. In previous experiments KRAS-mutant OVCAR-8 cells possessed significantly higher levels of pAKT and pERK1/2 compared to COV318 cells (data not shown). It was also confirmed that treatment with olaparib and/or FK866 could block or substantially reduce the level of PAR in both cell lines. Interestingly, OVCAR-8 cells had higher basal levels of PAR. γ H2AX levels were assessed, however at this time point no DNA damage could be detected (data not shown). Overall, these results suggest it is unlikely that inhibition of ERK1/2 or AKT signalling contribute towards synergy of combined olaparib/FK866 treatment, but treatment with FK866 alone is sufficient to block PARylation by reducing NAD^+ availability for PAR synthesis.

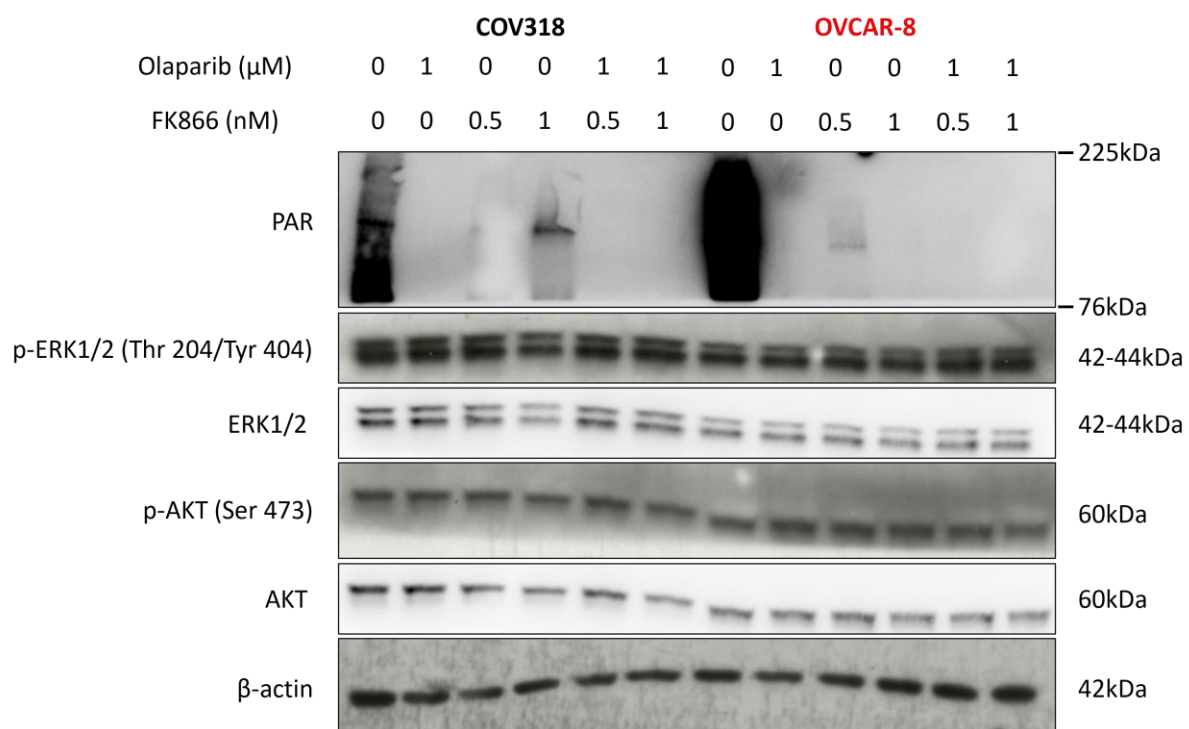


Figure 25. Olaparib and FK866 have no influence on AKT or ERK1/2 signalling but block PARylation

COV318 (KRAS-wild type, black) and OVCAR-8 (KRAS-mutant, red) cells were treated for 24 hours with vehicle, $1\mu\text{M}$ olaparib, FK866 (0.5 and 1nM) or the combination and lysates were extracted for western blotting analysis. Total and phosphor ERK1/2 and AKT proteins were measured, along with overall PARylation. β -actin was used as a loading control. Data represents one independent experiment.

2.4. Discussion

In recent years evidence is growing to support the use of a PARPi/NAMPTi combination in diseases such as TNBC⁹⁸, Ewing's sarcoma⁹⁹, and more recently in HGSOC models with intrinsic or acquired PARPi-R¹⁰⁰. However, it is hard to ignore the fact that NAMPTis have struggled in clinical trials (as monotherapies) due to dose limiting toxicity preventing the use of sufficiently high doses/optimal schedules to achieve clinical efficacy^{117,122,144}. Although preclinical evidence suggests combining NAMPTis with other agents (e.g., PARPis or chemotherapeutic agents) is likely to improve therapeutic opportunities, to widen the therapeutic window and maximise the therapeutic efficacy of NAMPTis there is a need to identify biomarkers that are predictive of NAMPTi response, to guide their use as monotherapies but also in combination with agents such as PARPis¹¹⁷. To date only *BRCA1/2* mutation status has been shown to be a possible biomarker to guide the combinations use⁹⁸. However, preliminary bioinformatic analysis (performed by Dr Adrian Benito-Mauricio) highlighted that EOC tumours harbouring *KRAS*-activating mutations are exquisitely sensitive to the NAMPTi FK866 (unpublished data). Cross-talk mechanisms exist between RAS-RAF-MEK-ERK and PI3K-AKT-mTOR signalling cascades, and pathway convergence can occur on factors such as c-Myc²⁷⁵, which are known to regulate *NAMPT* expression (and in some instances its dependence for NAD⁺ biosynthesis)^{116,147}. For example, the oncogenic *BRAF*^{V600E} mutation¹⁴⁷ and loss of *PTEN*¹⁸⁹ have both been shown to upregulate *NAMPT* expression. Considering this, we suspected activation of RAS/PI3K-signalling could create a "metabolic bottleneck" that could be exploited with a NAMPTi.

In each EOC subtype mutations in RAS/PI3K-signalling occur: HGSOC (*NF1* or *PTEN* loss); LGSOC (*BRAF*, *KRAS*, *NRAS* and *ERBB2* mutations); MOC (*KRAS* mutation or *ERBB2* amplification); CCOC and EnOC (*PI3KCA* and *PTEN* mutations)⁶. Furthermore, in the ARIEL2 (NCT01891344) clinical trial it was reported dysregulated RAS/PI3K signalling could cause resistance to rucaparib⁵³, highlighting the need identify combination strategies that could improve the efficacy of PARPis in this group of patients.

In this chapter, using a panel of ten human EOC cell lines I show that cells harbouring a *KRAS/PI3K* mutation are exquisitely sensitive to the NAMPTi FK866 (in 2D and 3D). This coincided with a greater reduction in NAD⁺ (0.5nM FK866) and ATP (5nM FK866) levels after 24- and 48-hours treatment, respectively. Similar timings of NAD⁺ and ATP depletion have

been observed previously¹²⁶. Interestingly, LC-MS/MS based metabolomics demonstrated that *KRAS*-mutant OVCAR-8 cells possess smaller β -NMN/ NAD^+ pools compared to *KRAS*-wild type COV318 cells, suggesting that oncogenic activation of *KRAS* signalling could increase the demand for β -NMN/ NAD^+ . Previous studies have demonstrated that *KRAS*-mutant tumours have lower intracellular NAD^+ concentrations^{182,183}. Furthermore, LC-MS/MS based metabolomics showed that FK866 treatment (1nM) led to a greater reduction in β -NMN/ NAD^+ levels after 24 hours treatment in OVCAR-8 cells, suggesting *KRAS*-mutations leads to dependence on NAMPT activity to maintain NAD^+ pools. Interestingly, overexpression of wild-type *KRAS* is not sufficient to upregulate *NAMPT* expression¹⁹⁰. However, given oncogenic mutations in the next step of the pathway (i.e., *BRAF*^{V600E}) upregulates *NAMPT* expression leading to it becoming the master regulator of NAD^+ synthesis via transcriptional regulation (e.g., via MYC, MAX, STAT3-5, HIF-1 α and NF- κ B^{116,147,184-186}), it is plausible oncogenic *KRAS*-mutations could have a similar effect. This does highlight the need to characterise how individual RAS/PI3K pathway alterations (e.g., *KRAS*^{G12C}, *PIK3CA*^{E545K}, *PTEN* loss or *NF1* loss) influence basal NAD^+ availability and the transcriptional regulation of *NAMPT* (e.g., using CRISPR-Cas9 gene editing or by overexpression mutations of interest), ideally in an ovarian background.

The expression of *NAMPT* and *NAPRT*, should be profiled in the panel of EOC cells, along with *NAPRT* inhibitor sensitivity, to better characterise whether they are reliant on the salvage and/or Preiss-Handler pathway¹¹⁶. Given *NAMPT*is have been shown to induce cell death (e.g., via apoptosis, necrosis or autophagy) by increasing intracellular ROS and inducing the loss of MMP¹³¹ it would be interesting to investigate if this more readily occurs in EOC cells with RAS/PI3K alterations. Particularly as *KRAS*-mutant tumours can become reliant on NADK activity (NAD^+ -dependent enzyme) for survival, as its loss reduces their capacity to cope with oxidative stress^{180,181}. More comprehensive metabolic profiling should be undertaken using plate-based assays (e.g., ECAR and OCR assays could assess mitochondrial and glycolytic activity, respectively)^{127,129} as ovarian tumours lacking *BRCA1/2* mutations are reported to exhibit *NAMPT*-dependent glycolysis¹²⁷. But also, through more comprehensive global metabolomics. However, it should be noted that global metabolomics studies have been conducted with *NAMPT*is in diseases such as ovarian cancer^{112,135-138}. Finally, basal AKT and

ERK1/2 phosphorylation should be profiled in the EOC panel to investigate whether activation of either signalling axis correlates with FK866-sensitivity.

Another important finding is that the combination of a NAMPTi (FK866) and PARPi (olaparib) is synergistic at specific doses in EOC cells (excluding COV318 cells) in 2D, in agreement with recently published evidence that NAMPTis could sensitise intrinsically PARPi-R HGSOc cells to PARPis¹⁰⁰. However, in 3D culture RAS/PI3K-mutation status appeared to be a more important determinant of sensitivity to the combination as A2780, HEY-A8 and OVCAR-8 cells remained exquisitely sensitive to the combination, unlike COV318, KURAMOCHI and OVSAHO cells. It is well established that cells cultured in 3D can display different drug responses due to alterations in their physical (e.g., arrangement of surface receptors and transporters) and physiological properties (e.g., differences in cell stage, metabolism, or pH and oxygen levels throughout a spheroid)²⁷⁴. This highlights the importance of testing drugs in 3D culture where possible. Importantly synergy/potentiation can be rescued by supplementing media with the NAD⁺ precursor β -NMN, as shown previously⁹⁹. However, in A2780 cells it was not possible to completely rescue cells despite supplementing media with 250 μ M of β -NMN. This could suggest that inhibiting the catalytic activity of NAMPT contributes towards the synergistic interaction beyond the depletion of NAD⁺ (e.g., eNAMPT has cytokine-like functions^{122,276}) or that this dose of β -NMN was not sufficient to replenish β -NMN/NAD⁺ pools in A2780 cells. Since NAD⁺ assays were not performed at the endpoint of rescue assays it is unclear whether the NAD⁺ pool is completely rescued over the course of the assay. Alternatively, β -NMN could be unstable at 37°C. However, this appears to be an unlikely explanation as a complete rescue could be observed in the other cell lines. Furthermore, albeit at a lower temperature (RT) and not in cells, β -NMN has been reported as stable at RT over the course of 2 weeks²⁷⁷.

Low doses of FK866 (<1nM) and olaparib (1 μ M) could not upregulate caspase 3/7 activity in COV318, KURAMOCHI or OVCAR-8 cells as monotherapies. However, in combination significant upregulation of caspase 3/7 activity could be observed in the *BRCA2*- and *KRAS*-mutant KURAMOCHI and OVCAR-8 cell lines, respectively. This confirms the combination upregulates apoptosis, as has previously been observed⁹⁸⁻¹⁰⁰, and again supports the use of the combination in a *KRAS*-mutant EOC background. Importantly, using LC-MS/MS based metabolomics we confirmed that inhibiting PARP-activity (prevents NAD⁺ consumption) does not have a greater impact on the NAD⁺ pool than inhibiting NAMPT, as shown previously⁹⁹.

Furthermore, we show treatment with 0.5-1nM of FK866 alone or in combination with 1 μ M of olaparib could block PARylation. Unfortunately, we did not quantify levels of PAR (e.g., using an ELISA based assay), but one would expect by limiting the availability of NAD⁺, combined treatment with a NAMPTi and PARPi should decrease PARylation and/or MARYlation below what can be achieved with a PARPi alone, as demonstrated by Heske and colleagues⁹⁹. This does highlight the need to quantitatively assess the influence of the combination on the level of PARP-trapping since further reductions in PARylation due to NAD⁺ depletion could lead to greater trapping. Interestingly, an ATR-FTIR spectroscopic approach that measures the dissociation kinetics of PARP-1 from biotinylated DNA strand break-mimicking oligonucleotides demonstrated that NAD⁺ levels correlate with PARP-1/DNA dissociation kinetics²⁷⁸.

Given PARP activity²⁷⁹ and NAD⁺ levels¹²⁸ are reported to directly regulate RAS/PI3K-signalling we were surprised that changes in ERK1/2 or AKT phosphorylation were not observed following treatment with olaparib and/or FK866. For example, FK866 treatment has previously been shown to inhibit the phosphorylation of ERK1/2, mTOR, p70S6K (after 24 hours), but also AKT (after 48 hours)¹²⁸. This could suggest that the combination does not interfere with the RAS/PI3K-signalling cascades to mediate its cytotoxic effects. However, it cannot be completely ruled out as additional timepoints/doses were not tested, and this experiment was not replicated. Furthermore, we did not detect γ H2AX after 24 hours treatment with the selected doses of olaparib/FK866. In previous studies higher doses of NAMPTis were used (5-10nM) to demonstrate that NAD⁺ depletion exacerbates γ H2AX accumulation after 24-48 hours combination treatment⁹⁸⁻¹⁰⁰. However, since OVCAR-8 cells are so sensitive to FK866 doses >1nM were typically not assessed. Despite this, γ H2AX levels should be assessed in the EOC panel (e.g., by WB or IF) using appropriate timepoints and doses of FK866, to investigate whether the combination leads to earlier/greater induction of γ H2AX in KRAS/PI3K-mutant EOC cells. In addition, given studies in ovarian cancer cell lines have demonstrated that NAD⁺ availability can regulate *BRCA1* gene expression through an NAD⁺-dependent 'metabolic switch' (i.e., an increased NAD⁺/NADH ratio leads to loss of CtBP at the *BRCA1* promoter increasing *BRCA1* transcription, and vice versa^{133,134}), it could be interesting to investigate whether treatment with the combination reduces *BRCA1* levels to contribute to any potential increases in DNA damage.

To date the influence of the combination on cell cycle progression has not been assessed⁹⁸⁻¹⁰⁰. However, given NAMPTi^{129,185} and PARPi^{280,281} have been shown to induce marked arrest in the G2/M phase of the cell cycle individually, it is likely the combination could have a similar effect. Markers of replication stress could also be assessed (e.g., R-loop formation²⁸²) along with replication fork progression (e.g., DNA fibre assays³⁷). In each context it would be important to investigate whether the combination has a greater/earlier effect in *KRAS/PI3K*-mutant EOC cells.

Further mechanistic insight could be gained by performing global assays (e.g., RNA-seq or proteomics) in a *KRAS/PI3K*-mutant EOC cell line. To date a RPMA (reverse phase protein microarray) based proteomics and phosphoproteomics examination of a NAMPTi/PARPi combination has been performed in Ewing sarcoma⁹⁹. In addition to some expected markers (e.g., γ H2AX, cleaved caspases-3 and -7), cleavage of PARP, and increased phosphorylation of the stress-activated protein kinases SAPK/JNK and p38 MAPK was observed. In all cell lines tested we did not observe cleavage of PARP-1 by western blotting (data not shown). It would be interesting to investigate whether we could observe an increase in phosphorylation of stress activated protein kinases, as their prolonged activation is known to correlate with apoptotic cell death⁹⁹. In contradiction to this, transient activation of these protein kinases is associated with cell survival, and FK866 has previously been shown to downregulate pp38MAPK and pSAPK/JNK levels¹²⁸. In addition, although different NAMPTi/PARPi combinations have been tested in the literature⁹⁸⁻¹⁰⁰, it would be important for us to investigate whether different PARPi (e.g., with differing trapping activity) and NAMPTi (e.g., OT-82 due to its favourable toxicology profile^{129,154,155}) combinations are synergistic, to confirm this is a class effect rather than a drug-specific effect. One could also argue it will be necessary to test the combinations efficacy in a suitable PDX model²⁸³.

To conclude, findings from this chapter revealed an unreported association between *KRAS/PI3K*-mutations and sensitivity to FK866 alone or in combination with olaparib. This suggests that combining FK866 with olaparib may benefit a wide range of EOC patients. However, since cell lines possess multiple mutations, isogenic models must be used to assess the combinations efficacy following the activation of RAS- or PI3K-signalling cascades and this will be investigated further in Chapter 3.

CHAPTER 3

Evaluating the combined effect of FK866 and olaparib in isogenic models of HGSOC

Chapter 3 – Evaluating the combined effect of FK866 and olaparib in isogenic models of HGSOC

3.1. Background

As discussed in **section 1.7.2.** transplantable murine models of HGSOC were previously developed by Walton and colleagues^{268,269} using CRISPR-Cas9 gene editing to introduce mutations that commonly occur in the disease. The following mutants were included in our study: ID8 *Trp53*^{-/-}, *Trp53*^{-/-}; *Nf1*^{-/-}, *Trp53*^{-/-}; *Pten*^{-/-}, *Trp53*^{-/-}; *Brca1*^{-/-} and *Trp53*^{-/-}; *Brca2*^{-/-}. The *Nf1* and *Pten* knockout models were of particular interest because loss of these tumour suppressor genes has been shown to upregulate RAS and PI3K signalling, respectively²⁶⁸. It should also be noted that loss of *Pten* can impede homologous recombination repair and sensitises cells to PARPis^{284,285}. Importantly, these isogenic lines can be injected into immunocompetent mice, unlike traditional xenografts, and model peritoneal metastasis and ascites development which is observed in EOC²⁶³. As such, ID8 models were used to investigate whether loss of *Nf1* or *Pten* increases NAD⁺ demand and sensitivity to the olaparib/FK866 combination.

3.2. Materials & Methods

3.2.1. Cell culture

The ID8 panel of cells were a gift from Professor Iain McNeish^{268,269} that included: *Trp53*^{-/-}, *Trp53*^{-/-}; *Nf1*^{-/-}, *Trp53*^{-/-}; *Pten*^{-/-}, *Trp53*^{-/-}; *Brca1*^{-/-} and *Trp53*^{-/-}; *Brca2*^{-/-} clones. For *in vivo* studies ID8 cells were cultured in phenol red-free DMEM supplemented with 25mM glucose, 4% FBS, 2mM L-glutamine, 100 units/mL penicillin, 100µg/mL streptomycin, 1mM sodium pyruvate (ThermoFisher, 11360070) and 1x Insulin-Transferrin-Selenium (ThermoFisher, 41400045). For *in vitro* studies ID8 cells were adapted to low glucose (5.6mM) over the course of three passages. After adaptation, cells were cultured in phenol red-free DMEM supplemented with 5.6mM glucose, 10% foetal bovine serum, 2mM L-glutamine, 100 units/mL penicillin, 100µg/mL streptomycin and 1mM sodium pyruvate.

For SRB and caspase assays ID8s were seeded at a density of 500 cells/well. In these experiments, cells were treated for 72- and 48-hours, respectively. For the NAD/NADH-Glo™ Assay, ID8 cells were seeded at a density of 2000 cells/well. Excluding mentioned differences

in seeding densities and timepoints, methods were consistent with those described in **section 2.3**.

3.2.2. Immunofluorescence

For immunofluorescence assays, ID8 *Trp53*^{-/-} (2000 cells/well) and *Trp53*^{-/-}; *Pten*^{-/-} (1000 cells/well) were treated for 48-hours with vehicle, cisplatin (Teva, EAS664), FK866 and/or olaparib. At endpoint cells were washed with 100µL of PBS, before being fixed for 15 minutes (RT) with 50µL of 4% PFA (ThermoFisher, 11586711) diluted in PBS. Plates were washed with 100µL of PBS and permeabilised for 20 minutes with 50µL of 0.1% Triton-X100 (Sigma-Aldrich, T8787) diluted in PBS. After two PBS washes, a blocking step was performed (1% BSA and 2% FBS in PBS) for 30 minutes at RT. 1% BSA (in PBS) alone or with the γH2AX (1:500) antibody (**Table 5**) was added to appropriate wells and plates were incubated overnight at 4°C. The next day, wells were washed three times with PBS and one time with 1X TBS-T. Next, cells were incubated in the dark (RT) for 2 hours with 50µL of 1% BSA (in PBS) staining solution containing: Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (1:500, Invitrogen, #A11001) and 1 µg/mL DAPI (Sigma-Aldrich, #9542). Wells were then washed three times with PBS and one time with 1X TBS-T, before adding 200µL of PBS to each well. Prior to imaging AbsorbMax™ film (Sigma-Aldrich, Z722537) was placed on top of 96-well plates to minimise background fluorescence. Images were then taken on an IN Cell™ Analyser 1000 (GE Healthcare Life Sciences) and the accompanying INCell analyser 3.5 software. A 20x objective lens was used and 25 fields were captured in each well.

CellProfiler V4.0.6 (Broad Institute of MIT and Harvard) was used to analyse γH2AX foci per nuclei, as described by Dr Yuewei Xu²⁶⁴. Nuclei were detected using the following parameters: typical diameter = 25-100 pixel units, threshold strategy = global, thresholding method = Otsu (Two classes, 1.318 smoothing scale, 10 smoothing filter). γH2AX foci were detected using the following parameters: typical diameter 3-15 pixel units, threshold strategy = global, thresholding method = Robust Background (1.3488 smoothing scale, 2 smoothing filter). Foci per nuclei were automatically detected by Cell Profiler. Secondary only control wells were included to ensure false positives weren't being detected and cisplatin treatment was used as a positive control. Graphs were generated using the GraphPad Prism 10.0.0 software.

3.2.3. Subcellular fractionation of cell lysates (PARP trapping assay)

1.5x10⁶ ID8 *Trp53*^{-/-}; *Pten*^{-/-} cells were seeded into T150 flasks. The following day, cells were treated with vehicle, olaparib and/or FK866. After 24 hours treatment, cell lysates were extracted using a method previously described by Murai and colleagues³³. On the day of extraction, cells were washed with PBS and detached from flasks using TrypLE™ Express (ThermoFisher, 12604013). Trypsin was neutralised with media and cells were centrifuged for 5 minutes at 500 x g (RT). Media was aspirated and cells were washed with ice-cold PBS. The cell suspension was then transferred into 1.5mL Eppendorf tubes and centrifuged at 500 x g for 3 minutes (4°C). Prior to this, reagents in the Subcellular Protein Fractionation Kit (ThermoFisher, 78840) were thawed and prepared following manufactures instructions, as described in **Table 6**. Each reagent was supplemented with a Halt™ Protease Inhibitor Cocktail. After centrifugation, the Subcellular Protein Fractionation Kits protocol was followed, using volumes required for a packed cell volume of 60μL (**Table 6**) to extract soluble nuclear extract and chromatin-bound nuclear extract fractions. Each fraction was quantified using the Pierce™ BCA Protein Assay (See section 2.3.9.). Western blotting was then performed with some minor adjustments from **section 2.3.10**. In brief, SurePAGE™, Bis-Tris gels were used (GenScript, M00657) with the Tris-MOPS-SDS Running buffer (GenScript, M00138). For this assay, PARP-1, α-Tubulin, Histone H3 and DNA Topoisomerase I antibodies were used (**Table 5**). DNA Topoisomerase I was imaged after stripping the PARP-1 membrane section with Restore™ Western Blot Stripping Buffer (ThermoFisher, 21059), according to manufactures instructions.

Table 6. Reagent volumes for different packed cell volumes

Packed cell volume (μL)	CEB (μL)	MEB (μL)	NEB (μL)	NEB (μL) + CaCl ₂ , MNase (μL)	PEB (μL)
10	100	100	50	50	50
60	600	600	300	300	300

For HeLa cells, 2x10⁶ cells is equivalent to 20μL packed cell volume, in this experiment 6x10⁶ cells were used. MNase = Micrococcal Nuclease, CEB = Cytoplasmic Extraction Buffer, MEB = Membrane Extraction Buffer, NEB = Nuclear Extraction Buffer, CaCl₂ = Calcium Chloride and PEB = Pellet Extraction Buffer.

3.2.4. In vivo studies using the ID8 TP53^{-/-}; PTEN^{-/-} murine HGSOc model

In vivo experiments complied with UK welfare guidelines and were conducted under personal and project license authority in dedicated animal facilities. ID8 *Trp53*^{-/-}; *Pten*^{-/-} cells were cultured as previously described^{268,269}. After a 7-day acclimatisation period ear clipping was undertaken (so mice could be identified) and 5x10⁶ cells were injected into 6–8-week-old

female C57BL/6 mice by intraperitoneal (I.P.) injection. Mice were monitored daily by observation of weight and overall health. A vehicle solution was prepared in a tissue culture hood by mixing 3.5% DMSO and 6.5% Tween 80 (Sigma-Aldrich, P4780) in 0.9% saline (NaCl in dH₂O; Sigma-Aldrich, S9888), after mixing the solution it was filtered using a vacuum pump. The vehicle solution was used to prepare a 50mg/kg olaparib drug suspension and a 10mg/kg FK866 drug suspension. Each suspension was vigorously vortexed and sonicated to break down particulates, and drug solutions were inspected using a haemocytometer to ensure large particles didn't remain. Drugs were stored as aliquots at -20°C and thoroughly centrifuged on the day of use.

After two weeks, omental tumours had formed and mice were treated with vehicle, olaparib and/or FK866. Separate dosing strategies were used for the endpoint (toxicology) and the survival experiments. For the endpoint experiment, mice received 10 daily I.P. injections over the course of two weeks. Injections were administered on days (D) 14-18, on D19-20 mice had a drug holiday and on D21-25 the final injections were administered. The day after the final injection (D26) mice were culled by terminal anaesthesia and cardiac puncture. Organs (omental tumour, spleen and liver) were collected, weighed and snap frozen. Ascites were collected, centrifuged at 500 x g (4°C) and the supernatant was snap frozen. The pellet was then resuspended in freezing medium (10% DMSO in FBS) and slowly frozen at -80°C. When ascites weren't present a peritoneal lavage was performed with 5mL of sterile FACS buffer (50mL of 0.1M EDTA in PBS). Terminal blood was collected in syringes pre-coated with EDTA, transferred to Eppendorf tubes on ice and centrifuged at 500 x g for 5 minutes (4°C). The blood plasma was transferred to a new tube, centrifuged at 16,800 x g for 3 minutes (4°C) and the supernatant was snap frozen. The pellet was then resuspended in freezing medium and slowly frozen. For the survival experiment, mice received 8 daily I.P. injections over the course of two weeks. Injections were administered on D14-17, on D18-20 mice had a drug holiday and on D21-24 the final injections were administered. During the treatment period, mice received wetted food or HydroGel® (Clear H₂O, 70-01-1062) to prevent dehydration. On D28 tail or saphenous bleeds were performed. Blood was stored on ice and transported to the Royal Veterinary College Pathology & Diagnostic Laboratory to perform red blood cell (RBC) counts. Mice were culled upon reaching a human endpoint by cervical dislocation. Organs were collected and weighed, and ascites were collected.

3.2.5. Omental tumour metabolite extraction and Targeted HILIC LC-MS/MS analysis

Omental tumours were processed for LC-MS/MS assays with PhD student Yitao Xu. Roughly 10-15mg of omental tumour was weighed and placed into clean homogeniser tubes containing 0.8mL ice cold 80% Methanol and 0.1mm Precellys® Glass Beads (ThermoFisher, 12927835). 10µL of HILIC standards (**Table 8**) were spiked into each tube. Tissue was homogenised using the Precellys Evolution tissue homogeniser (Bertin) with an activated Cryolys Evolution cooling system (18,000 x g, 4 x 20 sec cycle with a 30 sec pause, two cycles). Tubes were centrifuged at 21,000 x g for 10 minutes (4°C). Supernatants were collected in Eppendorf tubes. An additional 800µL of 80% Methanol was added, previous steps were repeated, and extracted supernatants for each sample were pooled. Samples were then dried under nitrogen flow and stored at -80°C. A processing blank was also included during the extraction. Samples were then subjected to dual-phase extraction. 300µL of chloroform (ThermoFisher, 390760025)/methanol (2:1) was added to each sample, and tubes were vortexed. Tubes were vortexed and subsequently centrifuged at 21,000 x g for 10 minutes (4°C). Each samples aqueous (upper) and organic (lower) layers were transferred into separate LC-MS vials.

On the day of LC-MS/MS assays omental tumour (aqueous phase) samples were re-suspended in 100µL of H₂O and vortexed for 30 seconds and stored in an autosampler at 10°C. LC-MS/MS assays were performed using an Agilent 1290 Infinity LC system (Agilent Technologies) and a 4000 QTRAP triple quadrupole instrument (AB SCIEX) coupled to an ESI source (Turbo V).

Acquity UPLC® BEH Amide 1.7µm 2.1 x 150mm Column (Waters, 186004802) was used for HILIC Liquid Chromatography, with a column temperature of 50°C. To increase the columns lifetime a Acquity UPLC® BEH Amide 1.7µm VanGuard™ Pre-Column 2.1 x 5mm (Waters, 186004799) was used. Elution was performed at a flow rate of 500µL/min using the gradient described in **Table 8** and 5µL of sample was used per injection. Needle wash steps were performed with Acetonitrile:H₂O (1:1, v/v). For HILIC positive mode, mobile phase A was comprised of ACN and 0.1% formic acid (v/v). Mobile phase B contained H₂O, 20mM ammonium formate (Supelco, 70221) and 0.1% FA. For HILIC negative mode, mobile phase A comprised of ACN and 10mM ammonium hydroxide (Honeywell, 44273). Mobile phase B contained H₂O, 20mM ammonium acetate (Supelco, 73594) and 10mM ammonium hydroxide.

An NAD⁺ standard curve (0.025-10µg/mL) was included in the runs for quantification. Quality control pooled samples were used in all experiments, and metabolites out of a QC accuracy range of 70–130% were removed from subsequent datasets. Peak integration was performed using the SCIEX OS Software version 3.0. Data analysis was performed using the OptiMSR R package (Developed by Dr Cristina Balcells and PhD student Yitao Xu). MetaboAnalyst 5.0²⁸⁶ was used to normalise samples blank-corrected relative intensities to the total sum of metabolites and then data was auto-scaled (**Appendix Figure 13**). A heatmap was produced using Two-way hierarchical clustering. Principal component analysis was performed. Along with a one-way ANOVA test to identify metabolites that significantly changed (False discovery rate of 0.05 was used) in different omental tumours.

Table 7. HILIC UPLC gradient elution in positive and negative mode

Time (min)	Flow rate (µL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	500	95.0	5.0
1.00	500	95.0	5.0
8.00	500	50.0	50.0
9.00	500	50.0	50.0
9.10	500	95.0	5.0
15.00	500	95.0	5.0

Table 8. Preparation of labelled HILIC standard mix solution for omental tumour analysis

Labelled Standard	Concentration (µg/mL)	Source	Identifier
L-alanine-2,3,3,3-d ₄	30	Sigma	485845
L-isoleucine- ¹³ C ₆ , ¹⁵ N	5	Sigma	608092
Citric-2,2,4,4,-d ₄ acid	30	Sigma	485438
Glycine-1- ¹³ C, 2,2-d ₂	50	Sigma	608076
L-glutamine ¹³ C ₅	40	Sigma	604690
L-aspartic acid- ¹³ C ₄	5	Sigma	604852
L-glutamic acid- ¹³ C ₅	20	Sigma	604860
L-valine- ¹³ C ₅	20	Sigma	758159
Fumaric acid- ¹³ C ₄	10	Sigma	606014
Succinic acid- ¹³ C ₄	20	Sigma	491983
Sodium pyruvate-3- ¹³ C, 3,3,3-d ₃	30	Sigma	608483
L-serine- ¹³ C ₃ , ¹⁵ N, 2,3,3-d ₃	10	Sigma	749877
L-malic acid- ¹³ C ₄	5	Sigma	750484
Lactic acid ¹³ C ₃	50	Sigma	746258
Uracil ¹³ C, ¹⁵ N ₂	10	Sigma	608459
Uridine 5,6-d ₂	10	Santa Cruz Biotechnology	sc-213138
TMP ¹³ C ₁₀ , ¹⁵ N ₂	10	Sigma	648590
Carnitine d ₃	10	Sigma	617466
SAH d ₄	5	Santa Cruz Biotechnology	sc-296290

SAM ¹³ C ₅	5	Sigma	798231
tyrosine d ₄	10	Sigma	489808
Lysine HCl d ₄	10	Sigma	616192
nicotinamide ¹³ C ₆	5	Supelco	V-034
Creatinine d ₃	10	Supelco	C-165
nicotinate d ₃	5	Sigma	489077

Table 9. MRM parameters for the HILIC-positive UPLC-MS/MS assay

Metabolite	Q1	Q3	RT (min)	DP	CE
acetylcarnitine	204	85	4.7	56	29
acetylorntithine	175	115	5.8	40	20
Adenine	136	119	3.3	65	30
Alanine	90	44	5.7	40	20
arginine	175	70	7.2	55	30
Asparagine	133	74	6.2	35	23
Betaine	118	58	4.8	60	35
Carnitine	163	103	5.4	55	25
Carnitine, d3	165	103	5.4	55	25
Carnosine	227	110	7.4	50	30
Choline	104	60	3.1	55	25
citrulline	176	159	6.3	35	15
Creatine	132	90	5.7	45	19
cysteine	122	76	5.8	25	21
Cytidine	244	112	4.3	41	17
Cytosine	112.01	95	4	60	30
dimethylarginine (ADMA) 2HCl	203	70	6.7	50	35
Glutamine	147.01	84	6.1	40	25
Glycine	76	30	5.9	45	25
Glycine-1-13C,2,2-D2	79	32	5.9	35	20
Guanine	152	135	4.3	60	27
guanosine	284	152	4.7	40	20
Histamine	112	95	6.8	45	20
Histidine	156	110	7.4	45	21
Isoleucine	132	69	4.8	35	27
Kynurenine	209	146	4.7	40	30
L-alanine-2,3,3,3-d4	94	48	5.7	35	15
Leucine	132	86	4.7	40	15
L-glutamine 13C5	152	88	6.1	30	25
L-isoleucine-13C6,15N	139	92	4.8	40	15
l-Ornithine HCl	133	70	7.3	35	25
L-serine-13C3, 15N,2,3,3-D3	113	66	6.1	30	15
L-valine-13C5	123	76	5.1	40	17
Lysine	147	84	7.3	40	25
lysine HCl d4	151	88	7.3	40	25
Methionine	150	61	5	40	35
Methionine sulfoxide	166	74	6.1	40	23

MTA	298.3	136.1	3	62	25
NAAD	665.4	136.2	3	78	55
NAD1	664	136	20	70	70
NAD2	664	97	20	70	85
NAMN	336.5	124.1	3	50	18
niacinamide	123	79	1.3	66	43
nicotinamide 13C6	129	85	1.3	66	43
nitro-tyrosine	227	181	4.6	50	20
NMN	335	123	20	100	20
NR	255.2	123.2	3	45	13
Phenylalanine	166	120	4.6	45	19
phenylethylamine HCl	122	105	3.7	40	20
proline	116	70	5.1	45	23
Putrescine	89.1	72.1	3	35	15
s-adenosyl homocysteine	385	134	6.3	51	27
s-adenosyl methionine iodide	399	250	7.6	51	23
SAH d4	389	136	6.3	51	27
serine	106	60	6.1	35	15
Serotonin HCl	177	160	4.3	35	20
thiamine HCl	265	122	5	46	19
Threonine	120	74	5.8	35	17
thymine	127	110	1.6	60	23
TMAO	76	58	4.3	56	27
trans 4-OH proline	132.01	86	5.7	45	20
Tryptophan	205	146	4.7	40	27
tyrosine	182	136	5.2	35	20
tyrosine d4	186	140	5.2	35	20
Valine	118	72	5.1	40	15

Table 10. MRM parameters for the HILIC-negative UPLC-MS/MS assay

Metabolite	Q1	Q3	RT (min)	DP	CE
4-Deoxyl-erythronic acid sodium salt hydrate	119	57	5.86	-35	-22
aKG	145	101	5.16	-31	-15
AMP	346	79	5.61	-55	-50
Aspartic acid	132	88	5.44	-30	-20
ATP	506	159	6.78	-70	-50
Citric acid	191	87	6.94	-40	-25
Citric-2,2,4,4,-d4 acid	195	88	6.95	-30	-24
CMP	322	139	5.97	-45	-32
CTP	482	384	6.07	-55	-35
D-(–)-3-Phosphoglyceric acid 2Na	185	97	6.11	-40	-22
dADP	410	79	4.98	-60	-80
dAMP	330	79	5.94	-55	-70
dATP	490	159	6.32	-70	-50
dCTP	466	159	6.5	-70	-50

D-Fructose 1,6-biphosphate 3Na hydrate	339	97	5.77	-45	-30
d-glu-6-H ₂ PO ₃ disodium salt hydrate	259	199	6.24	-35	-20
dGTP	506.01	159	6.85	-60	-50
Dihydroxyacetone phosphate dilithium salt	169	79	5.86	-25	-36
DL-isocitric acid trisodium hydrate	191	85	6.94	-50	-30
dUMP	307	195	5.76	-50	-24
dUTP	467	159	6.5	-55	-45
GDP	442	159	6.67	-55	-50
glucuronic acid	193	113	4.88	-35	-20
Glutamate	146	102	5.34	-40	-20
glycerol 3 phosphate lithium salt	171	79	5.66	-40	-30
GMP	362	79	6.08	-70	-60
GTP	522	424	7.38	-60	-35
IDP	427	159	6.15	-55	-35
IMP	347	79	5.84	-55	-60
ITP	507	79	6.8	-75	-90
L-aspartic acid-13C ₄	136	91	5.44	-35	-18
L-glutamic acid-13C ₅	151	106	5.34	-45	-20
L-glutathione oxidised	611	306	6.51	-40	-34
L-glutathione reduced	306	143	6.51	-40	-26
L-malic acid-13C ₄	137	74	5.36	-30	-22
malic acid	133	71	5.36	-30	-15
NAD	662	540	5.8	-40	-25
NADH ₃₉₇	664.2	397	5	-100	-45
NADH _{par}	664.2	664.2	5.33	-100	-10
NADP	742	408	6.48	-45	-46
NADPH	744	408	5.92	-100	-50
Oxaloacetic acid	131	87	5.13	-25	-18
Phosphoenolpyruvate monosodium hydrate	167	79	6.07	-35	-16
Phospho-L-serine	184	97	6.2	-30	-20
TMP 13C ₁₀ , 15N ₂	333	79	5.8	-55	-60
TTP	481	159	6.32	-60	-35
UDP	403	159	5.89	-55	-40
UDP-N-acetyl glucosamine sodium salt	606	159	5.65	-80	-70
UMP	323	79	5.75	-50	-60
UTP	483	159	6.97	-80	-50

3.2.6. Omental tumour lysate extraction and western blotting

15-25mg of omental tumour tissue was weighed and transferred into homogeniser tubes containing 0.1mm Precellys® Glass Beads and 100µL of RIPA buffer (supplemented with 2X Halt™ Protease and Phosphatase Inhibitor Cocktail) per 10mg of tissue. Tissues were broken down using the same method described in **section 3.3.5**. After homogenisation samples were vortexed for 30 seconds and placed on ice for 5 minutes (repeated twice more). Tubes were

then centrifuged at 20,000 x g for 20 minutes (at 4°C). Supernatants were collected without taking up any glass beads. A protein assay (see **section 2.3.9.**) and western blotting (see **section 2.3.10.** and **Table 5**) was performed as described in previous sections.

3.3. Results

3.3.1. *Pten* and *Brca1/2* loss sensitises ID8 cells to FK866

In the following experiments, a panel of isogenic ID8 cell lines^{268,269} were used to investigate whether genetic mutations that commonly occur in HGSOc sensitise cells to the NAMPTi FK866. This included the loss of PTEN, which leads to elevated PI3K/Akt pathway activation. The loss of NF1, which leads to RAS activation. ID8 cells harbouring a BRCA1/2 knock-out (KO) were also included, since patients with these mutations are typically treated with PARPis. Before assessing FK866 sensitivity, the influence of NF1 and PTEN KO on ERK1/2 and AKT signalling was confirmed by western blotting (**Figure 26A**, experiment performed by PhD student Yitao Xu). This demonstrated that PTEN KO upregulated AKT phosphorylation, without upregulating ERK1/2 phosphorylation. By comparison, NF1 KO upregulated ERK1/2 phosphorylation, without upregulating AKT phosphorylation.

Interestingly, ID8 *Trp53*^{-/-}; *Nf1*^{-/-} cells (~26.9nM) were more resistant to FK866 when compared to the *Trp53*^{-/-} (~21.9nM) model (**Figure 26B**). Whereas *Trp53*^{-/-}; *Pten*^{-/-} cells (~16.8nM) were more sensitive, suggesting activation of AKT rather than ERK1/2 is a determinant of FK866 sensitivity. The *Trp53*^{-/-}; *Brca1*^{-/-} and *Trp53*^{-/-}; *Brca2*^{-/-} models were the most susceptible to FK866 treatment with IC₅₀ values of 11.2nM and 9.1nM, respectively.

To investigate whether increased FK866 sensitivity was due to greater NAD⁺ pool depletion, as was observed in the human EOC cell line panel (**section 2.4**), NAD⁺ assays were performed following 24 hours treatment with vehicle or FK866 (**Figure 26C**, experiment performed by PhD student Yitao Xu). From this it was revealed that FK866 could significantly deplete NAD⁺ pools in all models tested, but the loss of *Nf1* or *Pten* did not increase the level of NAD⁺ depletion. Taken together, these data suggest that activation of AKT or ERK1/2 signalling alone is not sufficient to become dependent on NAMPT for NAD⁺ pool maintenance.

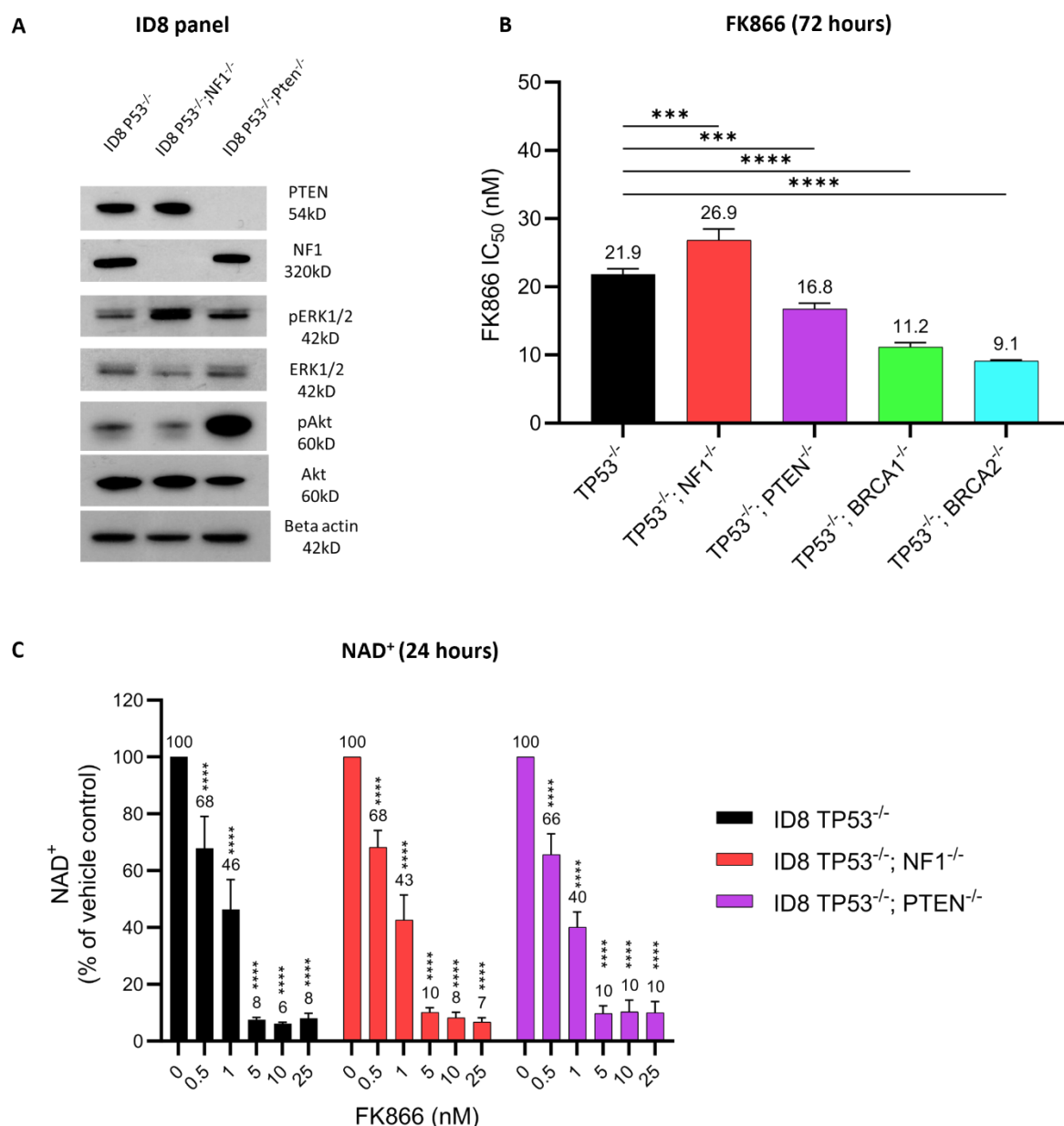


Figure 26. Influence of FK866 on ID8 cell lines

(A) Basal AKT and ERK1/2 signalling in ID8 cell lines. β -actin was used as a loading control. Data represents one independent experiment. Cell lysis and western blotting were performed by PhD Student Yitao Xu. **(B)** ID8 cell lines FK866 sensitivity after 72 hours treatment. Cell proliferation was measured using the SRB assay, for full dose response curves see **Appendix Figure 8**. For (b) data represents three independent experiments $\pm$ 95% confidence intervals. **(C)** ID8 cells were treated for 24 hours with vehicle (0.1% DMSO) or FK866 (0.5 to 25nM). NAD⁺ content was measured using the NAD/NADH-GloTM assay. Data was normalised to cell biomass (SRB assay). This experiment was carried out by PhD student Yitao Xu. Data represents three independent experiments $\pm$ SD. For (b-c) statistical significance between vehicle and monotherapy groups was determined using one-way ANOVA

followed by Dunnett's multiple comparisons test (*p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001).

3.3.2. FK866 synergises with olaparib in ID8 cells

The ID8 panel was treated with olaparib and FK866 to investigate whether loss of *Pten* or *Nf1* increased the benefit of the combination (**Figure 27 and Appendix Figure 9**). *Trp53*^{-/-} cells were most resistant to olaparib treatment, followed by: *Trp53*^{-/-}; *Nf1*^{-/-} > *Trp53*^{-/-}; *Pten*^{-/-} > *Trp53*^{-/-}; *Brca2*^{-/-} > *Trp53*^{-/-}; *Brca1*^{-/-}. In all cell lines tested, co-treatment with FK866 could reduce olaparib IC₅₀ values. Importantly, co-treatment in the *Trp53*^{-/-}, *Trp53*^{-/-}; *Nf1*^{-/-} and *Trp53*^{-/-}; *Pten*^{-/-} models could reduce IC₅₀ values to levels observed in olaparib treated *Trp53*^{-/-}; *Brca1/2*^{-/-} models. However, similar reductions in IC₅₀ values were achieved in the three BRCA-proficient models (**Appendix Figure 9**), despite the fact *Trp53*^{-/-}; *Pten*^{-/-} cells are intrinsically more sensitive to FK866 monotherapy treatment.

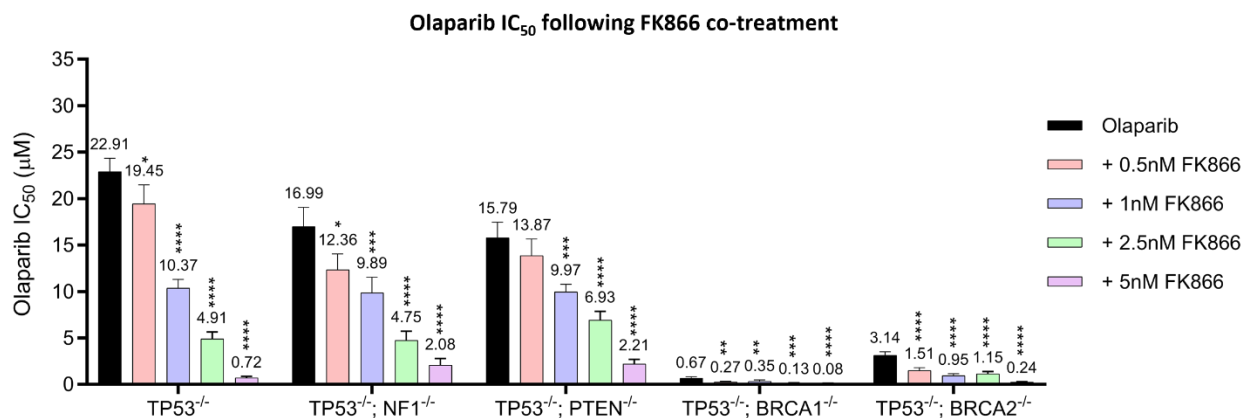


Figure 27. Influence of olaparib and FK866 on ID8 cell lines

ID8 cell lines were treated for 72 hours with olaparib and FK866. Cell proliferation was measured using the SRB assay, for full dose response curves and % change in IC₅₀ values see **Appendix Figure 9**. Data represents three independent experiments ± 95% confidence intervals. Statistical significance between groups was determined using one-way ANOVA followed by Dunnett's multiple comparisons test (*p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001).

To investigate whether the combination was synergistic in the ID8 panel, 6 x 6 matrix assays were performed (PhD student Yitao Xu, personal communication). In each cell line synergy (ΔBliss > 10) could be observed. However, in the *Trp53*^{-/-}; *Nf1*^{-/-} and *Trp53*^{-/-}; *Pten*^{-/-} models synergy could be achieved at a larger number of doses.

3.3.3. β -NMN partially rescues ID8 cells from olaparib and FK866

Next, we investigated whether β -NMN media supplementation could rescue ID8 cells treated with olaparib and FK866, as was observed in the EOC cell line panel (**Section 2.4.5.**). Since *Trp53*^{-/-}; *Brca1/2*^{-/-} models are intrinsically more sensitive to olaparib they received a lower dose (1 μ M) of the PARPi than the HR-proficient models (10 μ M). Excluding *Trp53*^{-/-}; *Brca1*^{-/-} cells, the selected dose of FK866 had very little impact on cell proliferation. In all cell lines tested the combination reduced cell proliferation to a greater extent than either monotherapy group. In the *Trp53*^{-/-}, *Trp53*^{-/-}; *Nf1*^{-/-} and *Trp53*^{-/-}; *Pten*^{-/-} models a significant partial rescue could be achieved (**Figure 28**). In the *Trp53*^{-/-}; *Brca1/2*^{-/-} models a partial rescue could be observed but this did not reach significance. Taken together, these data could suggest the selected dose of β -NMN was insufficient to completely protect ID8 cells from the combination, likely due to incomplete replenishment of β -NMN pools.

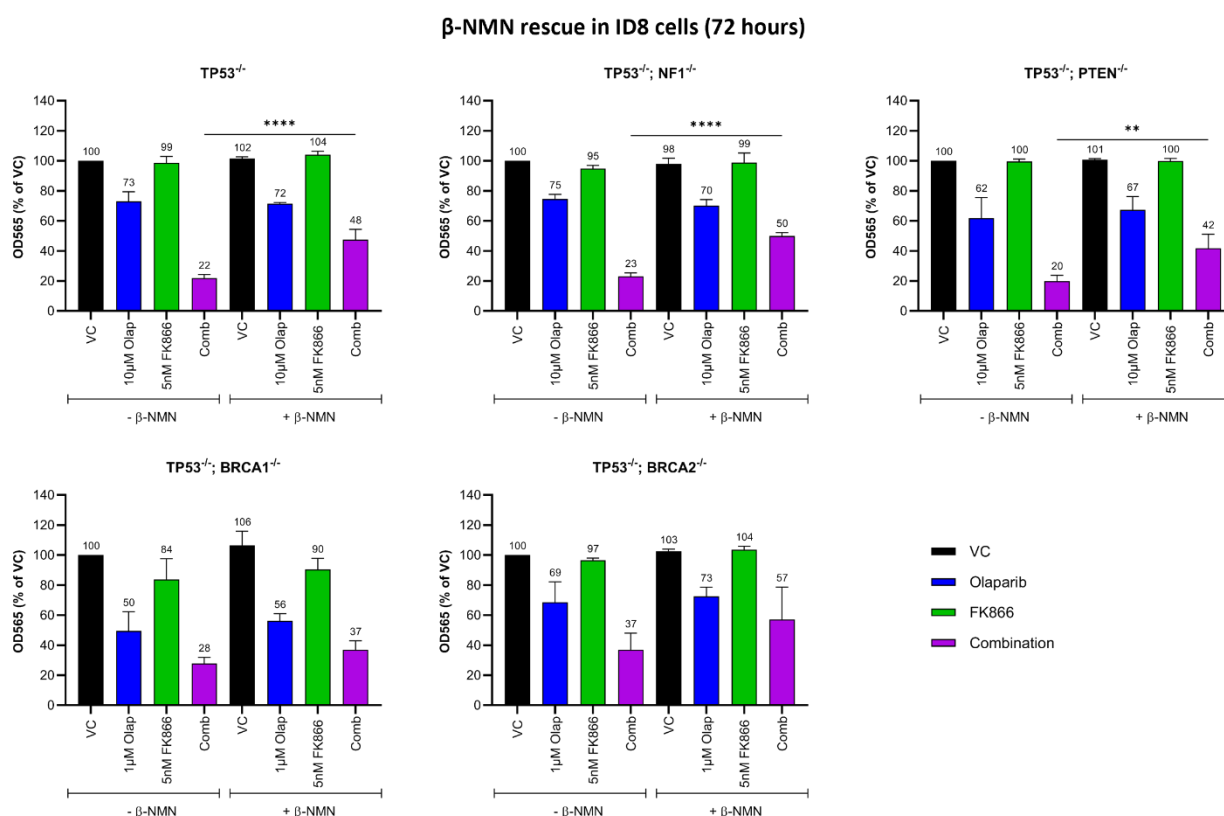


Figure 28. β -NMN media supplementation partially rescues olaparib and FK866 treated ID8 cells

ID8 cells were co-treated with olaparib (1 or 10 μ M) and FK866 (5nM) $\pm$ 250 μ M of β -NMN to investigate whether the NAD⁺ salvage pathway intermediate could promote survival. Cell proliferation was measured after 72 hours using the SRB assay. Data is the average of three independent experiments

± SD. Statistical significance was determined using one-way ANOVA followed by Šídák's multiple comparisons test ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3.3.4. Olaparib and FK866 significantly increases apoptosis in Pten-null cell lines

To further investigate the role of *Trp53*, *Nf1*, *Pten* and *Brca1/2* loss in sensitising cells to the combination, ID8 cells were treated with the combination and the induction of apoptosis was assessed (**Figure 29**). Excluding *Trp53*^{-/-} and *Trp53*^{-/-}; *Nf1*^{-/-} models, olaparib treatment was able to upregulate caspase 3/7 activity by >2 fold. In all models, FK866 monotherapy could not significantly upregulate apoptosis. In all cell lines tested, the combination significantly upregulated caspase 3/7 activity. Excluding *Trp53*^{-/-}; *Brca2*^{-/-} cells, caspase activity increased in a dose dependent manner (i.e., with increasing FK866 concentrations). Interestingly, *Trp53*^{-/-}; *Pten*^{-/-} cells had the greatest increase in caspase 3/7 activity, even exceeding the upregulation observed in HR-deficient *Trp53*^{-/-}; *Brca1/2*^{-/-} cells. These data suggest that loss of *Pten* sensitises cells to the combination.

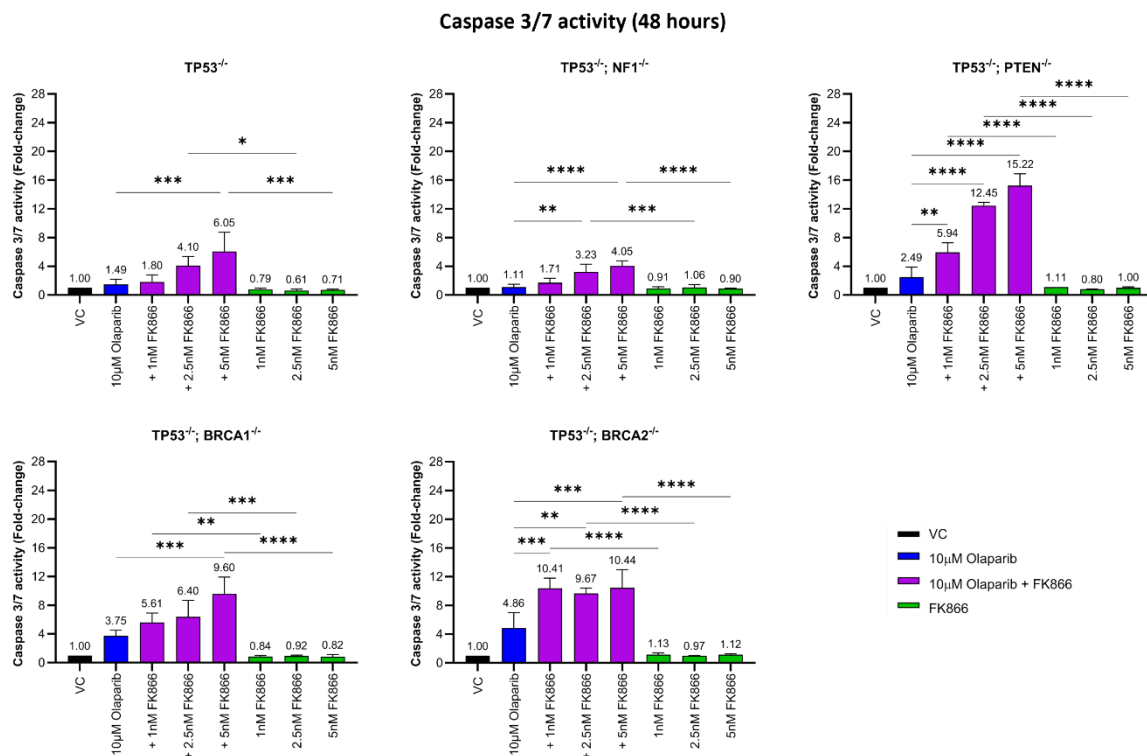


Figure 29. Combination treatment upregulates caspase 3/7 activity in ID8 cells

ID8 cells were co-treated with olaparib (10µM) and FK866 (1 to 5nM) for 48 hours to assess their influence on caspase 3/7 activity (apoptosis). This was measured using a Caspase-Glo® 3/7 assay. Data is the average ± SD of three independent experiments. Statistical significance was determined using

one-way ANOVA followed by Šídák's multiple comparisons test ($p < 0.05$, $**p < 0.01$, $*** p < 0.001$, $**** p < 0.0001$).

3.3.5. Olaparib and FK866 co-treatment increases γ H2AX foci formation in ID8 cells

Since there have been indications in the literature that loss of PTEN impedes homologous recombination repair and sensitises cells to PARPis^{284,285} we set out to investigate whether *Pten* null ID8 cells accumulate more DNA damage basally or following treatment with olaparib and/or FK866 (**Figure 30**). Under basal conditions both *Trp53*^{-/-} (~3.6 γ H2AX foci/nuclei) and *Trp53*^{-/-}; *Pten*^{-/-} (~4.5 γ H2AX foci/nuclei) models possess relatively high numbers of γ H2AX foci on average. FK866 treatment had very little impact on γ H2AX foci formation (~3.7 and ~4.9 on average, respectively). Olaparib treatment significantly upregulated γ H2AX foci formation (~5.0 and ~7.0 on average, respectively). Whilst the largest number of γ H2AX foci were observed in the combination treated groups (~5.9 and ~8.1 on average, respectively). Overall, these data confirm that olaparib and combination treatment upregulate γ H2AX foci formation; that *Pten* null ID8 cells accumulate more γ H2AX foci under basal and treated conditions; but differences in the level of γ H2AX foci accumulation do not necessarily explain the outcome from apoptosis assays.

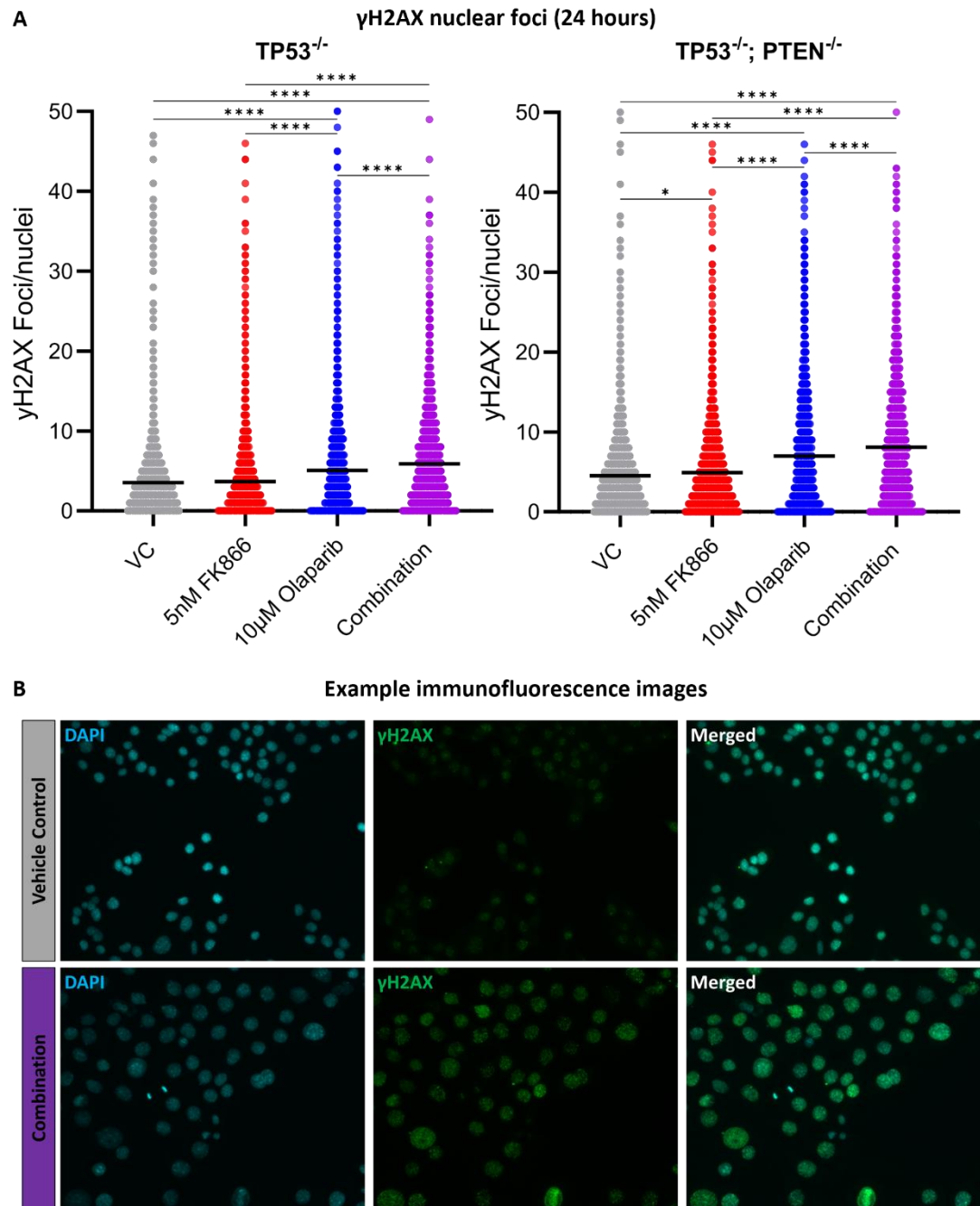


Figure 30. Combined olaparib and FK866 treatment increases γ H2AX foci formation in ID8 cells

ID8 cells were treated with vehicle (0.1% DMSO), olaparib (10 μ M), FK866 (5nM) or the combination for 24 hours before immunostaining for γ H2AX. **(A)** Scatter dot plots show the number of γ H2AX foci per cell. Data is pooled from three independent experiments. Since data are heavily skewed, the (non-parametric) Kruskal-Wallis test and Dunn's multiple comparisons test were used for statistical tests among groups ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). **(B)** Examples of immunofluorescence staining from vehicle (top panel) and combination (bottom panel) treated ID8-

Trp53^{-/-};Pten^{-/-} cells are shown. Images were recoloured and overlaid using ImageJ. Brightness and contrast were not adjusted.

3.3.6. Increased PARP trapping could not be detected

Since combined treatment with a NAMPTi and PARPi can reduce PARylation to a greater extent than PARPi monotherapy⁹⁹ and this could increase the trapping of PARP on DNA, we investigated whether the combination of FK866 and olaparib could increase PARP-trapping using a subcellular fractionation approach, that has been described by Murai and colleagues³³. In their experiments the alkylating agent MMS was included to increase base levels of DNA damage to aid the detection of trapping. However, since inducing DNA damage (e.g., with MMS or H₂O₂) activates PARP and depletes NAD⁺ (**Appendix Figure 21**), we excluded DNA damaging agents as they wouldn't allow us to determine whether FK866 induced NAD⁺ depletion increases trapping alone or in combination with olaparib. 5nM FK866 and/or 10µM olaparib was selected since these doses have been shown to block PARylation in ID8 *Trp53^{-/-}; Pten^{-/-}* cells after 24 hours (PhD student Yitao Xu, personal communication). In this experiment, Histone H3 was only detected in the chromatin-bound fraction, confirming we successfully separated nuclear-soluble and chromatin-bound fractions (**Figure 31**). However, only a modest increase in PARP-1 levels could be detected in the chromatin-bound fraction following treatment with olaparib and/or FK866. This could suggest that the combination does not significantly increase PARP trapping or that at the selected dose/timepoint trapping isn't increased enough to be detected in the absence of a DNA damaging agent. However, it should be noted a positive control group (e.g., olaparib and 0.01% MMS) was not included in this experiment.

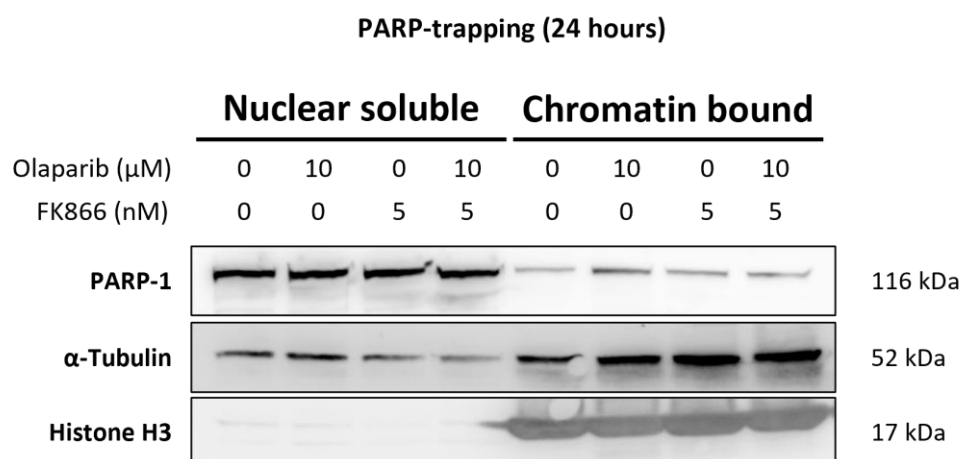


Figure 31. Combined olaparib and FK866 treatment does not drastically increase PARP-trapping

ID8 *Trp53*^{-/-}; *Pten*^{-/-} cells were treated for 24 hours with vehicle (0.1% DMSO), olaparib (10μM), FK866 (5nM) or the combination. Cell lysates were fractionated into nuclear-soluble and chromatin-bound fractions and western blot analysis was performed. Blots were probed with indicated antibodies. Histone H3 was used as a positive marker for chromatin-bound fractions.

3.3.7. The combination of FK866 and olaparib suppresses tumour growth in ID8-*Trp53*^{-/-}; *Pten*^{-/-} tumours *in vivo*

The first *in vivo* study was used to assess the tolerability of olaparib and/or FK866 in C57BL/6J mice injected with ID8-*Trp53*^{-/-}; *Pten*^{-/-} cells. Also, to assess the ability of the combination to reduce omental tumour weight, since ovarian tumours preferentially spread to this site during metastatic spread in patients²⁸⁷ and when using this mouse model^{268,269}. Drugs were resuspended in a vehicle solution containing 3.5% DMSO and 6.5% Tween-80 in saline since olaparib²⁶⁴ (at 50mg/kg) and FK866¹²⁸ (at 30mg/kg) were previously made up in this or a similar formulation for *in vivo* studies. Neither drug completely dissolved (as expected for olaparib²⁶⁴), but instead formed drug suspensions. In this model omental tumours typically formed 14 days after injecting ID8-*Trp53*^{-/-}; *Pten*^{-/-} cells (Dr Sarah Spear, personal communication). To minimise the risk of FK866-induced toxicity, mice went onto receive 10 I.P. injections in total (maximum dose of 30mg/kg FK866), with a 2 day drug holiday between injections 5 and 6, since similar protocols have been used for NAMPTis *in vivo*^{98,99,121,128,152} (Figure 32A).

Excluding FK866 (30mg/kg) and Combination B (20mg/kg of FK866) groups, a significant loss of body weight (≥ 10%) was not observed in the remaining groups (Appendix Figure 10). For

Combination B, bodyweight dropped to 86% (mouse 2) on day 25, but the following day this increased back to 91% and mice showed no signs of discomfort. By comparison, in the FK866 (30mg/kg) group body weight dropped to 90% (mouse 2, day 18) overnight or gradually over 3 days (mouse 3, day 25). In these instances, although the weight loss limit (20% loss) wasn't reached mice were displaying significant signs of discomfort (e.g., hunching, piloerection, laboured breathing, separation from cage mates, reduced activity, and squinted eyes), so were culled. An autopsy was performed and revealed both mice had faecal impaction, and mouse 2 had distended intestines full of a bile like substance. It was interesting to observe that in both mice drug toxicity became pronounced on the 5th and 10th day of drug treatment, suggesting 5-day treatment cycles may not be optimal for mice receiving FK866. Interestingly, the remaining FK866 (30mg/kg) mouse (mouse 1) and the Combination A (30mg/kg of FK866) group did not experience weight loss or show any signs of discomfort.

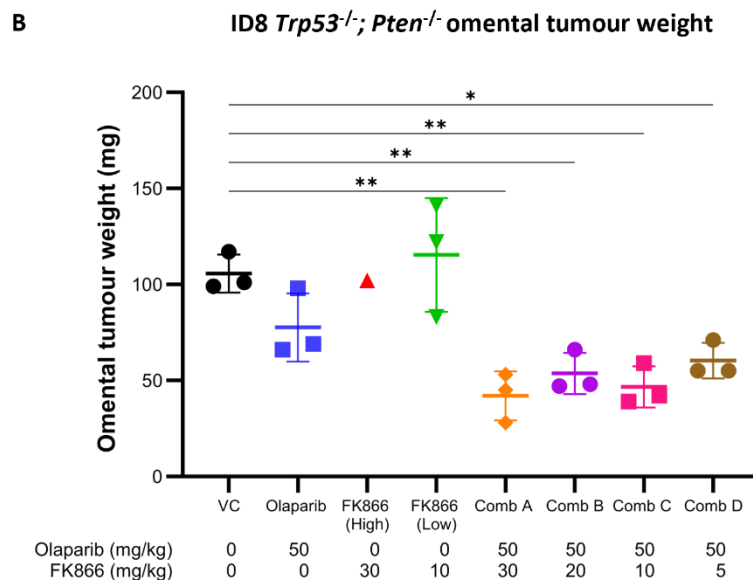
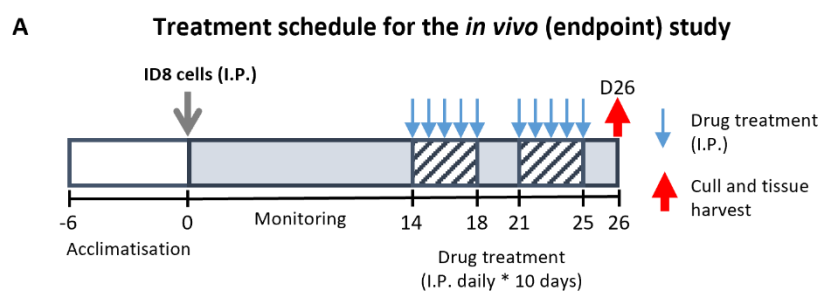


Figure 32. Combined olaparib and FK866 treatment reduces omental tumour weight in the HR-proficient ID8-*Trp53*^{-/-}; *Pten*^{-/-} model

(A) The timeline for the endpoint (toxicology) study is shown. ID8-*Trp53*^{-/-}; *Pten*^{-/-} cells were injected intraperitoneally (I.P.) into C57BL/6J mice and allowed to grow for 14 days (omental tumours form) before treating mice with vehicle, olaparib, FK866 or the combination for 10 days. For relative body weight changes in mice during drug administration see **Appendix Figure 10. (B)** Omental tumours were harvested and weighed 24 hours after the final drug treatment. Excluding the FK866 (high) group data are from three separate mice, error bars = SD. FK866 (low) data are from an independent experiment. One-way ANOVA and Dunnett's multiple comparisons test were used to calculate statistical differences among groups ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Unlike vehicle mice, none of the mice treated with olaparib and/or FK866 developed ascites by the endpoint. Olaparib treatment slightly reduced omental tumour weight (77.7mg on average) compared to vehicle mice (105.6mg on average), but this did not reach significance (**Figure 32B**). Mice treated with 10mg/kg (115.3mg on average) or 30mg/kg (102.0mg) of FK866 did not consistently observe a reduction in omental tumour weight, and in some instances omental tumours were larger than those found in vehicle mice. It seems unlikely that FK866 (10mg/kg) treatment would increase omental tumour size, so larger omental tumours are likely due to slight differences in tumour seeding, since 10mg/kg FK866 mice were from an independent experiment. Treatment with the combination of olaparib (50mg/kg) and FK866 (5-30mg/kg) significantly reduced omental tumour weight: Combination A (42.0mg on average) > Combination C (46.7mg on average) > Combination B (53.7mg on average) > Combination D (60.3mg on average). Although increasing the dose of FK866 could increase the combinations benefit, negligible differences were observed between groups receiving 10-30mg/kg of FK866. Taken together, these data show that in the ID8-*Trp53*^{-/-}; *Pten*^{-/-} model the combination of FK866 and olaparib can significantly reduce the size of omental tumours, 30mg/kg of FK866 is not a tolerable dose, 5-day treatment cycles may not be optimal for future *in vivo* experiments and very little benefit comes from increasing FK866 doses above 10mg/kg.

3.3.8. The combination of Olaparib and FK866 increases DNA damage *in vivo*

Western blot analysis was performed using omental tumours from mice treated with vehicle, olaparib (50mg/kg), FK866 (10mg/kg) and the combination (50mg/kg olaparib and 10mg/kg FK866) (**Figure 33**). Olaparib and FK866 treatment had an inconsistent influence on AKT phosphorylation, but p-AKT appeared to be upregulated in mice treated with the

combination. ERK1/2 phosphorylation was blocked in one olaparib (mouse 2) and one combination (mouse 1) treated omental tumour, but in the remaining tumours p-ERK1/2 remained unchanged. RPA32 phosphorylation was probed for, however, bands were either undetectable or non-specific bands were observed (data not shown). Interestingly, treatment with the combination appeared to reduce the total level of RPA32 protein expression. An upregulation in Chk1 phosphorylation could be observed in two olaparib (mouse 1 and 3) and all combination treated omental tumours. The greatest increase in p-Chk1 was observed in the combination group. Basal expression of γ H2AX was high in vehicle treated omental tumours but could be upregulated following treatment with the combination. ATM and ATR were probed for but were either undetectable or non-specific bands were observed (data not shown). Taken together, these data suggest that the combination activates Chk1 and increases DNA damage (γ H2AX) *in vivo*.

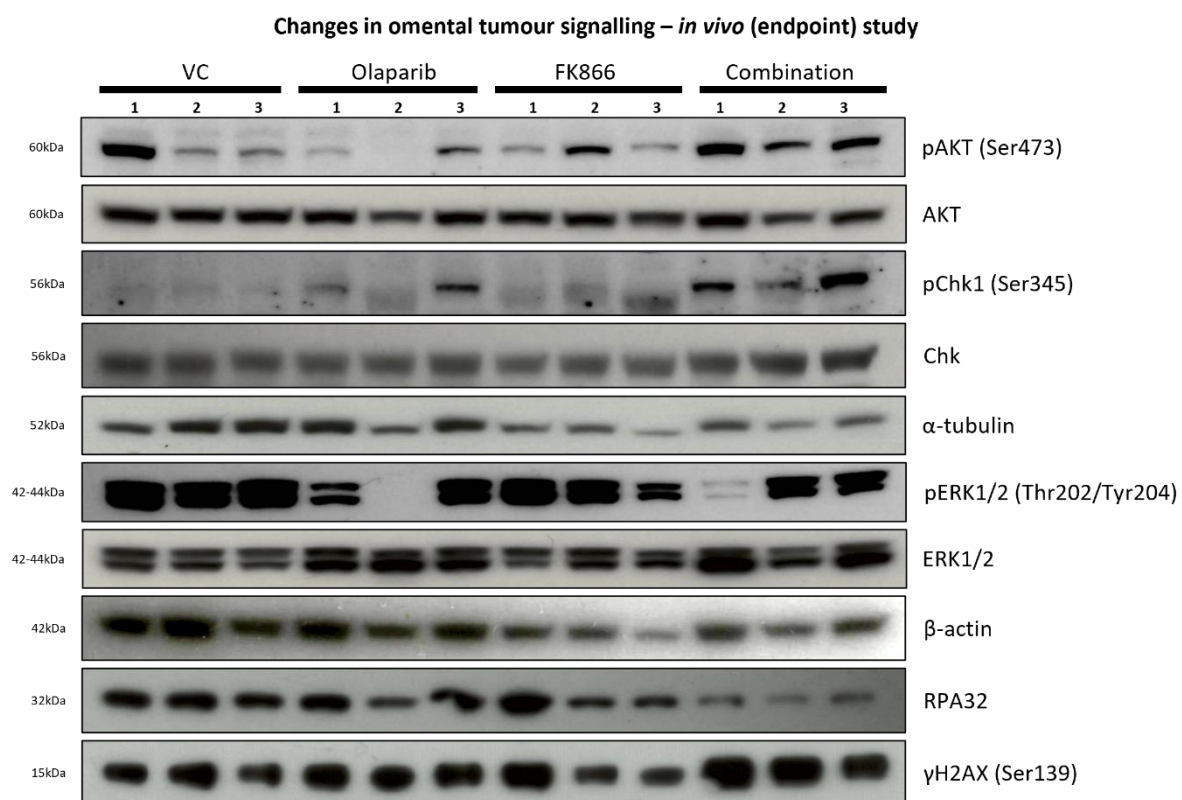


Figure 33. Changes in omental tumour signalling following combined olaparib and FK866 treatment

Mice were treated with vehicle, olaparib (50mg/kg), FK866 (10mg/kg) or the combination. Omental tumours were harvested, total protein was extracted and examined by western blotting. β -actin and α -Tubulin were used as loading controls. Tumours were extracted from mouse 1-3 in each treatment

group. 10mg/kg FK866 tumours were harvested in a separate independent experiment. PhD student Yitao Xu assisted in tissue homogenisation and re-imaging some sections.

3.1.1. The combination of olaparib and FK866 induces metabolic alterations in omental tumours

An in-house HILIC-UPLC MS/MS analytical method was used to measure up to 112 analytes (**Tables 9 and 10**). Metabolites that were detectable and within the QC accuracy range of 70-130% were included (**Appendix Figure 13**) in subsequent analysis. Hierarchical clustering highlighted that four clusters formed. In clusters 1 and 2 (left of **Appendix Figure 11**) olaparib (50mg/kg) and vehicle treated omental tumours do not separate based on treatment received, and the short distances between branches suggests these samples are similar. By comparison, in clusters 3 and 4 (right of **Appendix Figure 11**) combination and FK866 (10mg/kg) treated samples form distinct groups based on the treatment received, and distances between branches are greater, suggesting that samples from different treatment groups are more dissimilar. The differences between each group are further highlighted by principal component analysis (PCA) (**Appendix Figure 12**). FK866 and combination groups form distinct clusters, whereas olaparib and vehicle groups overlap considerably.

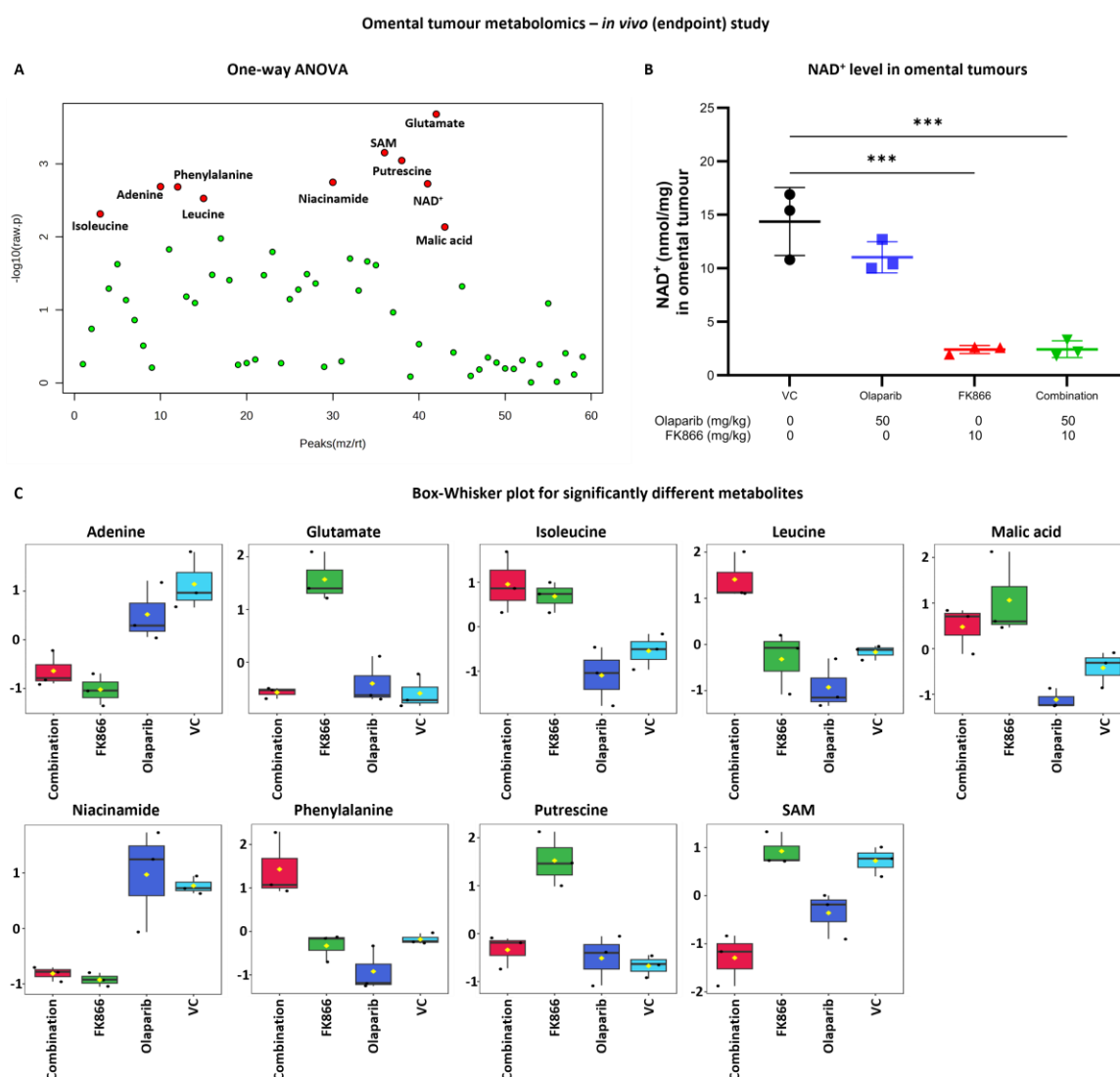


Figure 34. Significantly altered intracellular metabolites detected in omental tumours during the endpoint *in vivo* study

Vehicle, olaparib (50mg/kg), FK866 (10mg/kg) and combination (50mg/kg olaparib and 10mg/kg FK866) treated omental tumours were homogenised and metabolites were extracted (in collaboration with PhD student Yitao Xu). Please note, FK866 (10mg/kg) omental tumours were harvested in an independent *in vivo* experiment, but metabolite extraction was performed at the same time. Targeted metabolomics analysis was performed and **(A)** one-way ANOVA analysis was undertaken using Metaboanalyst (v.5.0.). 10 molecules (red dots) had an FDR < 0.05. Green dots indicate insignificant changes in metabolites. **(B)** Analyst (v1.6.2, SCIEX) software was used to calculate NAD⁺ levels (molar concentration/per mg of omental tumour) from a standard curve included in the same run. Data is the mean of three tumour samples experiments ± SD. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test ($p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). **(C)** Box plots of significantly altered ($p < 0.05$) metabolites (excluding NAD⁺) were

generated using Metaboanalyst (v.5.0.). The Y-axis represents each metabolites normalised concentration distribution.

One-way ANOVA analysis (**Figure 34A**) revealed that 10 metabolites were significantly changed ($FDR < 0.05$) in omental tumours following treatment: isoleucine, adenine, phenylalanine, leucine, niacinamide (also called nicotinamide), S-Adenosyl methionine (SAM), putrescine, NAD^+ , glutamate and malic acid. Molar concentrations (nM/mg) of NAD^+ in omental tumours were calculated (**Figure 34B**), and from this it was confirmed FK866 (10mg/kg) could significantly reduce NAD^+ levels alone or in combination with olaparib (50mg/kg). Surprisingly, a slight reduction of NAD^+ was observed in olaparib treated tumours but this did not reach significance.

Box plots were produced for the 9 remaining metabolites with significant changes (**Figure 34C**). For Adenine, treatment with olaparib slightly reduces the metabolites concentration, but treatment with FK866 and the combination led to larger reductions. Glutamate and putrescine are not affected by olaparib or combination treatment but appear to be elevated in FK866 treated omental tumours. Similar trends were observed for isoleucine, leucine, and malic acid. Treatment with olaparib slightly reduced their concentration and combination treatment increased their concentration. However, FK866 treatment only increased the concentration of isoleucine and malic acid, not leucine. Niacinamide concentrations are reduced following treatment with FK866 or the combination, but on average are elevated following olaparib treatment. Phenylalanine is unaffected by FK866 treatment, is reduced following olaparib treatment but elevated following combination treatment. Finally, SAM is unaffected by FK866 treatment but reduced by olaparib and combination treatment.

3.1.2. The combination of olaparib and FK866 improves survival outcomes in mice bearing ID8-*Trp53*^{-/-}; *Pten*^{-/-} tumours

Pilot studies demonstrated that olaparib (50mg/kg) and/or FK866 (≤ 20 mg/kg) were likely to be safe and tolerable *in vivo* (section 3.4.7.), though minor tweaks in the treatment schedule should be incorporated (**Figure 35A**). Based on earlier findings, we speculated that 20mg/kg of FK866 plus 50mg/kg olaparib was likely to inhibit tumour growth with minimal risk of adverse events. So, we set out to treat mice with vehicle, olaparib (50mg/kg), FK866 (20mg/kg) and two doses of the combination (50mg/kg olaparib plus 10mg/kg or 20mg/kg of FK866). In the previous endpoint experiment, mice treated with 30mg/kg of FK866

experienced dose limiting toxicity events on days 5 and 10 of their treatment cycles. Considering this, the treatment schedule was also adjusted, so mice received 8 I.P. injections in total, with a 3-day drug holiday after the 4th I.P. injection. Despite these changes, mice receiving 20mg/kg FK866 alone or in combination with olaparib experienced unexpected toxicity (e.g., weight loss) during the first four I.P. injections (**Appendix Figure 14**). As a result, in the 20mg/kg combination group mouse 5 (day 18) and mouse 6 (day 17) were culled as they were showing significant signs of discomfort (e.g., hunching, piloerection, laboured breathing, separation from cage mates, reduced activity, and squinted eyes). In the 20mg/kg FK866 group mouse 2 (day 17) and mouse 5 (day 18) were also culled. In surviving mice, the dose of FK866 was reduced from 20mg/kg to 10mg/kg for the final four I.P. injections and doses were skipped if weight loss was observed. However, due to the challenges faced in 20mg/kg FK866 monotherapy and combination groups this data was excluded from survival analysis (**Figure 35**). As a result, the first survival experiment did not contain a suitable FK866 monotherapy arm (10mg/kg), so this was repeated in an independent experiment. Significant weight loss was not observed in the remaining groups. Furthermore, red blood cell (RBC) count analysis (**Appendix Figure 15**) demonstrated 10mg/kg FK866 treatment did not significantly reduce RBC counts when compared to olaparib treated mice, suggesting the combination is unlikely to worsen olaparib-induced anaemia.

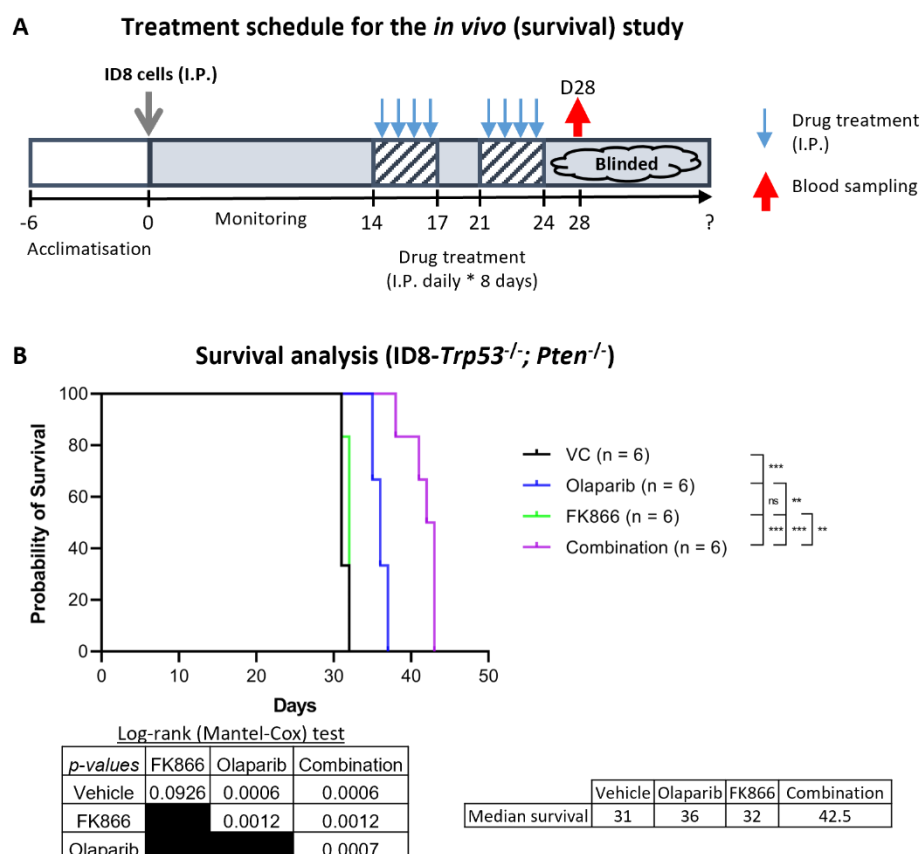


Figure 35. FK866 improves olaparib response in ID8-*Trp53*^{-/-}; *Pten*^{-/-} *in vivo*

(A) Timeline for the survival experiment. Mice were injected with ID8 tumours (ID8-*Trp53*^{-/-}; *Pten*^{-/-}) and randomised into different groups for drug treatment. Researchers were blinded after the final drug treatment until the study's completion for unbiased determination of humane endpoint. **(B)** Kaplan-Meier curves showing survival proportion of mice injected with ID8-*Trp53*^{-/-}; *Pten*^{-/-} tumours. 24 mice were randomised into four groups, *n* = 6 each. Mice were treated with vehicle, olaparib (50mg/kg), FK866 (10mg/kg) and combination (50mg/kg olaparib and 10mg/kg FK866). Mice receiving 20mg/kg FK866 alone or in combination with olaparib were excluded from analysis due to dose limiting toxicity (See **Appendix Figure 14** for body weight changes during treatment). FK866 (10mg/kg) data was obtained in a separate survival experiment. The log-rank (Mantel-Cox) test was used to determine statistical differences between survival curves. Bonferroni correction was used for the multiple comparisons test (*p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

Perhaps unsurprisingly, treatment with FK866 (10mg/kg) did not significantly improve survival outcomes compared to the vehicle group (*p* = 0.0926, not significant), as median survival only improved from 31 to 32 days (**Figure 35B**). However, olaparib (50mg/kg) treatment did provide a moderate survival benefit, increasing the median survival to 36 days (*p* = 0.0006,

significant). The combination of FK866 (10mg/kg) and olaparib (50mg/kg) provided the greatest survival benefit, with a median survival of 42.5 days. This was a significant improvement compared to vehicle control ($p = 0.0006$) and olaparib monotherapy ($p = 0.0007$). Overall, these findings suggest that FK866 improves olaparib responses in mice harbouring ID8-*Trp53*^{-/-}; *Pten*^{-/-} tumours.

3.2. Discussion

It should be recognised there are limitations with our selection of models. First, the epithelial origin of ID8 cells does not represent the fallopian tube origin of most HGSOC^{288,289}. Considering this, it would be wise to validate our experiments in additional genetically engineered mouse models of HGSOC, such as the HGS2 model²⁹⁰. Second, in the human EOC panel KRAS/PI3K-mutant cancer cell lines typically lacked a *TP53* mutation (i.e., excluding OVCAR-8 cells), so these tumours would be defined as type I EOC (i.e., LGSOC, MOC, EnOC, and CCOC) rather than type II (i.e., HGSOC)^{291,292}. As such, it would be ideal to knock-in oncogenic mutations of interest (e.g., *KRAS*^{G12V}, *BRAF*^{V600E}, or *PIK3CA*^{E545K}) in *Trp53*-wild type ID8 cells²⁶⁹ to characterise FK866 and/or olaparib responses. Indeed, MRes student Mi Chween²⁹³ attempted to address this problem using CRISPR-Cas9 gene editing to knock in the oncogenic *KRAS*^{G12V} mutation in ID8 cells. *Kras* guide 6 from a study conducted by Platt and colleagues²⁹⁴ was selected. However, in our hands the guides on-target activity (guide efficiency) was too low to generate knock in clones. It should be noted that ID8 cells stably expressing *KRAS*^{G12V} have been created and could be used to test the combination in a type I-like EOC background^{295,296}. Despite these caveats, given HGSOC is the most common subtype of EOC (~70% of cases)⁶, it was essential we tested the combinations influence in RAS/PI3K-mutant HGSOC cells (i.e., loss of NF1 or PTEN), so the ID8 panel was selected.

In this chapter I show that loss of *Pten*, *Brca1*, and *Brca2* increase FK866 sensitivity, whereas to our surprise loss of *Nf1* reduced FK866 sensitivity. Interestingly, ID8 cells were intrinsically more resistant to FK866 compared to human EOC cell lines, both in terms of cell proliferation and depletion of the NAD⁺ pool. Furthermore, despite differing FK866 sensitivity differences in NAD⁺ depletion could not be observed between ID8 *Trp53*^{-/-}, *Trp53*^{-/-}; *Nf1*^{-/-} and *Trp53*^{-/-}; *Pten*^{-/-} cells. This could suggest that ID8 cells are not dependent on NAMPT for NAD⁺ biosynthesis and could instead rely on the Preiss-Handler pathway, as can be observed in

ovarian tumours¹¹⁶. However, when using ID8 models in 2D culture authors have previously reported some genetic mutations (e.g., *Pten* loss) have modest effects^{297,298}, but differences become more apparent in 3D culture or *in vivo*. A similar phenomenon was observed with ID8 *Kras*^{G12V} cells^{295,296}. We did attempt to perform experiments in 3D (i.e., using different seeding densities, timepoints, plate formats and endpoint assays). Although all ID8 clones tested formed spheroid-like clusters in low-attachment plates *in vitro* (data not shown), optimal conditions for experiments were not identified. For example, *Trp53*^{-/-}; *Brca1*^{-/-} and *Trp53*^{-/-}; *Brca2*^{-/-} spheroids did not consistently respond to olaparib treatment, even though we know these models respond in 2D and *in vivo*²⁶⁴. Another possible explanation for ID8 cells being intrinsically more resistant to FK866 is the fact that after adaptation to low glucose, they are still cultured in the presence of 1mM sodium pyruvate (i.e., the only difference in media composition compared to the human EOC panel). Preliminary work previously conducted by Dr Adrian Benito-Mauricio demonstrated that supplementing human EOC cells media with 1-2mM pyruvate had no influence on FK866 sensitivity in TOV21G and OVCAR-8 cells, but a modest rescue (~20% increase in proliferation) could be observed in COV504 and COV644 cells (unpublished data). Taken together, it would be worth characterising NAMPT/NAPRT expression (and sensitivity to NAPRT inhibitors) in the ID8 panel, particularly as there are suggestions loss of PTEN expression upregulates NAMPT expression in the literature¹⁸⁹. It would also be worth investigating whether differences between ID8 models become more apparent in the absence of pyruvate. Finally, ATP levels should be quantified following FK866 treatment since in the human EOC panel we observed more pronounced ATP depletion in KRAS/PI3K-mutant cells, but based off of the NAD⁺ data this would appear to be unlikely.

Another important finding is that in 2D culture co-treatment with non-lethal doses of FK866 sensitised cells to olaparib irrespective of mutational background, in agreement with data from our human EOC cell line panel (**chapter 2**), and a previous study testing the combination in HGSOc¹⁰⁰. Through matrix assays we confirmed the interaction was again synergistic, and importantly in ID8 *Trp53*^{-/-}; *Nf1*^{-/-} and *Trp53*^{-/-}; *Pten*^{-/-} cells synergy could be observed at a greater number of doses. As was observed in the A2780 cells, combination treatment could only be partially rescued when supplementing media with 250µM β-NMN. Given 5nM FK866 alone had little-to-no influence on cell proliferation in ID8 cells, it would be important to quantify NAD⁺ levels at the end of the rescue assay to understand if β-NMN/NAD⁺ isn't being

rescued completely, or if NAMPT is contributing towards the combinations synergy beyond NAD⁺ depletion^{122,276}.

Importantly, the combination was shown to increase caspase 3/7 activity demonstrating the combination upregulates apoptosis. The largest increase in caspase activation was observed in ID8 *Trp53*^{-/-}; *Pten*^{-/-}, *Trp53*^{-/-}; *Brca2*^{-/-} and *Trp53*^{-/-}; *Brca1*^{-/-} models (in order). It must be noted that *PTEN* loss can cause a HRD, and the increase in caspase activation could be due to its synthetic lethal interaction with PARPi treatment^{284,285,299}, which could be enhanced by reducing NAD⁺ (and PAR) levels. However, when quantifying γH2AX levels in ID8 *Trp53*^{-/-} and *Trp53*^{-/-}; *Pten*^{-/-} cells by immunofluorescence, only slightly more γH2AX could be observed in *Trp53*^{-/-}; *Pten*^{-/-} cells following combination treatment. This could suggest that increased DNA damage doesn't contribute towards the synergy, but it is possible that *Trp53*^{-/-}; *Pten*^{-/-} cells have reduced capacity to deal with similar levels of DNA damage. Alternatively, since *PTEN* loss increases baseline replication stress³⁰⁰, and theoretically the combination should increase this further (i.e., due to a more potent reduction of PARylation^{37,99}), perhaps this contributes to the increased caspase activation. However, given we do not see much more DNA damage in these cells and DNA fibre assays were not performed, it appears to be an unlikely explanation. Interestingly, loss of *PTEN* and deletion of the metabolic regulator PDHK1 (pyruvate dehydrogenase kinase 1) is synthetic lethal due to the loss of MMP³⁰¹. Given FK866 has been shown to induce the loss of MMP, it would be interesting to investigate if this occurs following combination treatment, and if ID8 *Trp53*^{-/-}; *Pten*^{-/-} cells are more susceptible¹²⁶.

We did attempt to investigate the combinations influence on PARP-trapping using a fractionation assay³³. Typically, in these assays MMS-treatment is included to increase the base level of DNA damage, so that PARP-trapping can be readily detected in chromatin bound fractions. Unfortunately, MMS treatment wasn't compatible with our experiment design since MMS significantly depletes NAD⁺ and would make it challenging to distinguish between the influence of FK866 and MMS on PARP-trapping. Considering this, it would be interesting to test the combinations influence on trapping using an alternative approach³⁰².

An important observation is that the combination significantly reduced the growth of ID8 *Trp53*^{-/-}; *Pten*^{-/-} tumours *in vivo* and this corresponded with survival more than doubling compared to olaparib monotherapy treatment. Although the combination has been shown to

sensitise PARPi-R HGSOc xenograft tumours to olaparib¹⁰⁰, this is the first study to demonstrate that the combination could benefit *PTEN* null tumours. This is particularly significant as dysregulated PI3K signalling has been reported to cause resistance to rucaparib in clinical trials⁵³, so the combination could improve the efficacy of PARPis in this group of patients. Importantly, the combination (i.e., 50mg/kg olaparib and 10mg/kg FK866) was tolerable, and high on-target activity was observed (i.e., NAD⁺ was efficiently depleted). We did attempt to assess PARylation in omental tumours following treatment with olaparib and/or FK866, but basal PARylation was too low to detect any differences. It should be noted, *in vivo* experiments assessing the combinations efficacy using ID8 *Trp53*^{-/-} cells have not been performed. Doing so could help us better understand how *Pten*^{-/-} contributes to the combination benefit. In addition, it could also be worth performing an *in vivo* experiment in another *Pten*-null model (e.g., HGS2-*Trp53*^{-/-}, *Pten*^{-/-} and *Brca2*^{-/-})²⁹⁰, ideally in the absence of a *BRCA1/2*-knock out.

Metabolic analysis of omental tumours revealed that FK866 and the combination significantly depleted adenine. This was an interesting observation as FK866 has previously been shown to reduce NAD⁺ below a critical threshold, leading to adenine depletion, and this metabolic trigger primed mitochondrial apoptotic signalling pathways¹²⁵. Treatment with olaparib, significantly depleted SAM in omental tumours, and this was more pronounced following combination treatment. Given SAM is the sole methyl donor for the methylation of DNA, histones and other proteins, this could suggest the combination alters omental tumours epigenetic landscape³⁰³ and AS^{253,255}. The amino acids leucine, isoleucine and phenylalanine were elevated following combination treatment, indicating that protein synthesis has decreased, or their uptake has increased^{136,194}. Finally, although NAMPTis such as FK866 have been shown to increase ROS accumulation¹²⁶, we did not observe any change in the reduced glutathione (GSH)/oxidised glutathione (GSSG) ratio. Given GSH is a major antioxidant that neutralizes ROS, leading to the accumulation of GSSG (i.e., indicative of oxidative stress)^{130,304}, this could suggest that oxidative stress does not contribute towards the combinations synergy. However, it is important to recognise that although the GSH/GSSG molar ratio can be used to measure oxidative status in tissues, we may not have used an appropriate extraction method to confidently assess it by LC-MS/MS³⁰⁵.

Importantly, western blotting experiments with omental tumour samples confirmed that the drug combination increased γ H2AX levels. However, to our surprise basal levels of γ H2AX were high in omental tumours, perhaps as a result of *Pten*-loss induced HRD^{284,300}. We also observed an increase in Chk1 phosphorylation at serine 345 (ATR-dependent) following treatment with the combination, suggesting the combination promotes DNA damage-induced cell cycle arrest^{306,307}. We did investigate whether the combination altered the phosphorylation of RPA32, but this wasn't detectable in any condition. However, a decrease in total RPA32 expression could be observed. Given RPA32 plays a critical role in the DNA-damage and replication-stress responses, its reduced expression could suggest genomic instability has increased. For example, RPA depletion has been shown to inhibit the formation of RAD51 nuclear foci, leading to persistent unrepaired DSBs³⁰⁸. Unlike in the human EOC cell lines (i.e., COV318 and OVCAR-8 cells) where the combination had no influence on ERK1/2 phosphorylation, treatment with olaparib and the combination had a sporadic influence on its phosphorylation, with a reduction in pERK1/2 being observed in some samples. By comparison, pAKT levels were consistently higher in combination treated samples. This could possibly be a response to presence of DNA damage, as AKT serine 473 phosphorylation has previously been observed in response to ionizing radiation and DNA-damaging agents (e.g., doxorubicin)³⁰⁹. Alternatively, the combination could be upregulating the feedback activation of PI3K and AKT, as has been shown previously following NAMPTi treatment^{104,126,128}.

In summary, evidence from this chapter demonstrates that FK866 treatment significantly improves olaparib response in *Pten*-null HGSOC tumours.

CHAPTER 4

Optimising a Chemical Proteomics method to study the intracellular ADP-ribosylome using LC-MS/MS based metabolomics

Chapter 4 – Optimising a Chemical Proteomics method to study the intracellular ADP-ribosylome using LC-MS/MS based metabolomics

4.1. Background

As discussed in **section 1.7.3.**, previous studies using “clickable” 6Yn-NAD⁺ precursor probes (e.g., 6Yn-ProTide and 6Yn-Ad) provide conflicting evidence for their utility to study ADP-ribosylation. Several challenges are associated with these probes. Firstly, they must be converted into 6Yn-AMP > 6Yn-ADP > 6Yn-ATP > 6Yn-NAD⁺ (**Figure 11**) before they can be utilised by PARPs for ADP-ribosylation. As a result, if poor labelling occurs it could be challenging to identify where metabolic bottlenecks occur without robust metabolomics. Secondly, 6Yn-ATP can be used for AMPylation²⁷⁰, preventing its incorporation into the NAD⁺ pool and utilisation by PARPs for ADP-ribosylation. Although some metabolomics experiments have been performed using “clickable” 6Yn-NAD⁺ precursor probes (e.g., 6Yn-ProTide and 6Yn-Ad)^{232,270} these studies typically only measure one part of the biosynthetic pathway, do not identify conditions that could boost the synthesis of 6Yn-NAD⁺, and do not measure the concentration of endogenous metabolites (e.g., ATP or NAD⁺) so it is impossible to quantify the incorporation of 6Yn-ATP or 6Yn-NAD⁺ into endogenous metabolic pools (i.e., ATP and NAD⁺). As such, we developed targeted RP and HILIC UPLC-MS/MS methods for intracellular metabolic analysis of 6Yn-NAD⁺ biosynthesis intermediates, to optimise the conversion of 6Yn-Ad or 6Yn-ProTide probes into 6Yn-NAD⁺ for the chemical proteomic detection of ADP-ribosylated proteins.

4.2. Materials & Methods

4.2.1. Cell culture and drug preparation

COV318, KURAMOCHI, OVSAHO, HEY-A8, OVCAR-8 and HEK293T (a gift from Professor Ed Tate) cells were used for subsequent experiments. EOC cell lines were cultured as described previously (see **section 2.3.1.**). HEK293T cells were cultured in DMEM (ThermoFisher, 11995065) supplemented with 4.5 g/L glucose, 2mM L-glutamine, 110mg/L sodium pyruvate, 10% foetal bovine serum, 100 units/ mL penicillin and 100µg/mL streptomycin. In some experiments, T47D and MDA-MB-435 cells were used, in these instances tissue culture and sample preparation was performed by Dr Simeon Draganov for subsequent metabolomics assays.

Purified stock compounds were synthesised by Dr Simeon Draganov, as described in his thesis²⁴². These compounds included: NR, NRH, 6Yn-Ad, 6Yn-ProTide, 6Yn-AMP, 6Yn-ATP and 6Yn-NAD. For permeabilization experiments, a separate stock of 6Yn-NAD (BioLog, N 051) was purchased to ensure sufficient quantities were available for experiments.

4.2.2. Transient overexpression of NMNAT1 and PARP-1

The NMNAT-1 plasmid was provided by Professor Ed Tate's laboratory as a purified stock in H₂O. The pCMV-PARP1-3xFlag-WT plasmid (Addgene, 111575) was obtained from Addgene as a bacterial stub. To make adequate stocks of the PARP-1 plasmid, the bacterial stub was streaked onto Lysogeny broth (LB) Agar plates with 50µg/mL kanamycin (prepared in house). Sterility was maintained by working near a Bunsen burner. Plates were incubated overnight at 37°C and individual colonies were selected and transferred into 500mL flasks containing LB and 50µg/mL kanamycin. Each flask was placed in a shaker (~250rpm) overnight (~18 hours) at 37°C. The following day, bacterial cultures were transferred into centrifuge tubes and spun at 5,000 x g (4°C) for 10 minutes. Supernatants were discarded and plasmid purification was performed using the EndoFree® Plasmid Maxi Kit (QIAGEN, 12362). Plasmid concentrations were measured on a NanoDrop® ND-1000 UV-Vis Spectrophotometer (ThermoFisher). Sanger sequencing was carried out by GENEWIZ.

HEK293T cells were seeded onto 6 well plates at a density of 400,000 cells/well. The following day, cells were transfected with NMNAT-1 (2000ng) or PARP-1 (2000ng) plasmids using a lipofectamine™ 2000 (ThermoFisher, 11668019) kit, following manufacturer's guidelines. Cells that weren't transfected with plasmid were treated the same, so still received lipofectamine treatment. The following day, cells were treated with compounds such as: 6Yn-ProTide, 6Yn-Ad, β-NMN, NR, NRH, MMS (Sigma-Aldrich, 129925), cisplatin or the PARG inhibitor PDD00017273 (Selleckchem, S8862). Where indicated, to induce PARylation cells were treated with 2mM hydrogen peroxide (H₂O₂; Supelco, 106097) in PBS at 37°C for 10 minutes prior to extraction. At endpoint, cells were prepped for western blotting (see **sections 2.3.9. and 2.3.10.**) and/or metabolomics assays (see **section 2.3.8.**), as described previously.

4.2.3. Targeted LC-MS/MS analysis for the metabolism of 6Yn-Ad/ProTide analogues

To measure the intracellular conversion of 6Yn-Ad and 6Yn-ProTide analogues LC-MS/MS assays were performed using an Agilent 1290 Infinity LC system (Agilent Technologies) and a

4000 QTRAP triple quadrupole instrument (AB SCIEX) coupled to an ESI source (Turbo V). To detect 6Yn-Ad, 6Yn-Protide, 6Yn-AMP, 6Yn-NAD, NAD⁺ and β -NMN a ACQUITY UPLC[®] HSS T3 Column was used for RP Liquid Chromatography (positive mode) as described in section 2.3.8. To detect ATP and 6Yn-ATP an Acquity UPLC[®] BEH Amide 1.7 μ m 2.1 x 150mm Column was used for HILIC Liquid Chromatography (negative mode) as described in **section 3.3.5**. MRM parameters are described in **Table 11**. The Analyst (v1.6.2, SCIEX) software was used to perform peak integration. Purified stock compounds were used to produce a calibration curve (0.025-10 μ g/mL) for each run. Using this and cell counts, molar concentrations per million cells were calculated for each metabolite. Graphs were generated using the GraphPad Prism 10.0.0 software.

Table 11. MRM parameters for the HILIC UPLC-MS/MS 6Yn-NAD⁺ assay

Metabolite	Q1	Q3	RT (min)	DP	CE	Detected in cells?
<u>6Yn-Ad_1</u>	306.3	174.3	5.09	55	40	✓
6Yn-Ad_2	306.3	135.1	5.09	55	60	✓
<u>6Yn-AMP_1</u>	386.3	174.3	3.64	70	28	✓
6Yn-AMP_2	386.3	108.4	3.64	70	92	✓
6Yn-AMP_3	386.3	97.3	3.64	70	50	✓
<u>6Yn-ProTide_1</u>	623.1	432.5	7.62	180	26	✓
6Yn-ProTide_2	623.1	288.3	7.62	180	36	✓
6Yn-ProTide_3	623.1	174.2	7.62	180	48	✓
6Yn-ProTide_4	623.1	97.2	7.62	180	58	✓
6Yn-NAD_1	702.3	580.3	3.73	93	25	✓
6Yn-NAD_2	702.3	562.2	3.73	93	28	✓
6Yn-NAD_3	702.3	466.3	3.73	93	37	✓
<u>6Yn-NAD_4</u>	702.3	174.2	3.73	93	66	✓
<u>NAD_1</u>	664.0	136.0	0.88	70	70	✓
NAD_2	664.0	97.0	0.88	70	85	✓
<u>β-NMN_1</u>	335.0	123.0	0.68	100	20	✓
β -NMN_2	357.0	235.0	0.68	50	14	X
6Yn-ATP_1	544.2	466.3	5.73	-95	-25	✓
<u>6Yn-ATP_2</u>	544.2	159.0	5.73	-95	-46	✓
<u>ATP_1</u>	506.2	408.2	6.25	-70	-25	✓
ATP_2	506.2	159.1	6.25	-70	-50	✓

Main ions are underlined

4.2.4. Transient permeabilization of HEK293T cells and treatment with 6Yn-NAD

250,000 KURAMOCHI cells were seeded on 6 well plates. The following day cells were transfected with 100 μ M of 6Yn-NAD and 20 μ M of N-[1-(2,3-Dioleoyloxy)propyl]-N,N,Ntrimethylammonium methylsulfate (DOTAP; Roche, 11 202 375 001), adapting a method described by Lehner et al (2022)²³¹. After a 30 minutes incubation at 37°C, vehicle or

olaparib (final concentration of 10 μ M) was spiked directly into wells. Cells were left for an additional 30 minutes, media was aspirated and cells were treated with 2mM H₂O₂ (in PBS) or vehicle (in PBS) for 10 minutes. After this, metabolites were extracted (see **section 2.3.8.**) as previously described and LC-MS/MS assays (see **section 4.3.3.**) were performed to quantify the uptake of 6Yn-NAD. The Analyst (v1.6.2, SCIEX) software was used to perform peak integration. Purified stock compounds were used to produce a calibration curve (0.025-10 μ g/mL) for each run. Using this and cell counts, molar concentrations per million cells were calculated for each metabolite. Graphs were generated using the GraphPad Prism 10.0.0 software.

4.3. Results

4.3.1. Ovarian cancer cell lines convert 6Yn-ProTide and 6Yn-Ad into 6Yn-NAD

To investigate whether 6Yn-Ad and 6Yn-ProTide analogues are metabolically incorporated into intracellular adenosine metabolism and the NAD⁺ salvage pathway their conversion into 6Yn-AMP and 6Yn-NAD⁺ was measured (**Figure 36**). In this experiment ovarian cancer cell lines (i.e., COV318, HEY-A8 and OVCAR-8) were treated for 24 hours with either compound. All three cell lines internalised 6Yn-Ad/ProTide analogues and converted them into 6Yn-AMP. OVCAR-8 cells synthesised the highest concentration of 6Yn-AMP. COV318 and HEY-A8 produced comparable levels of 6Yn-AMP. The highest level of 6Yn-AMP synthesis was generally observed following treatment with 500 μ M of 6Yn-Ad. However, in OVCAR-8 cells it appeared slightly more was synthesised following 250 μ M 6Yn-Ad treatment, though differences were negligible. 6Yn-ATP could not be measured as we lacked a pure standard for method development at the time of this experiment.

Interestingly, despite synthesising the most 6Yn-AMP, OVCAR-8 cells produced the least 6Yn-NAD⁺ following 6Yn-Ad treatment. Furthermore, despite producing ~10 μ mol/million cells more 6Yn-AMP following 6Yn-ProTide treatment (compared to COV318 and HEY-A8 cells) OVCAR-8 cells only synthesised more 6Yn-NAD⁺ than COV318 cells. HEY-A8 cells produced the most 6Yn-NAD⁺ following 6Yn-ProTide treatment, and 6Yn-Ad treatment led to comparable levels of 6Yn-NAD⁺ synthesis. Finally, following 6Yn-Ad treatment COV318 cells produced the most 6Yn-NAD⁺ (~10,000pmol/million cells) in this experiment, and a dose dependent increase could be observed.

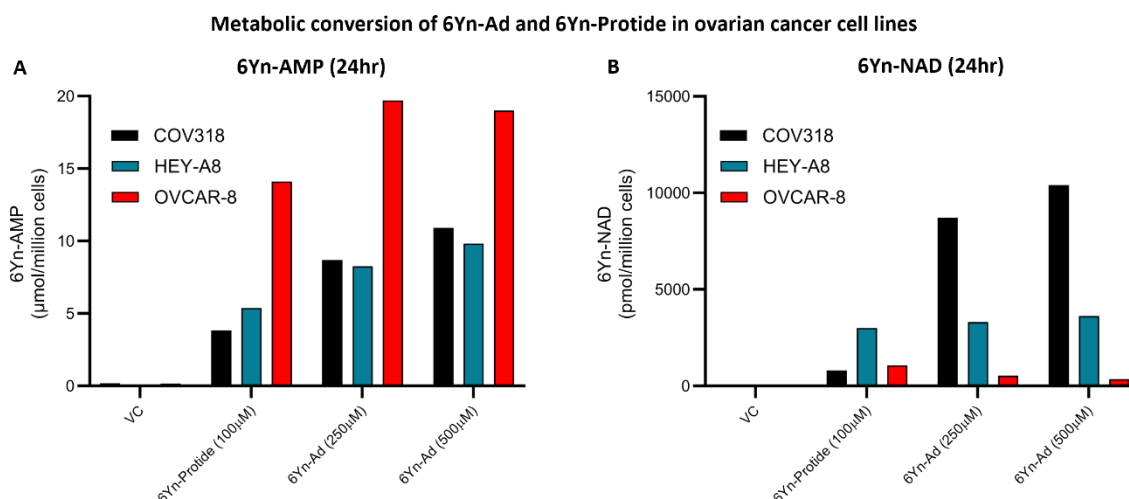


Figure 36. 6Yn-Ad and 6Yn-ProTide are converted into 6Yn-NAD⁺ in EOC cell lines

COV318, HEY-A8 and OVCAR-8 cells were treated for 24 hours with vehicle, 6Yn-Ad (250-500 μM) or 6Yn-ProTide (100 μM). Concentrations of the **(A)** 6Yn-AMP intermediate and **(B)** 6Yn-NAD⁺ are shown in mol/million cells. 6Yn-ATP was not measured as a standard was not available at the time of this experiment. Data is from one independent experiment.

4.3.2. Conversion from 6Yn-ATP to 6Yn-NAD is the rate limiting step in COV318 cells

Since COV318 cells produced the largest amount of 6Yn-NAD⁺, these cells were selected for a time course experiment to improve our understanding of 6Yn-Ad metabolism. 6Yn-Ad was readily taken up by COV318 cells, but intracellular concentrations gradually decreased over the course of 24 hours (**Appendix Figure 16**). 6Yn-AMP remained at ~3-4 μmol/million cells during the experiment, but concentrations appeared to start trending downwards at the final timepoint (**Figure 37**). After 6 hours, 6Yn-ATP concentrations peaked at ~0.6 μmol/million cells and appeared to start trending downwards at 24 hours. Interestingly, ~20% incorporation of 6Yn-ATP into the endogenous ATP pool could be achieved, suggesting the availability of 6Yn-ATP is not limiting 6Yn-NAD⁺ synthesis. However, after 6 hours the concentration of β-NMN fell by 2-4-fold but appeared to start increasing at the 24-hour timepoint. Finally, ~4000-5500 pmol/million cells of 6Yn-NAD⁺ was synthesised during the time course experiment. This equated to ~0.5-1% incorporation of 6Yn-NAD⁺ into the endogenous NAD⁺ pool. Taken together, these data indicate conversion of 6Yn-ATP into 6Yn-NAD⁺ is likely to be the pathways bottleneck and the availability of β-NMN could be a limiting factor.

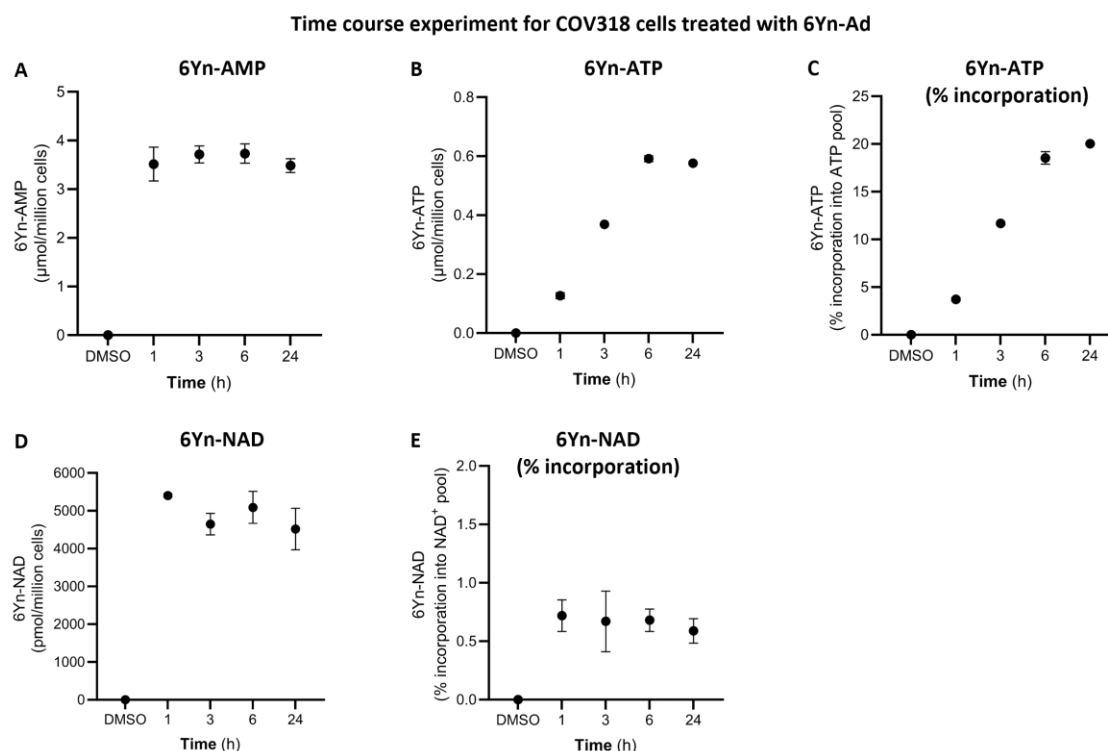


Figure 37. COV318 cells synthesise 6Yn-ATP but this is the bottleneck for 6Yn-NAD⁺ production

COV318 cells were treated with vehicle or 6Yn-Ad (250μM) for 1, 3, 6 and 24 hours. Concentrations of **(A)** 6Yn-AMP **(B)** 6Yn-ATP and **(D)** 6Yn-NAD⁺ are shown in mol/million cells. The % incorporation of **(C)** 6Yn-ATP and **(E)** 6Yn-NAD into their endogenous metabolic pools was determined. For NAD⁺, ATP, β-NMN and 6Yn-Ad concentration see **Appendix Figure 16**. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².

4.3.3. NMNAT-1 overexpression and β-NMN supplementation boost 6Yn-NAD⁺ synthesis

To investigate how to improve the conversion of 6Yn-ATP and β-NMN into 6Yn-NAD⁺ (catalysed by NMNAT-1), NMNAT-1 was overexpressed to explore whether increased availability of the enzyme could upregulate 6Yn-NAD⁺ synthesis. HEK293T cells were selected for subsequent experiments since they are easy to transfect. By western blotting we confirmed that NMNAT-1 is overexpressed 24 and 48 hours (**Figure 38**) after lipofectamine 2000 transfection. Surprisingly, overexpression (OE) of NMNAT-1 almost completely inhibited PARylation.

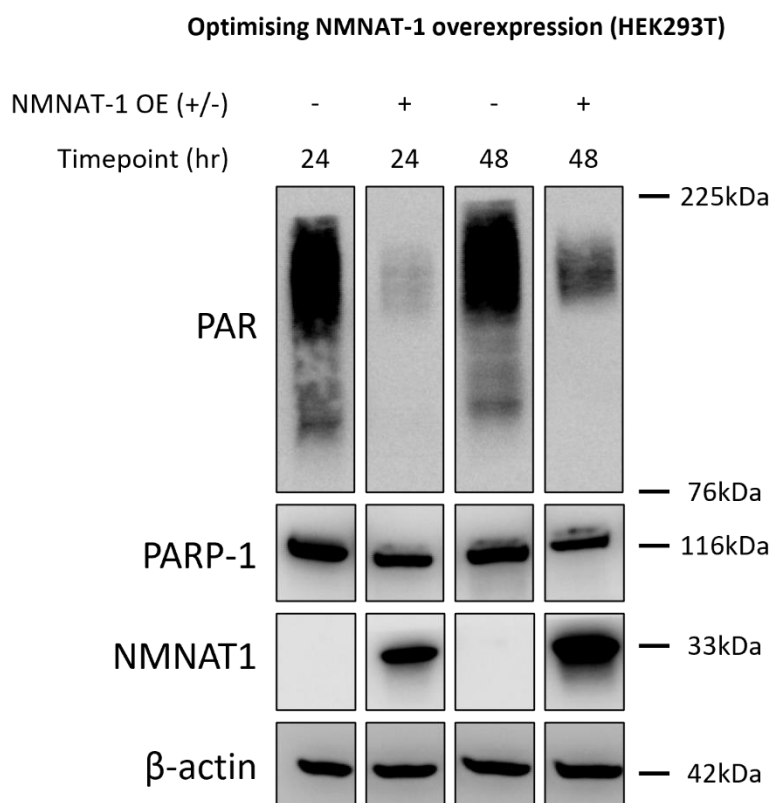


Figure 38. Confirmation of NMNAT-1 overexpression in HEK293T cells

HEK293T cells were cultured for 24-48 hours following the overexpression of NMNAT-1 and lysates were extracted. PARylation, PARP-1 and NMNAT-1 levels were assessed by western blotting. β -actin was used as a loading control. All sections of this image are from the same gel.

The influence of NMNAT-1 OE and β -NMN media supplementation on 6Yn-Ad metabolism was then assessed by metabolomics. Following NMNAT-1 OE the intracellular concentration of 6Yn-Ad increased by ~ 1.75 fold (**Appendix Figure 17**). NMNAT-1 OE also increased the intracellular concentration of 6Yn-AMP from $\sim 0.6\mu\text{mol}/\text{million cells}$ to $\sim 0.8\mu\text{mol}/\text{million cells}$ (**Figure 39**). However, NMNAT-1 OE decreased the concentration of 6Yn-ATP from $\sim 0.2\mu\text{mol}/\text{million cells}$ to $\sim 0.1\mu\text{mol}/\text{million cells}$. This equates to a decrease of 6Yn-ATP incorporation into the ATP pool from $\sim 4\%$ to $\sim 2.5\%$. It was confirmed that intracellular concentrations of β -NMN increased by at least 4-fold following media supplementation. However, β -NMN had little to no influence on the synthesis/consumption of 6Yn-AMP or 6Yn-ATP.

Importantly, β -NMN supplementation was able to boost the synthesis of 6Yn- NAD^+ from $919\text{pmol}/\text{million cells}$ to $1351\text{pmol}/\text{million cells}$. Furthermore, 6Yn- NAD^+ synthesis was

upregulated following NMNAT-1 OE (3863pmol/million cells) and this could be increased further with β -NMN supplementation (5892pmol/million cells). Overexpressing NMNAT-1 increased the incorporation of 6Yn-NAD⁺ into the NAD⁺ pool from ~0.4% to a peak of ~2%. However, although β -NMN supplementation and NMNAT-1 OE led to the greatest increase in 6Yn-NAD⁺ synthesis, because endogenous NAD⁺ synthesis also increased, incorporation into the NAD⁺ pool dropped slightly (i.e., to 1.39%).

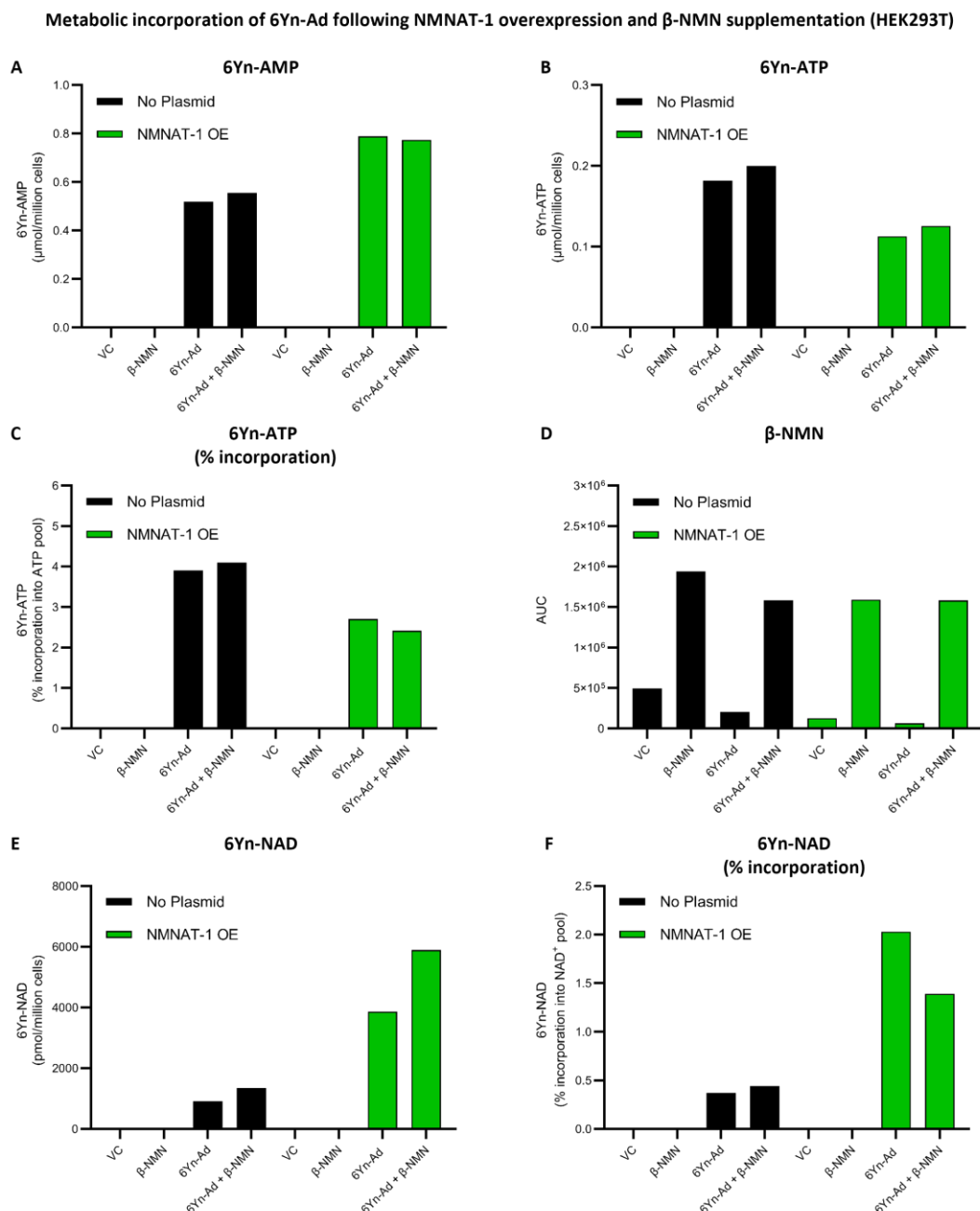


Figure 39. NMNAT-1 overexpression and β -NMN supplementation boost 6Yn-NAD⁺ production

HEK293T cells were treated for 24 hours with vehicle, β -NMN (500 μ M) and/or 6Yn-Ad (250 μ M) (where indicated NMNAT-1 was overexpressed 24 hours prior to treatment). Concentrations of **(A)** 6Yn-AMP **(B)** 6Yn-ATP and **(E)** 6Yn-NAD⁺ are shown in mol/million cells. **(D)** β -NMN concentrations are shown as the area under the curve (AUC). The % incorporation of **(C)** 6Yn-ATP and **(F)** 6Yn-NAD into their endogenous metabolic pools was determined. For NAD⁺, ATP and 6Yn-Ad concentration see **Appendix Figure 17**. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².

4.3.4. NRH boosts NAD⁺ synthesis to a greater extent than β -NMN

In recent years it has been suggested that NRH is a precursor in the synthesis of NAD⁺, and it can surpass NAD⁺ concentration increases caused by β -NMN or NR supplementation^{108,109}. To investigate whether this was the case HEK293T cells were treated for 1 and 24 hours with vehicle, NR, NRH or β -NMN (**Figure 40**, samples were generated by Dr Simeon Draganov). After 1-hour NRH was the only NAD⁺ precursor that was able to boost NAD⁺ synthesis. Furthermore, over the course of 24 hours NRH supplementation led to the largest increase in NAD⁺ synthesis.

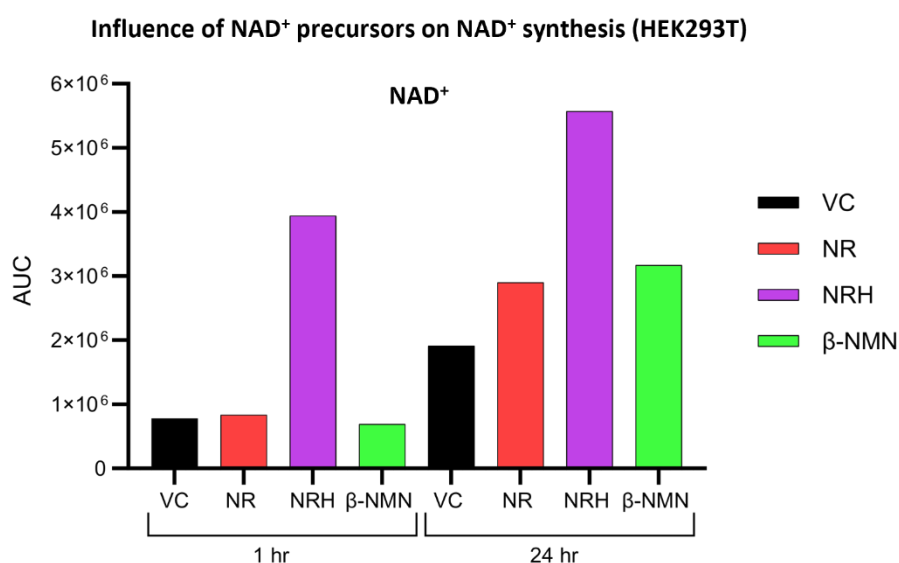


Figure 40. NRH efficiently boosts NAD⁺ synthesis in HEK293T cells

HEK293T cells were treated for 1 and 24 hours with vehicle, β -NMN (500 μ M), NR (500 μ M) or NRH (500 μ M). NAD⁺ concentrations are shown as the area under the curve (AUC). Data is from one independent experiment. Tissue culture, drug treatment and metabolite extraction was performed by Dr Simeon Draganov, this experiment has previously been published in his PhD thesis²⁴².

4.3.5. NRH boosts 6Yn-ATP consumption and 6Yn-NAD⁺ synthesis in 6Yn-ProTide treated cells

A pilot study carried out by Dr Simeon Draganov suggested that the greatest increase in 6Yn-NAD⁺ synthesis occurred following co-treatment with 6Yn-ProTide and NRH, instead of NR or β -NMN (**Appendix Figure 18**)²⁴². However, this experiment was only undertaken with basal levels of NMNAT-1. Considering this, HEK293T cells were co-treated with 6Yn-ProTide and NRH ($\pm$ NMNAT-1 OE), to characterise the influence of NMNAT-1 OE and NRH on 6Yn-NAD⁺ synthesis.

In this experiment, NMNAT-1 OE marginally increased the intracellular concentration of 6Yn-ProTide, and NRH co-treatment increased this further (**Appendix Figure 19**). NMNAT-1 OE led to a minor reduction in 6Yn-AMP (980 nmol/million cells to 935 nmol/million cells). Cells co-treated with 6Yn-ProTide and NRH had lower levels of 6Yn-AMP than cells treated with 6Yn-ProTide alone, with NMNAT-1 OE (935 nmol/million cells to 865 nmol/million cells) and without NMNAT-1 OE (980 nmol/million cells to 799 nmol/million cells) (**Figure 41**).

NMNAT-1 OE had the greatest influence on 6Yn-ATP levels, as overexpression reduced 6Yn-ATP by ~56.7% (171 nmol/million cells to 74 nmol/million cells) in cells treated with 6Yn-ProTide. This corresponds to a reduction of 6Yn-ATP incorporation into the ATP pool from ~9% to ~5.6%. Compared to treating with 6Yn-ProTide alone, co-treatment with NRH reduced 6Yn-ATP levels by 28.1% (171 nmol/million cells to 123 nmol/million cells) in cells with basal NMNAT-1 expression, and by 14.9% (74 nmol/million cells to 63 nmol/million cells) in cells overexpressing NMNAT-1. Interestingly, NRH was able to boost intracellular β -NMN levels by ~4-8 fold in cells with or without NMNAT-1 OE. This could suggest NRH is converted into reduced β -NMN and then β -NMN¹⁰⁹.

Following 6Yn-ProTide treatment and NMNAT-1 OE 6Yn-NAD⁺ concentrations increased by 115.4% (1473 pmol/million cells to 3173 pmol/million cells). This corresponds to an increase in 6Yn-NAD⁺ incorporation into the NAD⁺ pool from 0.26% to 0.46%. Co-treatment with NRH and 6Yn-ProTide increases 6Yn-NAD⁺ by 36.3% (1473 pmol/million cells to 2008 pmol/million cells) in cells with basal NMNAT-1 expression. However, in cells with NMNAT-1 OE NRH and 6Yn-ProTide co-treatment increases 6Yn-NAD⁺ by 46.6% (3173 pmol/million cells to 4653 pmol/million cells). Taken together, these data confirm that NRH supplementation and

NMNAT-1 OE can efficiently boost the synthesis of 6Yn-NAD⁺ following 6Yn-ProTide treatment.

Metabolic incorporation of 6Yn-ProTide following NMNAT-1 overexpression and NRH supplementation (HEK293T)

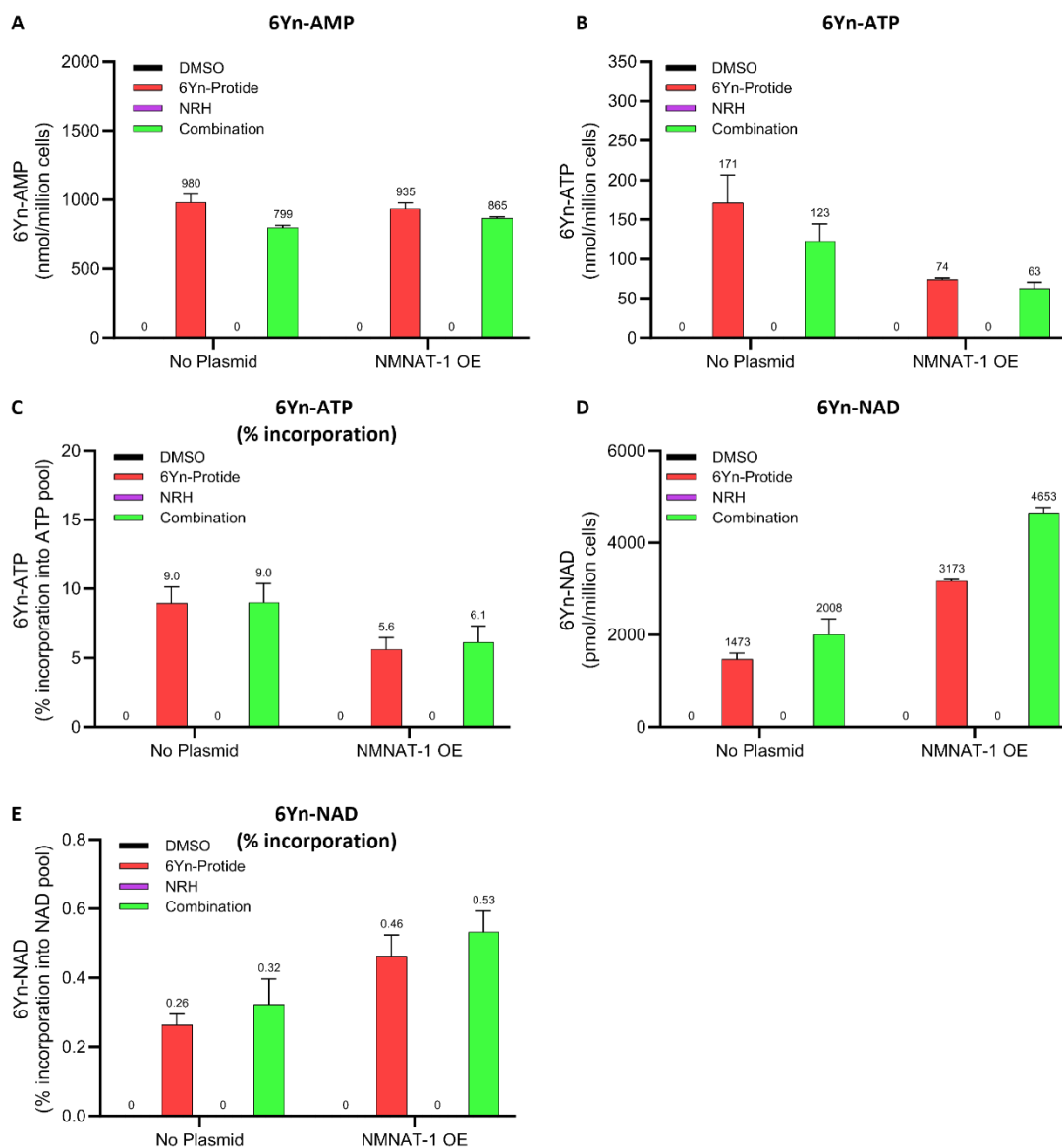


Figure 41. NRH efficiently boosts 6Yn-NAD⁺ synthesis in HEK293T cells

HEK293T cells were treated for 24 hours with vehicle, NRH (500μM) and/or 6Yn-ProTide (100 μM) (where indicated NMNAT-1 was overexpressed 24 hours prior to treatment). Concentrations of **(A)** 6Yn-AMP **(B)** 6Yn-ATP and **(D)** 6Yn-NAD⁺ are shown in mol/million cells. The % incorporation of **(C)** 6Yn-ATP and **(E)** 6Yn-NAD into their endogenous metabolic pools was determined. For β-NMN, NAD⁺, ATP and 6Yn-ProTide concentration see **Appendix Figure 19**. Data is from three independent experiments. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².

4.3.6. Influence of H₂O₂ on 6Yn-NAD⁺ consumption

Given basal levels of PARylation can be quite low in cell lines, we set out to identify conditions that could activate PARP, increase PARylation and potentially increase the utilisation of 6Yn-NAD⁺. To address this, HEK293T cells were treated with the PARG inhibitor (PARGi) PDD00017273, the alkylating agent MMS, H₂O₂ and cisplatin (**Appendix Figure 20**). As expected, inhibiting PARG activity prevented the degradation of PAR chains and increased levels of PAR that could be detected in HEK293T cells. 30 minutes treatment with 5mM MMS inhibited PARylation. However, this was most likely due to inappropriate timepoint/dose selection, given a metabolomics experiment performed in COV318 cells (**Appendix Figure 21**) demonstrated NAD⁺ would be depleted by ~89.2% under these conditions. This was comparable to 24 hours treatment with 5 nM FK866, so the availability of NAD⁺ was likely inhibiting PARylation. By comparison, 10 minutes treatment with H₂O₂ (2 mM) significantly upregulated PARylation of higher molecular weight proteins. Cisplatin treatment (24 hours, 5 or 10 µM) also upregulated PARylation, but PARylation had a greater impact on lower molecular weight proteins.

Since H₂O₂ is commonly used in the literature to boost ADP-ribosylation for the proteomic detection of ADP-ribosylated proteins^{203,209,212,213,221,232,310,311} and it can be conveniently incorporated into our experimental setup, the oxidative DNA damage-inducing agent (H₂O₂) was selected for subsequent experiments. Although we confirmed its influence on PARylation, we had not investigated its influence on 6Yn-ProTide metabolism. To address this, HEK293T cells were treated for 24 hours with vehicle, 6Yn-ProTide and/or NRH (± H₂O₂, 10 minutes prior to lysis), with and without NMNAT-1 OE. Treatment with H₂O₂ reduced the intracellular concentration of 6Yn-ProTide, regardless of if cells were co-treated with NRH or NMNAT-1 was overexpressed (**Appendix Figure 22**). Similarly, 6Yn-AMP levels generally decreased following H₂O₂ treatment (**Figure 42**), although following NMNAT-1 OE 6Yn-AMP levels varied in the absence of NRH supplementation. 6Yn-ATP followed the trend observed in previous steps following H₂O₂ treatment. In the absence of NRH this resulted in a reduction of 6Yn-ATP incorporation into the ATP pool, both with and without NMNAT-1 OE. By contrast, when media was supplemented with NRH, cells were treated with H₂O₂, and NMNAT-1 was overexpressed, a large increase of 6Yn-ATP incorporation into the ATP pool was observed.

Surprisingly, H₂O₂ treatment did not decrease the level of 6Yn-NAD⁺, in fact there was a slight increase in all conditions tested. By comparison, endogenous NAD⁺ was generally reduced following H₂O₂ treatment, and the largest reductions were observed when NRH supplementation was included. As a result, H₂O₂ treatment increased the incorporation of 6Yn-NAD⁺ into the NAD⁺ pool. In the absence of NRH, H₂O₂ increased this from ~0.06% to ~0.13% incorporation into the NAD⁺ pool. Whereas, in the presence of NRH it increased from ~0.04% to ~0.11%. Taken together, these data indicate that H₂O₂ treatment increases the consumption of 6Yn-ProTide, 6Yn-AMP and 6Yn-ATP, and this results in an increase in the synthesis of 6Yn-NAD⁺ and its incorporation into the NAD⁺ pool. However, this data does not suggest that H₂O₂ treatment increases the utilisation of 6Yn-NAD⁺ by PARP-1 for PARylation, since its concentration increases while endogenous NAD⁺ levels decrease.

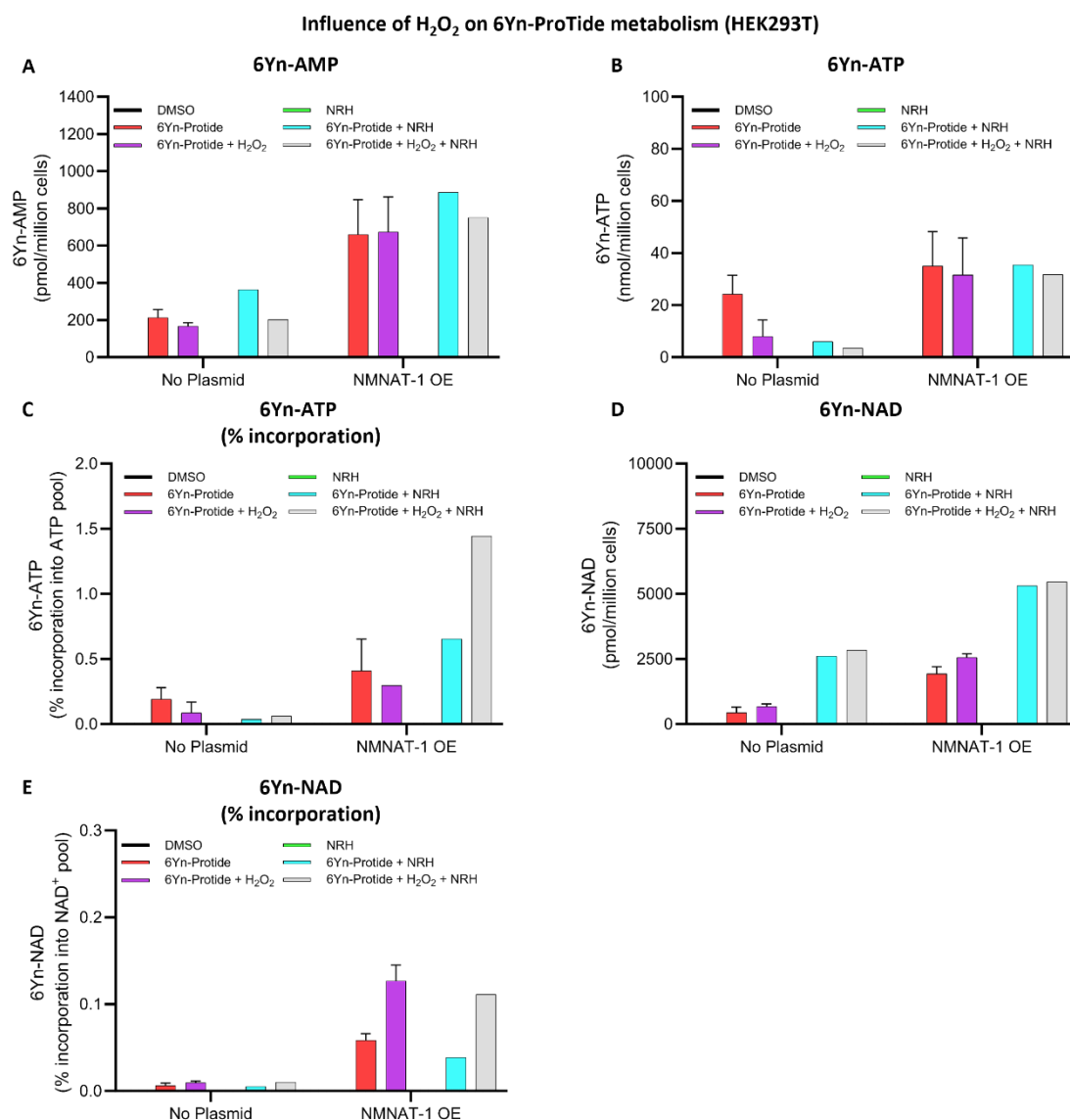


Figure 42. Influence of H₂O₂ on 6Yn-ProTide metabolism

HEK293T cells were treated for 24 hours with vehicle, NRH (500μM) and/or 6Yn-ProTide (100 μM) (± NMNAT-1 OE 24 hours prior to treatment). Where indicated, samples were treated with 2mM H₂O₂ in PBS 10 minutes prior to metabolite extraction. Concentrations of **(A)** 6Yn-AMP **(B)** 6Yn-ATP and **(D)** 6Yn-NAD⁺ are shown in mol/million cells. The % incorporation of **(C)** 6Yn-ATP and **(E)** 6Yn-NAD into their endogenous metabolic pools was determined. For β-NMN, NAD⁺, ATP and 6Yn-ProTide concentration see **Appendix Figure 22**. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².

4.3.7. ADP-ribosylated proteins can be detected by proteomics using 6Yn-ProTide

To investigate whether ADP-ribosylated proteins could be detected using any of the conditions tested in metabolomics assays, Dr Simeon Draganov treated HEK293T cells with vehicle, 6Yn-ProTide and/or NRH ($\pm$ NMNAT-1 OE), and where indicated cells were treated with H₂O₂ (in PBS prior to lysis) (**Figure 43**). Each step of this experiment was performed by Simeon Draganov and is covered in more detail in his PhD thesis²⁴².

Importantly, from these experiments it was demonstrated that enrichment of PARP-1 (0.9 Log₂ Fold change) could be detected in 6Yn-ProTide treated HEK293T cells, demonstrating that even with relatively low levels of 6Yn-NAD⁺ incorporation into the NAD⁺ pool, it is still utilised by PARP-1, enabling its detection by proteomics. Co-treatment with NRH (1.2 Log₂ Fold change) or H₂O₂ (1.3 Log₂ Fold change) marginally increased the enrichment of PARP-1. Combined treatment with 6Yn-ProTide, NRH and H₂O₂ significantly upregulated PARP-1 enrichment (2.5 Log₂ Fold change). However, the highest level of PARP-1 enrichment was observed following the same treatment, but with NMNAT-1 OE (3.9 Log₂ Fold change). Importantly, treatment with the PARPi olaparib reduced PARP-1 enrichment (1.8 Log₂ Fold change) in this condition, indicating inhibiting the catalytic activity of PARP-1 reduces labelling (ADP-ribosylation) with 6Yn-NAD⁺.

Treatment with 6Yn-ProTide alone led to the detection of 205 proteins that were likely to be ADP-ribosylated. Surprisingly, co-treatment with NRH reduced this to 175 proteins. Treatment with 6Yn-ProTide and H₂O₂ ($\pm$ NRH) led to the detection of 293 proteins. However, under these conditions when NMNAT-1 was overexpressed 339 proteins could be detected overall. Taken together, these data suggest that 6Yn-ProTide is a suitable probe to identify ADP-ribosylated proteins.

Detection of ADP-ribosylated proteins by proteomics (HEK293T)

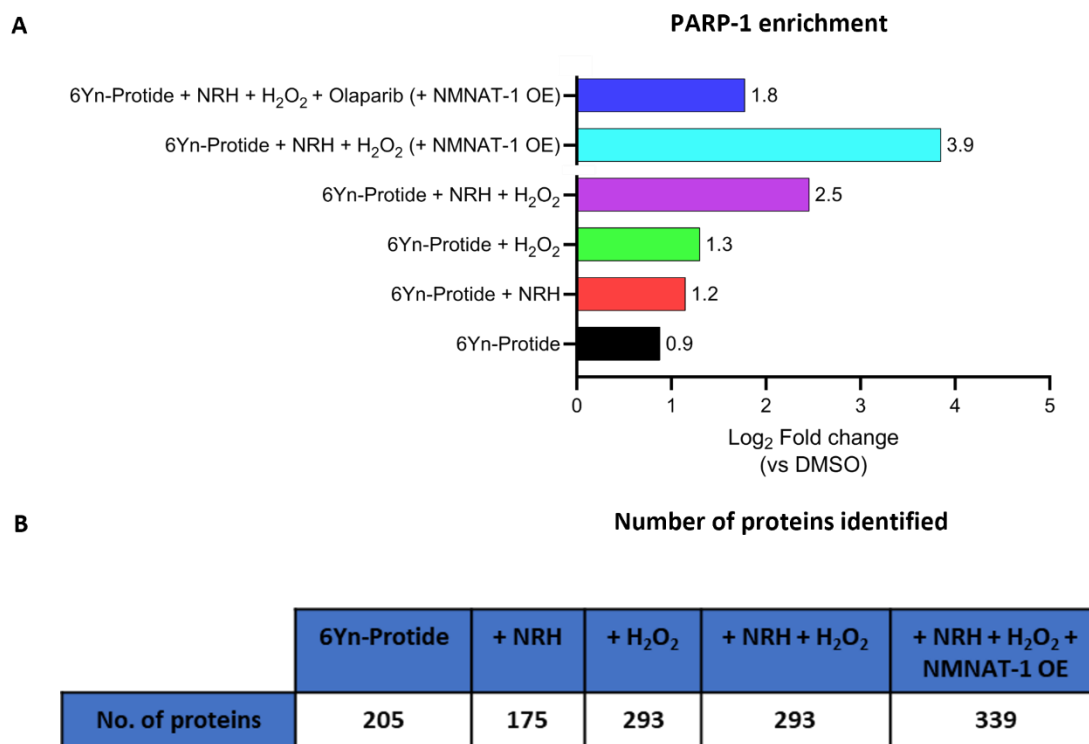


Figure 43. PARP-1 enrichment and protein enrichment following 6Yn-ProTide treatment

HEK293T cells were treated for with vehicle, NRH (500μM, 24 hr) and/or 6Yn-ProTide (100 μM,) (± NMNAT-1 OE 24 hours prior to treatment). Where indicated, samples were treated with 2mM H₂O₂ in PBS (6 minutes) or olaparib (1 μM, 24 hr). NMNAT-1 OE data is from a separate proteomic experiment. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴², and each step of sample preparation and data analysis was performed by Simeon.

4.3.8. 6Yn-Ad and 6Yn-ProTide metabolism in HR deficient KURAMOCHI cells

Given proteomics analysis demonstrated it is possible to detect ADP-ribosylated proteins without NMNAT-1 OE, we set out to investigate how readily the 6Yn-Ad and 6Yn-ProTide probes are converted into 6Yn-NAD⁺ in a HRD cell line, in an ovarian cancer background. Considering this, KURAMOCHI cells were treated for 24 hours with vehicle, 6Yn-Ad or 6Yn-ProTide alone, in combination with NRH or H₂O₂ (in PBS, 10 minutes). Uptake of 6Yn-Ad and 6Yn-ProTide was confirmed by metabolomics (**Appendix Figure 23**). Co-treatment with 6Yn-Ad and NRH reduced 6Yn-Ad levels by ~16.7%. Whereas co-treatment with H₂O₂ reduced 6Yn-Ad by ~83.3%. Interestingly, 6Yn-Ad could be detected in cells treated with 6Yn-ProTide. Given

cells are treated with pure stocks of 6Yn-ProTide it is most likely that synthesised 6Yn-AMP is converted back into 6Yn-Ad by 5'-Nucleotidase²³³ following 6Yn-ProTide treatment. In cells treated with 6Yn-ProTide, NRH co-treatment slightly increased the intracellular concentration of 6Yn-ProTide. Whereas co-treatment with H₂O₂ reduced the intracellular concentration of 6Yn-ProTide by ~43.4%.

To our surprise NRH and H₂O₂ co-treatment had an opposing influence on 6Yn-AMP/ATP in cells treated with 6Yn-Ad and 6Yn-ProTide (**Figure 44**). For example, co-treatment with 6Yn-Ad and NRH or H₂O₂ appeared to reduce the intracellular concentration of 6Yn-AMP and 6Yn-ATP. Whereas co-treatment with 6Yn-ProTide and NRH or H₂O₂ appeared to increase the intracellular concentration of 6Yn-AMP and 6Yn-ATP. A similar trend was observed when assessing 6Yn-NAD⁺ levels, and the incorporation of 6Yn-NAD⁺ into the NAD⁺ pool ranged from 0.4-1.6%. It should be noted that 6Yn-NAD⁺ levels were highly variable between replicates. However, KURAMOCHI cells consistently synthesised higher concentrations of 6Yn-NAD⁺ than were achieved in HEK293T cells under each condition tested. Taken together, these data suggest that it might be possible to detect ADP-ribosylated proteins in KURAMOCHI cells using 6Yn-Ad or 6Yn-ProTide probes.

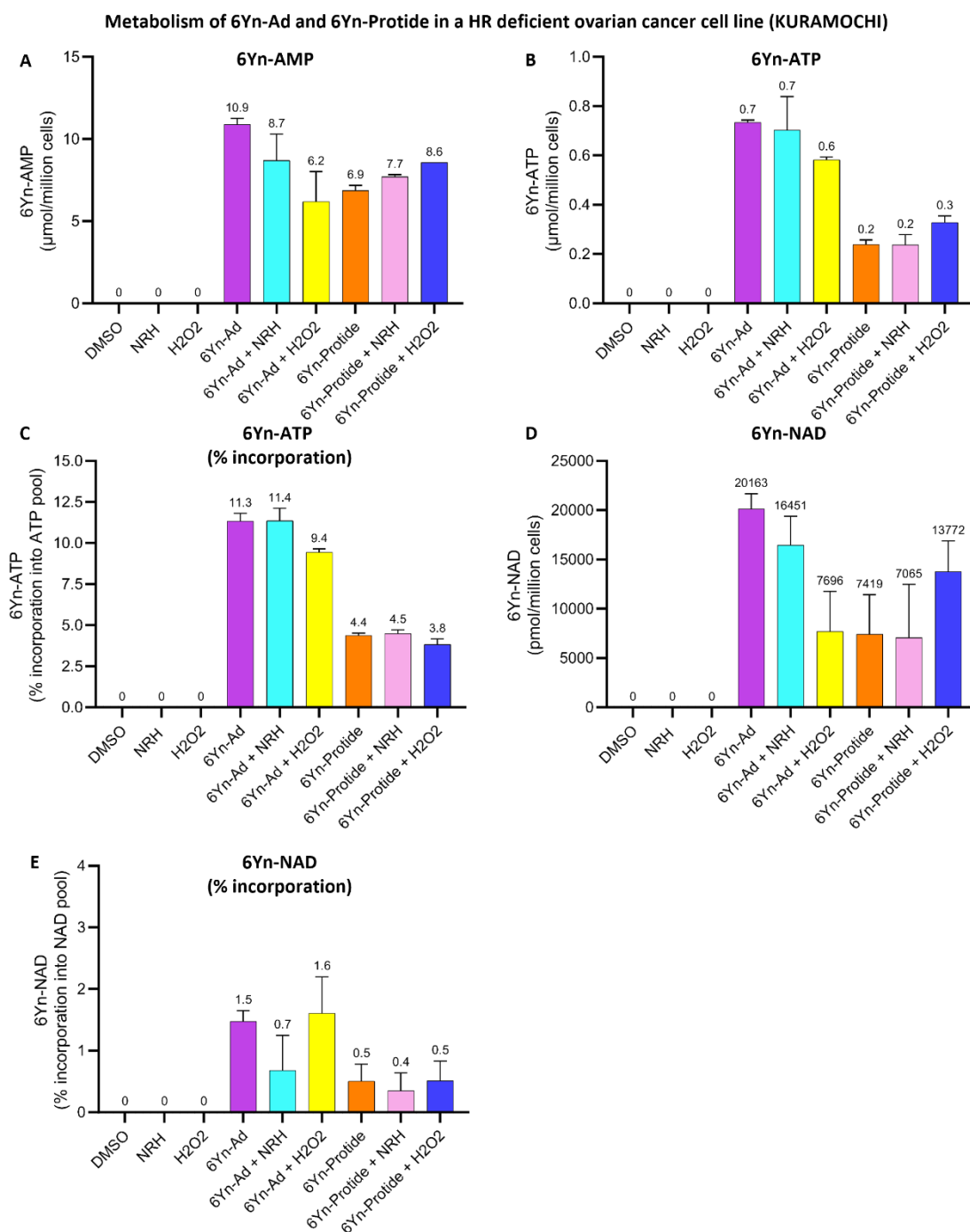


Figure 44. Metabolism of 6Yn-Ad and 6Yn-ProTide in HR deficient HGSOC KURAMOCHI cells

KURAMOCHI cells were treated for 24 hours with vehicle, 6Yn-Ad (500 μM) or 6Yn-ProTide (100 μM). Where media was supplemented with NRH (500 μM, 24 hr) or cells were treated with 2mM H₂O₂ (in PBS) for 10 minutes prior to metabolite extraction. Concentrations of **(A)** 6Yn-AMP **(B)** 6Yn-ATP and **(D)** 6Yn-NAD are shown in mol/million cells. The % incorporation of **(C)** 6Yn-ATP and **(E)** 6Yn-NAD⁺ into their endogenous metabolic pools was determined. For β-NMN, NAD⁺, ATP, 6Yn-Ad and 6Yn-ProTide concentration see **Appendix Figure 23**. Data is from one independent experiment.

4.3.9. Internalisation of the 6Yn-NAD⁺ analogue following DOTAP permeabilisation

Although metabolomics experiments suggest 6Yn-Ad and 6Yn-ProTide probes may be suitable to identify ADP-ribosylated proteins in KURAMOCHI cells (see **section 4.3.8.**) it may be necessary to significantly increase the incorporation of 6Yn-NAD⁺ into the NAD⁺ pool to increase the coverage of the proteomics assay. Since the overexpression of enzymes (e.g., NMNAT-1) can be challenging in ovarian cancer cell lines and this may not sufficiently boost 6Yn-NAD⁺ synthesis, a separate approach could be taken. For example, Lehner and colleagues demonstrated that it is possible to permeabilise HeLa cells with DOTAP and transfect a full length NAD⁺ analogue, without significantly impacting cell viability²³¹.

To investigate whether this approach could be applied in ovarian cancer cells, KURAMOCHI cells were permeabilised with 20 μ M DOTAP and treated with vehicle or 6Yn-NAD⁺ (100 μ M) for 30 minutes. Next, cells were treated with PBS alone or with 2 mM H₂O₂ (in PBS) for 10 minutes, and metabolites were extracted. Cell viability was measured in separate plates, and it was confirmed DOTAP permeabilization did not reduce the viability of cells at the dose tested. As expected, H₂O₂ treatment reduced the level of endogenous NAD⁺ (**Figure 45**). Whereas 6Yn-NAD⁺ levels did not drop following H₂O₂ treatment, suggesting the analogue is not being consumed. However, high levels of 6Yn-NAD⁺ incorporation into the NAD⁺ pool were achieved. In cells treated with 6Yn-NAD⁺ alone a 10.5% incorporation into the NAD⁺ pool was observed. Whereas co-treatment with 6Yn-NAD⁺ and H₂O₂ resulted in 17.6% incorporation into the NAD⁺ pool. These data confirm that it is possible to permeabilise KURAMOCHI cells with DOTAP to incorporate 6Yn-NAD⁺, though it is not yet clear if internalised 6Yn-NAD⁺ is utilised by PARP-1 for PARylation under these conditions.

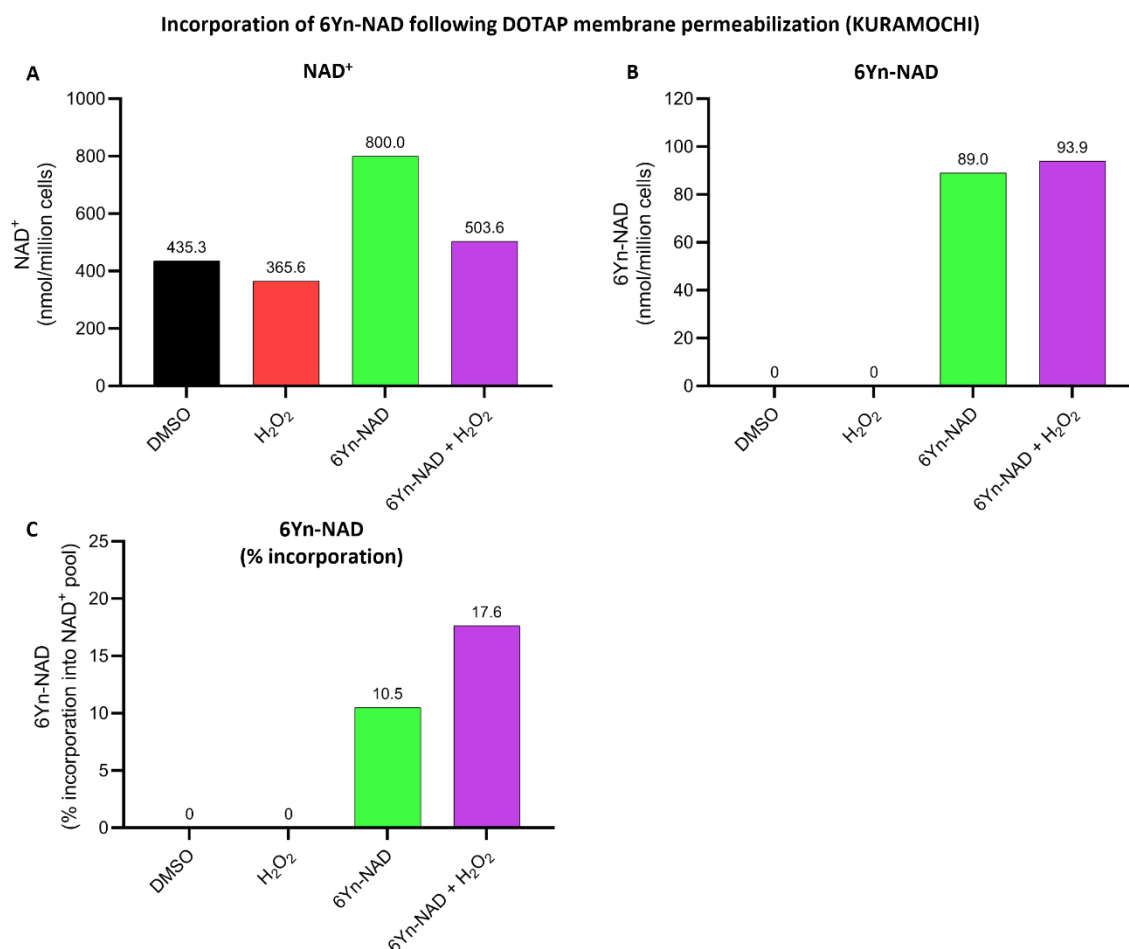


Figure 45. Incorporation of 6Yn-NAD⁺ into KURAMOCHI cells after DOTAP permeabilisation

KURAMOCHI cells permeabilised with 20μM of DOTAP. After 30 minutes cells were treated with vehicle or 6Yn-NAD (100μM) for 30 minutes. Where indicated, cells were treated with 2mM H₂O₂ (in PBS) or PBS alone for 10 minutes prior to metabolite extraction. Concentrations of **(A)** NAD⁺ and **(B)** 6Yn-NAD⁺ are shown in mol/million cells. The % incorporation of **(C)** 6Yn-NAD into their endogenous metabolic pools was determined. Data is from one independent experiment.

4.4. Discussion

“Clickable” NAD⁺ precursor probes such as 6Yn-Ad^{232,234} and 6Yn-ProTide²³⁵⁻²⁴⁰ have previously been used to study ADP-ribosylation and AMPylation, respectively. However, recent work by Dr Simeon Draganov demonstrated that non-specific probe-protein interactions (facilitated by the ribose ring in adenosine) can occur in lysates, leading to non-specific labelling if an additional protein precipitation step isn’t performed in cell lysates prior to click chemistry conjugation^{242,243}. Following the removal of non-specific labelling considerably lower levels of (suspected) ADP-ribosylated proteins are detectable by IGF.

Unfortunately this not only impacts the validity of previous proteomic studies using these probes to identify ADP-ribosylated proteins^{232,234}, but since the conversion of 6Yn-Ad/ProTide into 6Yn-NAD⁺ is a multi-step metabolic process it is challenging to optimise their use for proteomics using IGF/western blotting alone. In previous studies LC-MS/MS based metabolomics has been used to detect 6Yn-NAD⁺²³² and 6Yn-ATP²⁷⁰, respectively. However, in the study performed by Westcott and colleagues²³² the abundance of 6Yn-NAD⁺ wasn't measured, instead they only demonstrated 6Yn-NAD⁺ can be detected in a 6Yn-Ad treated sample (showed a single MS and MS/MS spectra). Although Kielkowski and colleagues²⁷⁰ quantify 6Yn-ATP levels in 6Yn-ProTide treated cells (i.e., chromatographic peak area peak area), like Westcott and colleagues²³², other steps in the biosynthetic pathway were not quantified. Furthermore, endogenous ATP and NAD⁺ metabolites were not measured, so the incorporation of 6Yn-ATP and 6Yn-NAD⁺ analogues within the endogenous ATP and NAD⁺ pools was not assessed. Considering this, there is a need to develop a metabolomics approach to (i) detect the intracellular conversion of precursor probes into 6Yn-AMP, 6Yn-ATP and 6Yn-NAD⁺, (ii) determine the relative incorporation of these metabolite analogues into the endogenous metabolite pools, and (iii) identify conditions that can boost the synthesis of 6Yn-NAD⁺ for proteomics^{242,243}.

In this chapter I show that 6Yn-Ad and 6Yn-ProTide analogues are converted into 6Yn-AMP, 6Yn-ATP, and 6Yn-NAD⁺ analogues using ovarian cancer cell lines (i.e., COV318, HEY-A8, KURAMOCHI and OVCAR-8) and in HEK293T cells. Importantly, this is the first time it has been demonstrated that the 6Yn-ProTide probe can be converted into 6Yn-NAD⁺, and the first time it has been shown that 6Yn-Ad/6Yn-ProTide probes can be converted into 6Yn-NAD⁺ in ovarian cancer cells. Interestingly, although COV318, HEY-A8 and OVCAR-8 all express similar levels of NMNAT-1 (converts 6Yn-ATP and β -NMN into 6Yn-NAD⁺) according to the human protein atlas, COV318 cells produce considerably more 6Yn-NAD⁺ following 6Yn-Ad treatment³¹². However, to our surprise COV318 cells produced less 6Yn-NAD⁺ following 6Yn-ProTide treatment in comparison to HEY-A8 and OVCAR-8 cells. One could argue this is because the least 6Yn-AMP was produced in COV318 cells following 6Yn-ProTide treatment. However, OVCAR-8 cells produced substantially more 6Yn-AMP, yet only synthesised slightly more 6Yn-NAD⁺ than COV318 cells. Unfortunately, in this pilot experiment we did not have access to a 6Yn-ATP standard, so it is unclear if the disparity between 6Yn-AMP and 6Yn-NAD⁺

levels occurs at the 6Yn-ATP step, since the metabolite wasn't quantified. These data highlight that intracellular conversion through the adenosine²³³ and NAD⁺ biosynthesis¹⁰⁷ pathways is complex, highly cell-dependent and potentially precursor probe dependent. Interestingly, KURAMOCHI cells can synthesise more than double the amount of 6Yn-NAD⁺ than COV318 cells following 6Yn-Ad treatment. This could be because KURAMOCHI cells possess ~1.41x more NMNAT-1 according to the human protein atlas³¹². Alternatively, since KURAMOCHI cells possess a *BRCA2*-mutation they may basally have a higher level of PARylation and increased demand for NAD⁺, potentially increasing the flux of our NAD⁺ precursor probes into the NAD⁺ pool. However, these results highlight our approach could be improved further by quantifying the expression of relevant enzymes in the biosynthetic pathway by western blotting, or by assessing expression levels using the human protein atlas³¹².

Another important observation is that conversion of 6Yn-ATP and β -NMN into 6Yn-NAD⁺, which is catalysed by NMNAT-1 in the nucleus, is the rate limiting step of the biosynthetic pathway. Encouragingly we show that this is targetable by supplementing media with β -NMN or NRH, the latter more efficiently boosts NAD⁺ synthesis, as has been reported previously¹⁰⁹. Furthermore, overexpression of NMNAT-1 also boosts the consumption of 6Yn-ATP and the synthesis of 6Yn-NAD⁺. Interestingly, although we found that overexpressing NMNAT-1 boosts 6Yn-NAD⁺ production (and its incorporation into the endogenous NAD⁺ pool), it also significantly inhibited PARylation. Although NMNAT-1 is known to support the catalytic activity of PARP-1 activity by promoting NAD⁺ synthesis, NMNAT-1 is also believed to stimulate ADP-ribosylation through an NAD⁺ synthesis independent mechanism (e.g., allosteric regulation)³¹³, one could speculate that overexpressing NMNAT-1 disrupts this NAD⁺ synthesis-independent activity to inhibit PARylation.

Encouragingly, H₂O₂ activated PARP (increased PARylation), decreased endogenous NAD⁺ levels, and increased the incorporation of 6Yn-NAD⁺ into the NAD⁺ pool. However, to our surprise this did not appear to increase the consumption of 6Yn-NAD⁺, which could suggest that endogenous NAD⁺ is preferentially used by PARP-1 for PARylation. This is a possibility because when assessing the catalytic activity of human PARP-1 with 6Yn-NAD⁺ (compared to NAD⁺), PARP1-catalyzed automodification (PARP activity) and hydrolysis (NADase activity) were ~10-10.8-fold and 0.86-9.82-fold lower, respectively^{220,221}. Alternatively, since 6Yn-NAD⁺ incorporation was typically low (maximum of 2% incorporation was observed), and so

endogenous NAD⁺ is significantly more abundant, it might be more likely that NAD⁺ is consumed by PARP-1 for ADP-ribosylation. However, since NAD⁺ biosynthesis is compartmentalised (e.g., nuclear, cytoplasmic and mitochondrial³¹⁴) it is possible that not all 6Yn-NAD⁺ is in the nucleus. If this were the case H₂O₂ treatment (and nuclear PARP-1 activation) would not necessarily have a significant impact on the level of overall 6Yn-NAD⁺. However, given overexpression of NMNAT-1 (nuclear enzyme) significantly boosts nuclear 6Yn-NAD⁺ production, under these conditions 6Yn-NAD⁺ compartmentalisation should not be a concern. Furthermore, through proteomics we demonstrated that 6Yn-NAD⁺ is utilised by PARP-1 as PARP-1 enrichment was observed (could be reduced with a PARPi) and protein labelling increased following H₂O₂-dependent PARP-activation.

Our most important observation was despite the low incorporation of 6Yn-NAD⁺ into the endogenous NAD⁺ pool, we identified conditions that boost 6Yn-NAD⁺ synthesis (i.e., NMNAT-1 overexpression and NRH supplementation) and activate PARP-1 (i.e., H₂O₂-treatment), improve the level of PARP-1 enrichment, and the number of potentially ADP-ribosylated proteins detected by proteomics, as discussed further here^{242,243}. Demonstrating that metabolomics can be employed to identify optimal conditions for the detection of ADP-ribosylated proteins using NAD⁺ precursor probes.

Despite the limitations associated with delivering NAD⁺ analogues intracellularly by permeabilising cell membranes (e.g., induction of cell stress activates PARP, making it difficult to study endogenous ADP-ribosylation)^{224,231}, we did assess the DOTAP transfection reagents ability to transfect KURAMOCHI cells with the full length 6Yn-NAD⁺ analogue. Using an approach described by Lehner and colleagues²³¹. Importantly, we show that substantially more 6Yn-NAD⁺ can be delivered into KURAMOCHI cells (10.5-17.6% incorporation into the NAD⁺ pool). However, even though more 6Yn-NAD⁺ was being incorporated into the NAD⁺ pool, we once again observed that H₂O₂ treatment led to the consumption of endogenous NAD⁺ but not 6Yn-NAD⁺. Again, suggesting that PARP-1 preferentially uses endogenous NAD⁺ for PARylation, or perhaps that the majority of transfected 6Yn-NAD⁺ is not in the nucleus. Suggesting that measurement of the nuclear 6Yn-NAD⁺ pool³¹⁵, visualisation of nuclear 6Yn-NAD⁺ dependent PARylation via fluorescence microscopy²³¹ and/or validation by IGF (e.g., ± PARPi-treatment) would be more informative than LC-MS/MS based metabolomics for optimising the transfection of full length NAD⁺ analogues.

In future it would be interesting to adapt this metabolomics workflow to optimise the metabolic incorporation of other precursor probes such as 2Yn-Ad²³⁴, 2Yn-ProTide²⁴², 8But-Yn-ProTide²⁴², and the ribose-functionalised NR6 probe²²¹ into their respective NAD⁺ pools. Interestingly, computational molecular docking experiments suggest that 8But-Yn-ATP and 2Yn-ATP analogues may not bind with as high affinity to NMNAT-1 as 6Yn-ATP²⁴², suggesting a chemical genetic (“bump-hole”) approach would be needed with both analogues. However, this was expected for 8But-Yn-analogues, as it should be converted into 8-Bu(3-yne)T-NAD⁺, which can only be utilised by PARPs following the mutation of a large “gatekeeper” amino acid in the active site (e.g., PARP-1^{L877A}, PARP-1^{I895A}, PARP-2^{L443A}, PARP-3^{L394A})^{219,230}. Measuring the conversion of the ribose-functionalised NR6 probe into NAD⁺ analogue 6 (possesses an azido substitution at 3'-OH of nicotinamide riboside) in live cells would be particularly interesting²²¹. Firstly, by having the functional group on the ribose ring rather than the adenine ring the probe would enter the NAD⁺ pool in fewer steps (i.e., NR > β-NMN > NAD⁺ versus Ad > AMP > ADP > ATP > NAD⁺). Secondly, by entering through the salvage pathway (as NR)¹⁰⁷, rather than having to enter via adenosine metabolism²³³ this precursor probe is not only considerably more likely to enter the NAD⁺ pool, but will not be used for AMPylation²³⁵⁻²⁴⁰. Although Zhang and colleagues demonstrate that human enzymes can convert NR6 into NAD⁺ analogue 6, the precursor probe was never tested in human cells²²¹. Given PAR-chains produced with NAD⁺ analogue 6 are PARG resistant it is possible the precursor probe could be cytotoxic^{221,225}.

It would also be interesting to perform a proteomics experiment using the HEK293T cell line following DOTAP permeabilization and 6Yn-NAD⁺ treatment, to directly assess whether this approach would outperform 6Yn-ProTide in the identification of ADP-ribosylated proteins. Furthermore, if this approach is optimal, it could be worth permeabilising ovarian cancer cell lines and treating them with 6Yn-NAD⁺ or other analogues, such as 8Yn-But-NAD⁺, to profile the activity of specific PARP enzymes using a chemical genetics (“bump-hole”) approach (i.e., following CRISPR-mediated knock in of appropriate “gatekeeper” mutations). Particularly as the “bump-hole” approach has only been used to profile the substrate activities of different PARP enzymes in cell lysates to date²²⁶⁻²³⁰.

In summary, this study successfully applies LC-MS/MS based metabolomics to monitor the metabolic conversion of 6Yn-Ad and 6Yn-ProTide probes into the ADP-ribosylation cofactor

6Yn-NAD⁺, in live cells. Using this approach, we identified conditions to maximise the enrichment of PARP-1 and boost the number of (potentially) ADP-ribosylated proteins that could be identified by proteomics.

CHAPTER 5

Assessing the impact of FK866 and olaparib on the transcriptome in HGSOC

Chapter 5 – Assessing the impact of FK866 and olaparib on the transcriptome in HGSOC

5.1. Background

As discussed in **section 1.7.4.**, very little is known about the influence of PARP activity and NAD⁺ availability on AS. Previously, groups have explored the influence of PARP-1 on AS using HeLa²⁵⁹, S2 *Drosophila*²⁵⁸ and more recently with KURAMOCHI²⁶⁴ cells. Although no studies have investigated how NAD⁺ availability could impact AS, since depleting NAD⁺ can inhibit the activity of NAD⁺ dependent enzymes such as PARPs and SIRT6, it is likely that a NAMPTi could alter AS through PARP-dependent and -independent mechanisms (e.g., by regulating the ADP-ribosylation of splicing factor and/or by regulating chromatin structure)^{253,255}. Considering this, we performed an RNA-sequencing experiment in a HGSOC background (using the COV318 cell line) to assess the influence of FK866 and/or olaparib on AS.

5.2. Materials & Methods

5.2.1. RNA Extraction

COV318 cells were seeded on 6 well plates at a density of 330,00 cells/well. The following day, cells were treated with 1µM of olaparib and/or 1nM of FK866. 24 hours later, wells were washed with PBS and RNA was extracted using an RNeasy Mini Kit (QIAGEN, 74106), following manufacturer's instructions. RNA concentration and quality (A_{260/280} and A_{260/230} ratios) a NanoDrop™ 2000 Spectrophotometer (ThermoFisher).

5.2.2. RNA Sequencing

Samples were transferred to the Imperial BRC Genomics Facility on dry ice, for library preparation and RNA-Sequencing. Mitochondrial and Ribosomal rRNA was removed by ribodepletion. 75 bases paired end sequencing was performed on a HiSeq4000 (Illumina), using two lanes to achieve a sequencing depth >50M (by merging data). Quality control checks were performed by the sequencing facility using FastQC³¹⁶. FK866 replicate 3 was not included in subsequent analysis steps as a sufficient sequencing depth was not achieved.

The GRCh38 reference genome (unmasked primary assembly) and GTF file (release 99) were downloaded from Ensembl. STAR 2.7.0a was used to generate genome indexes and run mapping jobs³¹⁷. FeatureCounts was used to perform read summarisation³¹⁸ and differential

expression (DE) analysis was performed using DESeq2³¹⁹. To identify alternative splicing events (ASEs) the SpliceFisher package was used³²⁰. For gene ontology (GO) enrichment analysis the gene ontology resource (<http://geneontology.org/>) was used.

5.3. Results

5.3.1. Olaparib, FK866 and the combination upregulate alternative splicing

Alternative splicing analysis was performed using the SpliceFisher³²⁰ algorithm. 24 hours treatment with 1 μ M olaparib caused 761 ASEs (in 570 separate genes) (**Figure 46**). 1064 ASEs (in 772 separate genes) were identified following 1nM FK866 treatment. Whereas treatment with the combination led to the identification of 1226 ASEs (in 813 separate genes). To our surprise, only 47 genes with ASEs were identified across all three conditions (**Figure 46**). Interestingly, 35 genes with ASEs (detected in at least two conditions) have previously been identified as determinants of PARPi sensitivity from CRISPR-screens^{36,94,321-323} (**Appendix Table 1**).

Influence of olaparib and/or FK866 on splicing (COV318)

A Number of detected alternate splice events (ASEs)

		Skipped exon	Alternative splice sites & mutually exclusive exons	Retained intron	Total events
COV318 (24 hours)	Olaparib (1 μ M)	560 (392)	16 (10)	185 (181)	761 (570)
	FK866 (1nM)	723 (497)	38 (18)	303 (289)	1064 (772)
	Combination	807 (483)	17 (9)	402 (366)	1226 (813)

B Venn diagram comparing ASEs detected following olaparib and/or FK866 treatment

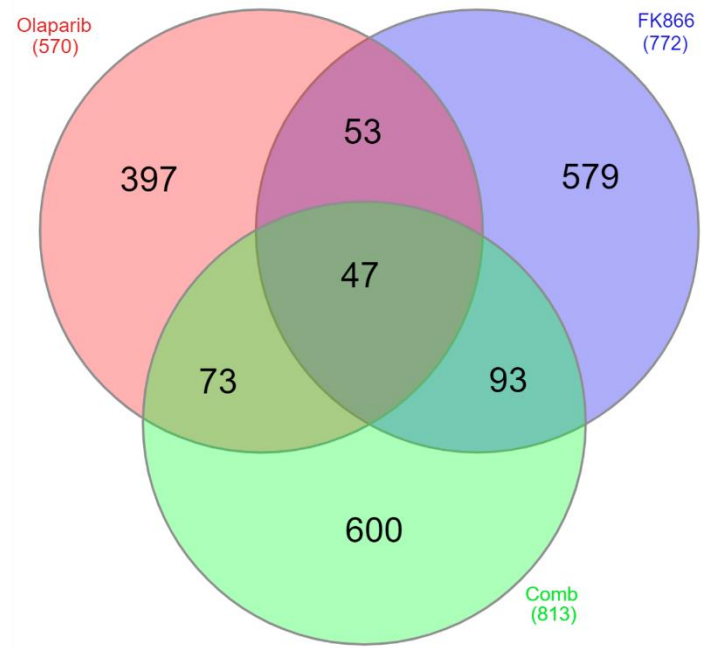


Figure 46. Influence of olaparib and/or FK866 treatment on alternative splicing in HGSOC

COV318 cells were treated for 24 hours with vehicle, olaparib (1 μ M) and/or FK866 (1nM) in technical triplicate. Total RNA was extracted for RNA-sequencing analysis and alternative splicing events (ASEs) (compared to vehicle groups) were detected using SpliceFisher³²⁰. **(A)** The number of exon skipping events, intron retention events, alternative splice sites or mutually exclusive exons is shown. The total number of ASEs is shown in each column, and in brackets duplicate ASEs were excluded (i.e., not including multiple ASEs in the same gene). **(B)** A Venn diagram comparing ASEs in each condition, duplicates were excluded for this analysis.

To improve our understanding of the biological processes that could be altered by these ASEs, gene sets that were identified from SpliceFisher analysis (following the removal of duplicates) were used for GO biological process (GOBP) enrichment analysis (<http://geneontology.org/>). The top 15 pathways (ranked by fold enrichment) are shown for each condition (**Figure 47**).

Following treatment with FK866 four different classes of biological process were altered: the regulation of gene expression, cell cycle, osmotic stress, and DNA repair. By comparison olaparib treatment altered 5 different classes of biological process: cell cycle, gene expression, organelle localisation, establishment or maintenance of cell polarity and DNA repair. Finally, combination treatment altered 7 different classes of biological process: nucleobase-containing compound transport, purine nucleoside monophosphate metabolism, gene expression, homotypic cell-cell adhesion, peptide biosynthetic process, maintenance of protein location and DNA repair.

Although these observations are interesting, it should be noted the presence of one or more ASEs in a gene does not guarantee the activity of a given pathway is altered. Furthermore, none of the detected events have been validated by PCR or confirmed to be translated by western blotting and/or proteomics³²⁴. In summary, a range of ASEs were identified in a HGSOC background following treatment with olaparib and/or FK866. These findings confirm that PARP activity and NAD⁺ availability can regulate alternative splicing. However, more work is needed to validate these events and investigate their functional significance.

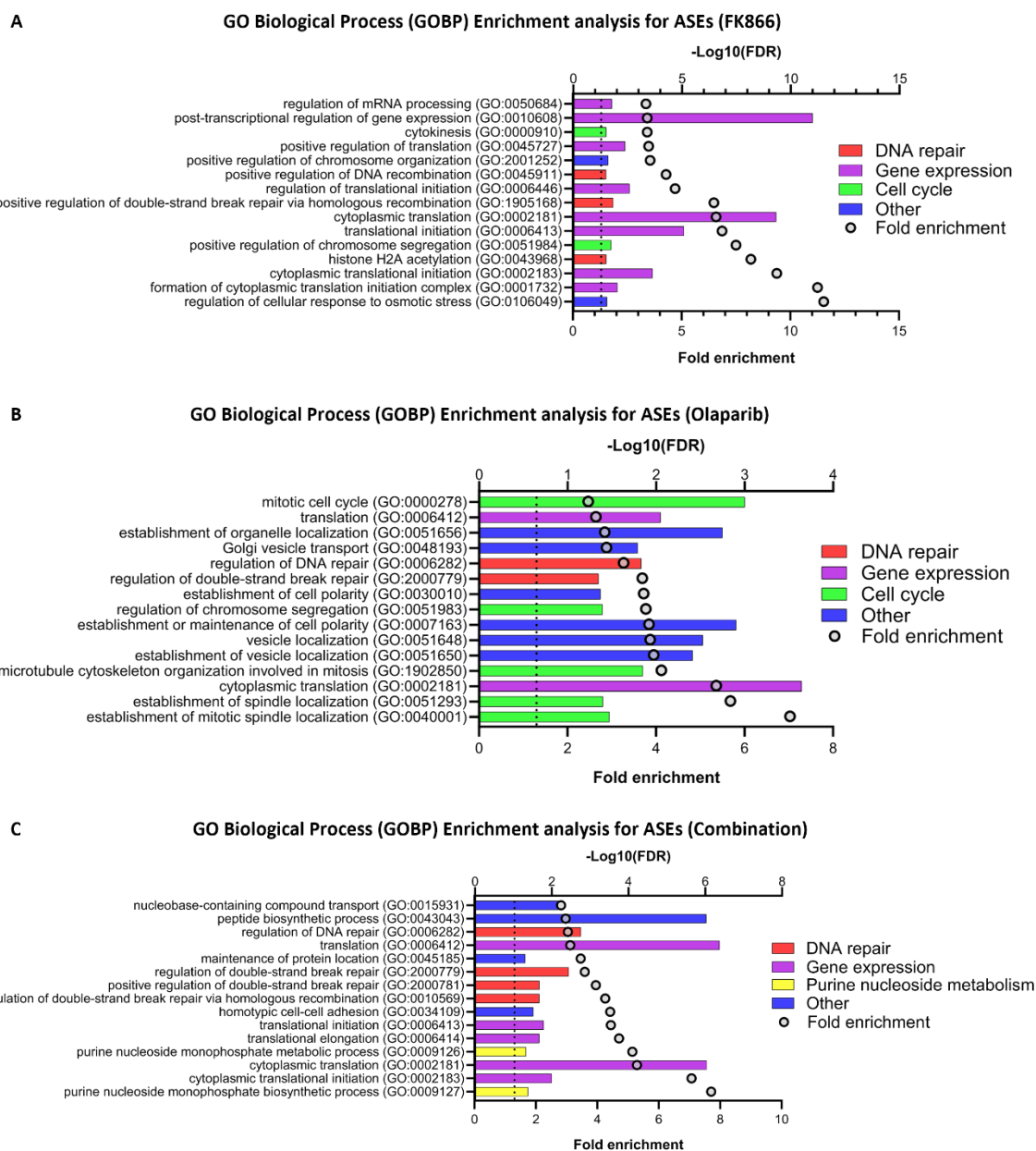


Figure 47. GO Biological Process (GOBP) enrichment analysis of alternative splicing events

COV318 cells were treated with vehicle, **(A)** FK866, **(B)** olaparib or the **(C)** combination for 24 hours and gene ontology (GO) analysis was performed on the detected ASEs to identify biological processes that are impacted relative to vehicle control samples. The top 15 GO terms based on biological process are ranked by fold enrichment. FDR < 0.05 was considered significantly enriched (threshold shown using a dotted black line). The top x-axis shows $-\text{Log}_{10}(\text{FDR})$ values and is visualised using bars on the graph. The bottom x-axis shows fold enrichment values and is visualised using open grey circles.

5.3.2. Gene expression was not significantly altered in selected conditions

To characterise the influence of PARP activity and NAD⁺ availability on gene expression, differential expression analysis was performed using the DESeq2 package³¹⁹. However, as illustrated in volcano (**Figure 48**) and PCA plots (**Appendix Figure 24**) under the selected conditions very few significant changes occurred. For example, in the PCA plot (**Appendix Figure 24**) very little distance separates each group. Vehicle and olaparib groups form three clusters, but do not separate according to treatment received. However, the FK866 and combination groups do separate into two clusters based on the treatment received.

In volcano plots (**Figure 48**) no genes were found to be significantly downregulated (FDR < 0.05, Log₂ fold change < -0.6) in any treatment group. Following FK866 treatment 7 genes were found to be significantly upregulated (FDR < 0.05, Log₂ fold change > 0.6): *ACTG2*, *C5orf46*, *Lnc-FILIP1L-1* and four novel transcripts (*Lnc-KCNE1B-155*, *-156*, *-157* and *-158*). Olaparib treatment significantly upregulated 13 genes: *AXL*, *CPA4*, *MMP1*, *OASL*, *SECTM1*, *SERPINE*, *SNORA57*, *S100A16*, *ZNF295-AS1* and four novel transcripts (*Lnc-KCNE1B-155*, *-156*, *-157* and *-158*). Whereas treatment with the combination only significantly upregulated four genes: *FOSL1*, *LINC01271*, *LINC01629* and *MIR12136*. Although significant changes could be identified, these data indicate that the selected conditions (e.g., drug concentrations/timepoint) were not optimal to identify changes in gene expression.

Influence of olaparib and/or FK866 on gene expression

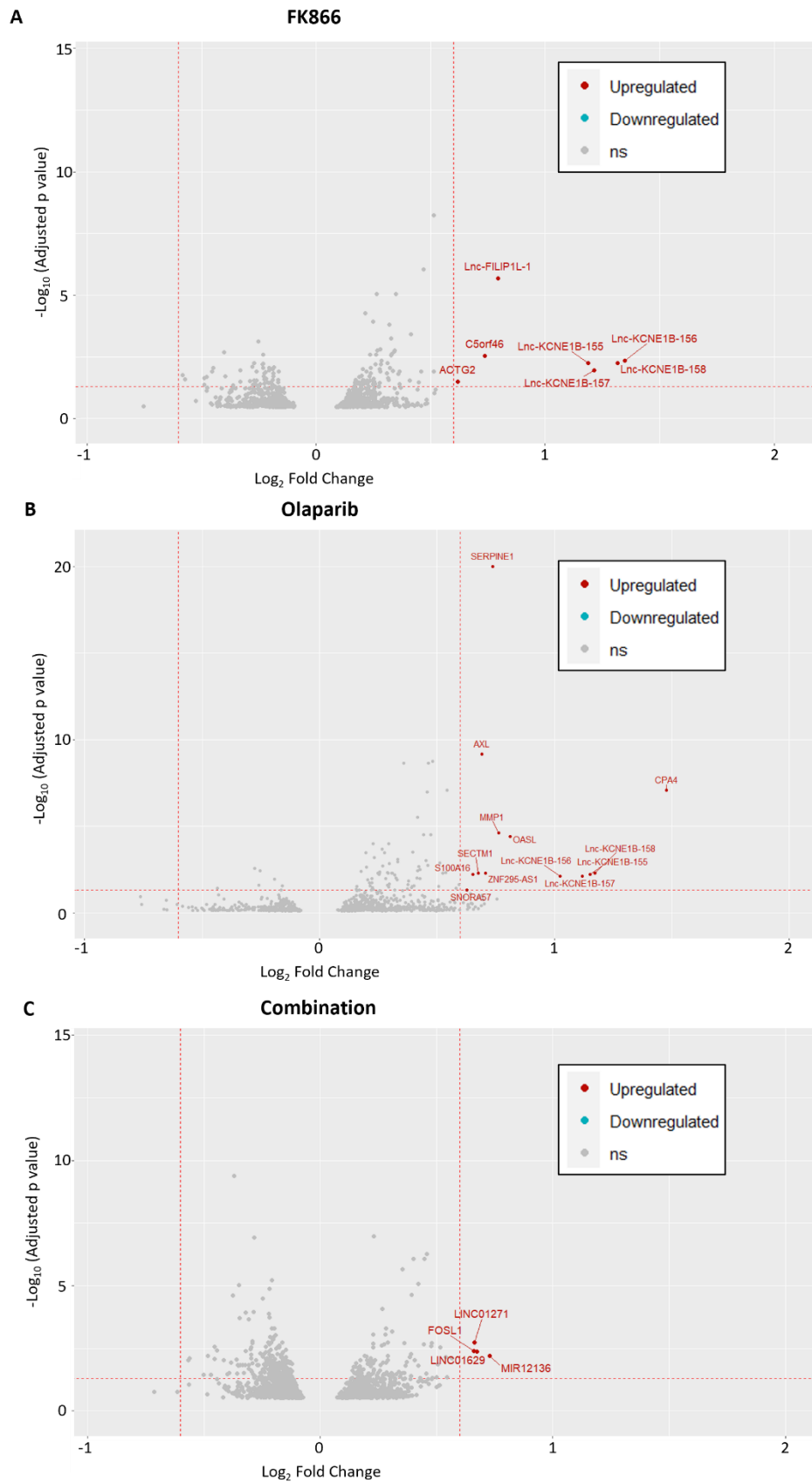


Figure 48. Selected olaparib and/or FK866 doses have very little influence on differential expression

COV318 cells were treated with vehicle, **(A)** FK866 (1nM), **(B)** olaparib (1 μ M) or the **(C)** combination for 24 hours and differential expression analysis was performed using DESeq2. The x-axis shows the log₂ fold change relative to vehicle control, and the cut off for log₂ fold changes of -0.6 or 0.6 are shown with vertical red lines. The y-axis shows the -log₁₀ (Adjusted p-value). FDR values < 0.05 were considered significant and the cut off is shown with the horizontal red line. Genes that are significantly upregulated and significant are shown in red. Genes that are significantly downregulated are shown in blue. PCA plots can be found in **Appendix Figure 24**.

5.4. Discussion

PARP is known to play an essential role in regulating ASEs through the ADP-ribosylation of splicing factors, and by regulating the structure of chromatin^{253,255}. By comparison, very little is known about how NAD⁺ availability regulates AS. NAD⁺ availability can alter the activity of PARPs, but also SIRT6 which regulate the structure of chromatin through coordinated histone acetylation and deacetylation²⁶⁵⁻²⁶⁷. As such, it is likely that NAD⁺ availability can alter AS through PARP-dependent and -independent mechanisms. Excluding a study recently completed by Dr Yuewei Xu²⁶⁴ (manuscript to be submitted) using *BRCA2*-mutant KURAMOCHI cells (HGSOC), studies investigating the role of PARP-1/PARYlation in AS have typically been performed in S2 *Drosophila*²⁵⁸ and HeLa²⁵⁹ cells. Whereas no studies have investigated the role of NAMPT in regulating ASEs. To improve our understanding of how PARP and/or NAMPT activity influence ASEs COV318 (HGSOC) cells were treated with olaparib, FK866, and the combination.

In this chapter I have shown that olaparib (570 genes), FK866 (772 genes) and the combination (1226 genes) promote ASEs in hundreds of individual genes. Furthermore, it was common for more than one ASE to be detected per gene, regardless of treatment condition. Although we anticipated overlap in the AS of genes across conditions since reducing NAD⁺ availability with a NAMPTi inhibits PARP activity⁹⁹, only 47 genes were identified as alternatively spliced across all three conditions. 73 genes were identified as alternatively spliced in the olaparib and combination groups, whereas 53 genes were identified in FK866 and olaparib groups. This could suggest these ASEs are PARP-dependent ASEs. Finally, 93 genes were identified as alternatively spliced in FK866 and combination groups. Interestingly,

of the genes with detected ASEs in at least two conditions (e.g., olaparib and combination groups), ~13% (35/266) were found in genes that are known determinants of PARPi sensitivity, based on CRISPR-screens^{36,94,321-323}. In the RNA-sequencing study performed by Dr Yuewei Xu^{263,264} 90% of the genes with ASEs (i.e., following treatment with indisulam) were found to be downregulated at the protein level, possibly through the activation of nonsense-mediated decay (e.g., AS can introduce 'poison exons' with premature stop codons)^{247,325}. Although with a different drug, it is plausible that ASEs occurring in determinants of PARPi-sensitivity reduce their protein expression and contribute towards the cytotoxic effect of olaparib and/or FK866 treatment. However, it is equally plausible that AS of these genes could be promoting PARPi-R and/or NAMPTi-R²⁴⁴.

GOBP enrichment analysis was performed for ASEs in each condition, and processes involved in DNA repair, cell cycle control and gene expression were highly enriched. Following olaparib treatment establishment of mitotic spindle localisation was the most highly enriched (fold enrichment) biological process, which is perhaps unsurprising since PARPs regulate a variety of mitotic functions³²⁶. Interestingly, following FK866 treatment regulation of cellular response to osmotic stress was the most highly enriched biological process. This could occur through NAMPTi-induced redox imbalance^{131,327} and/or the induction of oncosis¹³². However, the latter is unlikely to occur at this timepoint or dose, as ATP should not be depleted in COV318 cells after 24 hours treatment with 1nM FK866¹³². Finally, the most highly enriched biological process observed following combination treatment was purine nucleoside monophosphate biosynthetic process. This regulates the synthesis of metabolites such as guanosine monophosphate (GMP) and adenosine monophosphate (AMP). In previous studies FK866 has been shown to regulate purine metabolism (e.g., AMP, GMP, deoxyadenosine monophosphate (dAMP), cyclic AMP) in ovarian cancer cell lines¹¹². Furthermore, PARPis have been shown to downregulate purine metabolic pathways³²⁸.

In future a few issues must be addressed to improve our understanding of the ASE dataset. (i) Although ASEs were identified by RNA-Seq, previous proteomics studies have suggested that the majority of alternative transcripts may not even be translated into proteins, so it would be important to perform a paired proteomics experiment to identify which ASEs are translated^{324,329-331}. (ii) Proteomics data (or western blotting of selected proteins) could be used to determine whether olaparib and/or FK866 induced ASEs up or downregulate the

expression of proteins of interest. (iii) ASEs of interest should be visualised by sashimi plot³³² and validated by PCR. (iv) the functional consequences of individual splicing events should be investigated computationally³³³ or through experimental validation. (v) We should investigate whether the presence of more than one ASE in a gene following FK866 and/or olaparib treatment is functionally important. (vi) We could compare this dataset with previous studies^{258,259,264} to investigate if certain ASEs are consistently observed in different models, and if they are predictive of PARPi responses²⁴⁴.

Unfortunately, differential expression analysis provided very little insight into the influence of olaparib and/or FK866 treatment on gene expression changes. Importantly, data analysis was validated by a bioinformatician (Dr Clare Eckold), and quality control checks (e.g., FastQC and PCA plots) suggest the data is correct. Considering this, since COV318 cells are intrinsically resistant to FK866 and olaparib, it is likely the selected doses/timepoint were not optimal to observe large changes in gene expression. However, it should be noted we confirmed the selected doses of FK866/olaparib can reduce NAD⁺/PARylation levels. Regardless, some genes were significantly upregulated.

In FK866 and olaparib treated samples significant changes in the Novel Transcript, Similar To YY1 Associated Myogenesis RNA 1 YAM1 (i.e., *Lnc-KCNE1B-155*, *-156*, *-157* and *-158*) were consistently observed. These long noncoding RNAs (lncRNAs) are believed to play a role in the regulation of MAPK signalling, the actin cytoskeleton and cytokine receptor interactions³³⁴. In FK866 treated samples significant changes in *ACTG2* (actin gamma 2), *C5orf46* (Chromosome 5 Open Reading Frame 46), and *Lnc-FILIP1L-1* (Long non-coding RNA-Filamin A interacting protein 1-like) were also observed. *ACTG2* regulates the maintenance of the cytoskeleton and cellular motility³³⁵. *C5orf46* encodes a small exosomal protein that is overexpressed in some tumour types, and its expression can correlate with patient prognosis³³⁶. Whereas very little is known about the functional role of *Lnc-FILIP1L-1*.

A number of significant changes were observed following olaparib treatment: *AXL* (Tyrosine-protein kinase receptor UFO), *CPA4* (Carboxypeptidase A4), *MMP1* (Matrix Metalloproteinase 1), *OASL* (2'-5'-Oligoadenylate Synthetase Like), *SECTM1* (Secreted And Transmembrane 1), *SERPINE* (Serpin Family E Member 1), *SNORA57* (Small Nucleolar RNA, H/ACA Box 57), *S100A16* (S100 Calcium Binding Protein A16) and *ZNF295-AS1* (ZNF295 Antisense RNA 1).

Upregulation of *AXL* was an interesting observation since *AXL* inhibition/downregulation is known decrease the expression of DNA repair genes (i.e., causing a HR deficiency), sensitising cancer cells to PARPi³³⁷. Interestingly, activation of PARylation has previously been shown to upregulate MMP-1 levels³³⁸. High *OASL* expression is associated with low *ATM* (*ATM* Serine/Threonine Kinase) expression, the latter is a master transducer of DNA damage signals³³⁹. Interestingly, small nuclear RNAs (snoRNAs) are known to activate PARP-1 to control ribosome biogenesis and cell growth³⁴⁰. Although the role of *SNORA57* was not examined in the study by Kim and colleagues (e.g., the authors confirmed *SNORA73A*, *SNORA73B* and *SNORA74A* interact with PARP-1)³⁴⁰, this data could suggest *SNORA57* interacts with PARP-1. To my knowledge an association between PARP-activity and *S100A16*, *SECTM1*, *CPA4* or *ZNF295-AS1* has not been observed previously.

Finally, combination treatment led to significant changes in *FOSL1* (*FOS* Like 1, AP-1 Transcription Factor Subunit), *LINC01271* (Long Intergenic Non-Protein Coding RNA 1271), *LINC01629* (Long Intergenic Non-Protein Coding RNA 1629) and *MIR12136* (MicroRNA 12136). Interestingly, PARP-1 has been shown to regulate angiogenesis and wound healing by regulating the expression of *FOSL1*³⁴¹. However, PARP and/or NAMPT activity has not previously been associated with *LINC01271*, *LINC01629*, or *MIR12136*. In future it could be worthwhile to validate genes of interest by PCR, and their influence on PARPi and/or NAMPTi sensitivity could be explored. However, it would perhaps be more interesting to learn lessons from this experiment and perform a more optimal RNA-seq experiment. For example, it could be worthwhile to perform a similar experiment in a KRAS/PI3K-mutant EOC background, since the combination is highly efficacious in these cells, and could provide further mechanistic insight. In another in house RNA-seq experiment performed by Dr Yuewei Xu²⁶⁴ doses of olaparib (30µM) and/or indisulam were selected based on the upregulation of caspase 3/7 activity. For example, olaparib (1µM) and FK866 (1nM) doses could be increased to 10µM and 5nM, respectively, since data recently generated by PhD student Yitao Xu demonstrates these doses significantly upregulate caspase 3/7 activity in KRAS/PI3K-mutant EOC cells after 24-48 hours treatment.

Overall, the RNA sequencing data provided valuable insights into the influence of FK866, olaparib, and the combination on ASEs. However, additional work will be required to validate individual events and characterise the functional relevance of selected ASEs.

CHAPTER 6

Final discussion and future directions

Chapter 6 – Final discussion and future directions

6.1. Model for the synergy between FK866 and olaparib in RAS/PI3K-mutant tumours

In recent years pre-clinical evidence in TNBC⁹⁸, Ewing's sarcoma⁹⁹, and in HGSOC models with intrinsic or acquired PARPi-R¹⁰⁰, suggests that co-treatment with a NAMPTi (e.g., FK866) sensitises cells to PARPis (e.g., olaparib). The current model for synergy indicates the combination depletes β -NMN/NAD⁺, resulting in a significant reduction in PARylation (compared to PARPi monotherapy), increased levels of DNA damage (measured using γ H2AX) and phosphorylation of stress activated protein kinases (e.g., SAPK/JNK and p38 MAPK), resulting in the induction of apoptosis⁹⁸⁻¹⁰⁰. Unfortunately, dose limiting toxicity has hampered the progress of NAMPTis in clinical trials^{117,122,144}, so it will be necessary to identify biomarkers that can improve their therapeutic window and guide patient selection (e.g., in combination with PARPis)¹¹⁷. However, to date only *BRCA1/2* mutations have been shown to sensitise cancer cells to the PARPi/NAMPTi combination.

In this thesis, I present evidence indicating RAS/PI3K-activating mutations can sensitise EOC cells to the NAMPTi FK866. This included A2780, HEY-A8, OVCAR-8, TOV21G and ID8-*Trp53*^{-/-}; *Pten*^{-/-} cell lines, but not ID8-*Trp53*^{-/-}; *Nf1*^{-/-} cells. In human RAS/PI3K-mutants models increased sensitivity was associated with greater depletion of NAD⁺ and ATP pools following FK866 treatment. Furthermore, smaller β -NMN/NAD⁺ pools were observed in RAS/PI3K-mutants. In isogenic mouse models of HGSOC, since ID8-*Trp53*^{-/-}; *Pten*^{-/-} cells were more sensitive to FK866 compared to *Trp53*^{-/-} cells, unlike ID8-*Trp53*^{-/-}; *Nf1*^{-/-} cells, one could speculate that the PI3K-axis is a more important determinant of NAMPTi-sensitivity. However, it should be noted the ID8 panel did not perfectly model the human RAS/PI3K-mutant EOC cell lines. For example, ID8 models were intrinsically much more resistant to FK866, and differences in NAD⁺ pool depletion were not observed between different genotypes. However as discussed previously (**section 3.2**), differences between ID8 genotypes can be subtle in 2D culture²⁹⁵⁻²⁹⁸. In 2D culture co-treatment with non-lethal doses of FK866 sensitised human EOC cell lines to olaparib, irrespective of mutational background. However, greater caspase 3/7 activation could be observed in KRAS/PI3K-mutant cells (2D culture), and in 3D spheroids RAS/PI3K-mutations were an important determinant of sensitivity to the combination. In the

ID8 panel we again show that co-treatment with non-lethal doses of FK866 sensitised the panel to olaparib in 2D culture, irrespective of mutational background. However, when assessing caspase 3/7 activation, the greatest increase was observed in ID8-*Trp53*^{-/-}; *Pten*^{-/-} cells. Importantly, this translated to benefit *in vivo* as survival outcomes were significantly improved. Taken together, these data indicate that RAS/PI3K-mutant EOC tumours could benefit from treatment with the combination of a NAMPTi and PARPi. This is encouraging as RAS/PI3K-mutations are observed in each EOC subtype⁶, and dysregulated RAS/PI3K signalling is associated with PARPi-R in clinical trials⁵³, so the combination has the potential to improve PARPi efficacy in this group of patients.

Importantly, our data agrees with the current model for PARPi/NAMPTi synergy⁹⁸⁻¹⁰⁰. However, as highlighted previously (**sections 2.3 and 3.2**) some gaps remain in this study and others⁹⁸⁻¹⁰⁰. (i) It would be interesting to thoroughly investigate the influence of the combination on PARP-trapping, since co-treatment with a NAMPTi can reduce PARylation to a greater level than PARPi monotherapy⁹⁹, and evidence suggests NAD⁺ levels correlate with PARP-1/DNA dissociation kinetics²⁷⁸. (ii) The influence of the combination on cell cycle arrest should be assessed, since individually NAMPTis¹²⁹ and PARPis^{280,281} have been shown to induce marked arrest in the G2/M phase, but the combinations influence on the cell cycle hasn't been characterised to date. (iii) Given the rate of NAD⁺ and ATP depletion following NAMPTi-treatment can influence which mode of cell death cells use (e.g., apoptosis, autophagy or necrosis)^{117,132}, and human KRAS/PI3K-mutant cells had a high rate of ATP depletion following FK866 treatment, it would be important to characterize how FK866 and/or olaparib treatment upregulates different modes of cell death. (iv) Furthermore, since FK866 can induce mitochondrial dysfunction resulting in increasing levels of ROS and loss of MMP¹³¹ it would be interesting to investigate what influence the combination has on ROS and MMP, and if this contributes to the increased synergy observed in RAS/PI3K mutant lines. (v) Markers of replication stress could also be assessed (e.g., R-loop formation²⁸²) along with replication fork progression (e.g., DNA fibre assays³⁷). (vi) Finally, the combination should be tested in another *Pten*^{-/-} isogenic model of HGSOc²⁹⁰ or in an isogenic model representing type I-like EOC (e.g., wild-type *TP53* with *KRAS* or *PI3K* mutations)^{295,296}.

The RNA-sequencing study provided further insight into the influence of PARP activity on ASEs in HGSOc²⁶⁴. Whilst providing novel insight into the role of NAD⁺ availability/NAMPT activity

on AS. The influence of PARPi and/or NAMPTi on the regulation of gene expression was assessed, but since optimal conditions weren't used (e.g., doses/timepoint), and a KRAS/PI3K-mutant model wasn't selected, it is unlikely this data provides valuable mechanistic insights to support the use of a PARPi/NAMPTi combination. As discussed previously (**section 5.3**), ASEs of interest and differentially expressed genes could be validated by PCR/western blotting. Alternatively, an RNA-seq and/or quantitative global proteomics study could be performed in a KRAS/PI3K-mutant background, as this could provide novel mechanistic insights to explain why these cells benefit from treatment with the PARPi/NAMPTi combination.

6.2. Studying ADP-ribosylation using 6Yn-NAD⁺ precursor probes

In this thesis I also present evidence that metabolomics can be used to identify optimal conditions that favour the conversion of 6Yn-Ad/6Yn-ProTide probes into the “clickable” ADP-ribosylation co-factor, 6Yn-NAD⁺. My data highlight the importance of not only measuring the concentration of 6Yn-NAD⁺ precursor probes as they pass through the adenosine/NAD⁺ biosynthetic pathways, but also measuring endogenous metabolites (e.g., ATP and NAD⁺) to better understand how our probes are incorporated into their endogenous pools. Working with Dr Simeon Draganov^{242,243} we show that supplementing media with NRH, activating PARP-1 with H₂O₂ and/or overexpressing NMNAT-1 favours the incorporation of 6Yn-NAD⁺ into the endogenous NAD⁺ pool, resulting in increased labelling of PARP-1 and other (potentially) ADP-ribosylated proteins.

There are challenges associated with 6Yn-NAD⁺ precursor probes. As highlighted by Dr Simeon Draganov, adenosine-based probes can non-specifically label lysates if suitable adjustments aren't made to the sample preparation workflow (discussed in **section 4.3** and here^{242,243}). Furthermore, 6Yn-ATP can be used for AMPylation²⁷⁰, rather than being converted into 6Yn-NAD⁺ for ADP-ribosylation. Due to the number of steps involved in this metabolic pathway if inefficient labelling is observed, it can be unclear where metabolic bottlenecks are occurring without the use of metabolomics^{242,243}. Although the 6Yn-NAD⁺ analogue can now be purchased (e.g., from the BIOLOG Life Science Institute), the pre-cursor probes and intermediates in the biosynthetic pathway are not commercially available to my knowledge. Although it is possible to request these compounds be synthesised in house or by companies,

the chemical synthesis is expensive, challenging and results in low yields²⁴². Potentially limiting the availability of precursor probes and intermediates for use in labelling (proteomics) studies or to use as standards in metabolomics assays. Furthermore, data from Dr Simeon Draganov^{242,243} suggests that putative targets from proteomics assays cannot be validated using antibody-specific methods because (i) the level of labelling with the modified NAD⁺ analogue was below the level of detection by western blotting, or (ii) the considerable size of the biotin-ligated complex around the modification site, following click chemistry conjugation, prevents antibodies from binding to the protein(s) of interest. If this is the case novel PARylated proteins identified using this assay would likely have to be validated using co-immunoprecipitation (Co-IP)³⁴², without click chemistry.

Despite these challenges, we have shown 6Yn-NAD⁺ precursor probes can be used to identify known (e.g., PARP-1) and novel (potentially) ADP-ribosylated proteins^{242,243}. As discussed previously (**section 4.3**), in future (i) this metabolomics method could be adapted to optimise the incorporation of other NAD⁺ precursor probes into the NAD⁺ pool (e.g., 2Yn-Ad²³⁴, 2Yn-ProTide²⁴², 8But-Yn-ProTide²⁴², and the ribose-functionalised NR6 probe²²¹). (ii) ‘Hits’ identified in 6Yn-ProTide treated HEK293T cells should be validated by Co-IP. (iii) HEK293T cells should be permeabilised (with DOTAP) and treated with 6Yn-NAD⁺ to investigate whether more significant labelling of the ADP-ribosylated proteome can be achieved using this approach. (iv) The superior method should be used to characterise the PARylome in EOC cell lines (e.g., $\pm$ PARPi). For example, this approach could be used to investigate the influence of different mutations (e.g., KRAS/PI3K) or the acquisition of PARPi-R on the PARylome, or to investigate if all PARPis have the same impact on the PARylome (e.g., PARPis can inhibit the activity of other PARP enzymes³²).

6.3. Conclusions

This study highlights the importance of NAD⁺ metabolism in EOC, and suggests genetic alterations (e.g., KRAS/PI3K) introduce cellular bioenergetic requirements or “metabolic bottlenecks” that can be exploited by combining a NAMPTi and PARPi. Furthermore, we highlight that PARP activity and NAD⁺ availability (NAMPT activity) can influence ASEs in HGSOC. Finally, we have optimized a metabolomics assay that can be applied to identify suitable conditions for the detection of ADP-ribosylated proteins by proteomics, using

“clickable” pre-cursor NAD⁺ probes. In future this approach could be used to characterise the PARylome in EOC.

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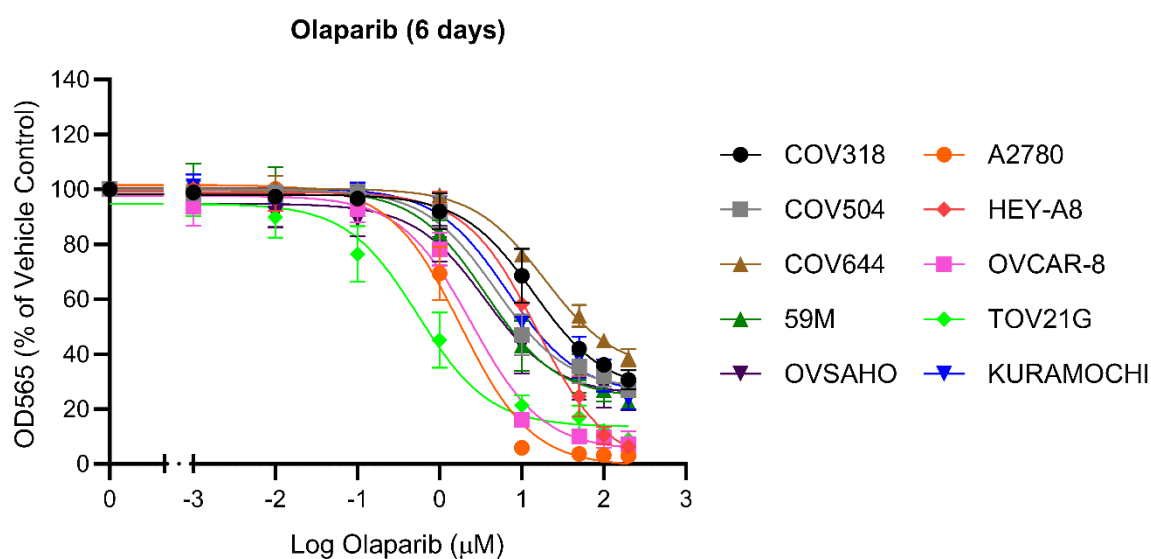
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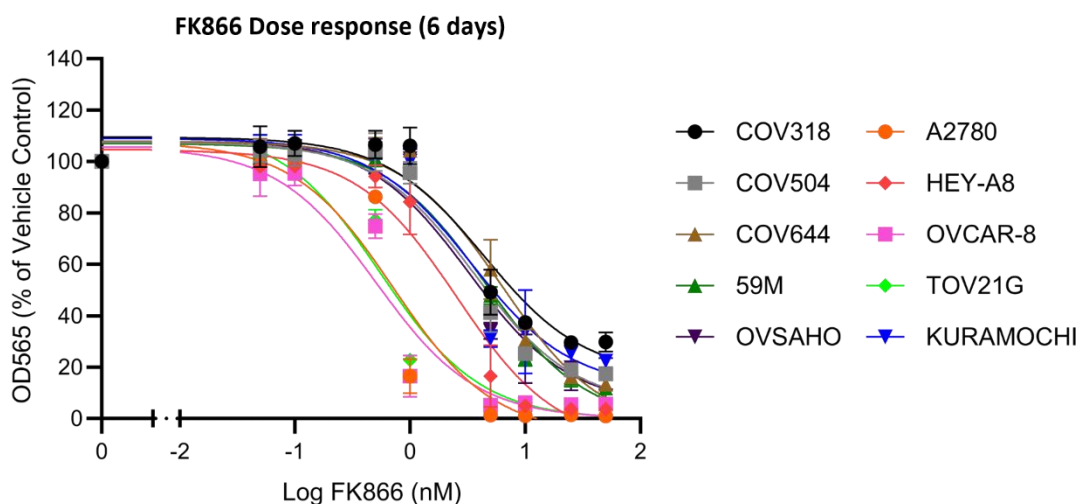
Appendix

Appendix Figures and Tables



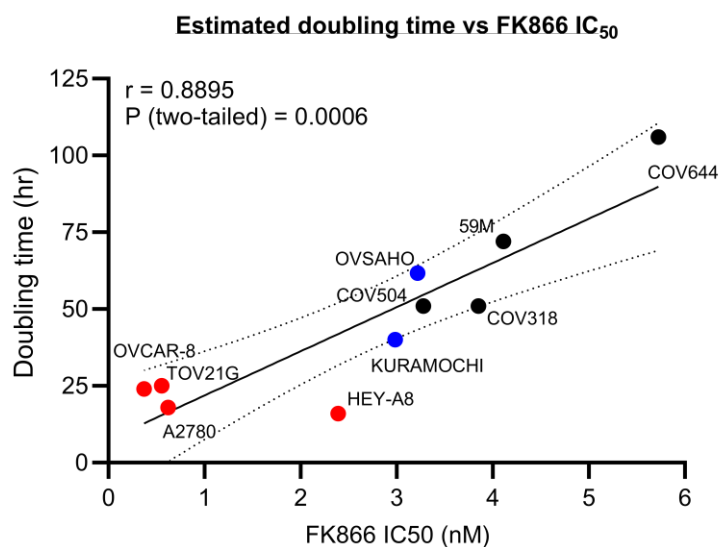
Appendix Figure 1. Olaparib sensitivity in EOC cell lines

EOC cell lines olaparib sensitivity after 6-days treatment. Cell proliferation was measured using the SRB assay. Data are presented as mean $\pm$ SD from three independent experiments.



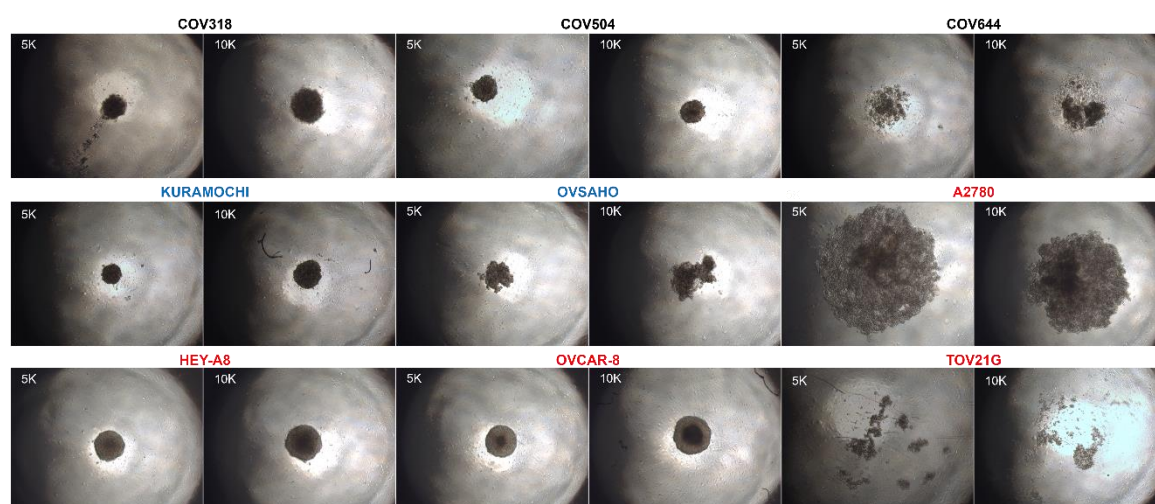
Appendix Figure 2. FK866 sensitivity in EOC cell lines

EOC cell lines FK866 sensitivity after 6-days treatment. Cell proliferation was measured using the SRB assay. Data are presented as mean $\pm$ SD from three independent experiments.



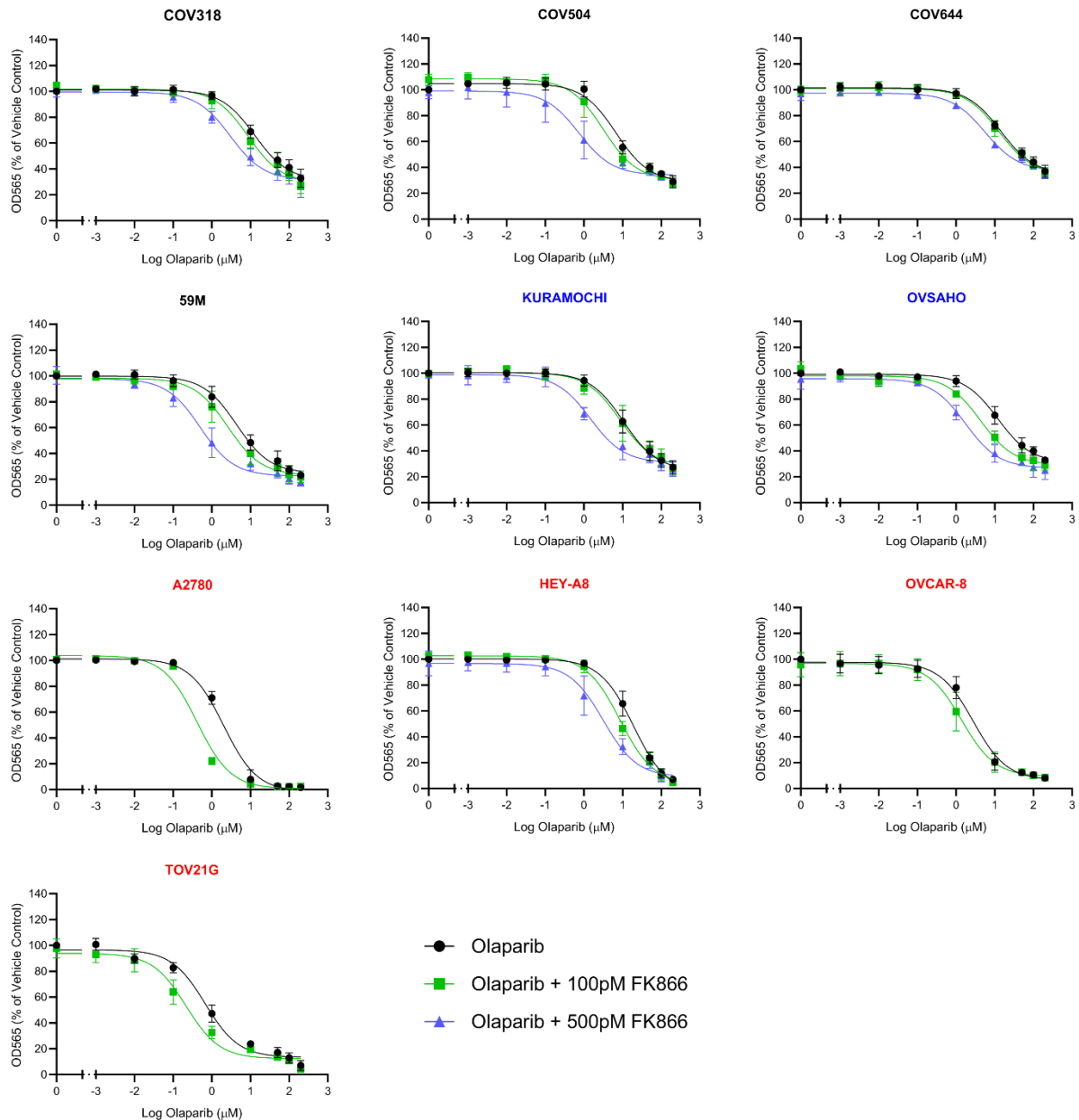
Appendix Figure 3. Relationship between EOC cell lines doubling time and FK866 sensitivity

Pearson correlation coefficient (R) and linear regression were used to analyse the relationship between each cell lines estimated doubling time and FK866 sensitivity (IC_{50}). r and p values are shown.



Appendix Figure 4. EOC cell line spheroid formation

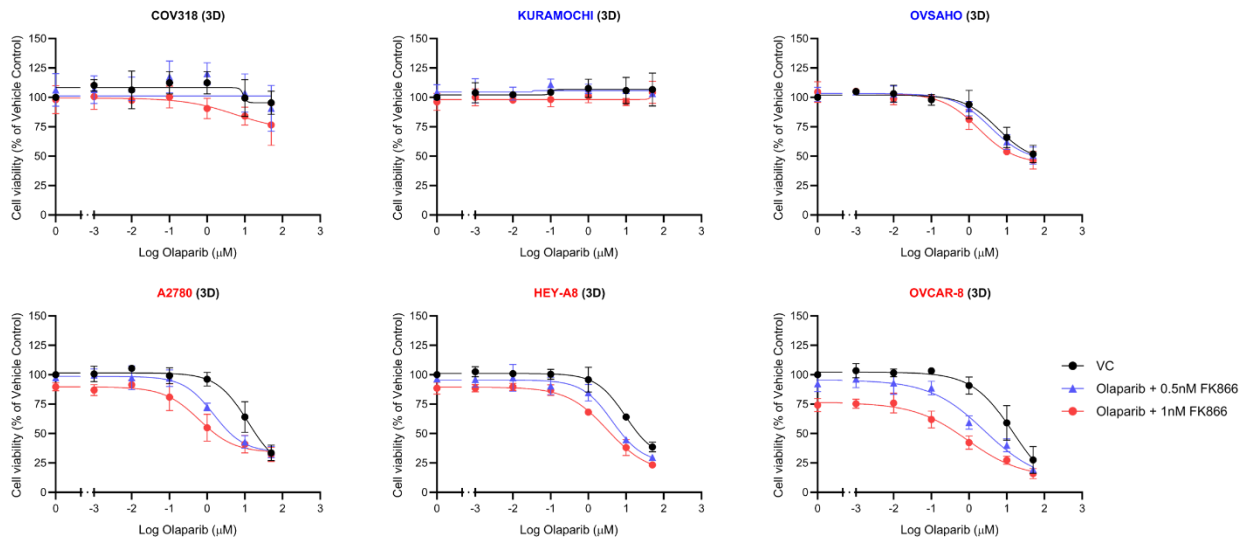
EOC cell lines were seeded on ULA 96 well plates at densities of 5,000 or 10,000 cells per well. 72 hours after seeding images were taken using an Eclipse TE2000-U Inverted microscope (Nikon) at 10X magnification. Red titles indicate cells have a mutation in KRAS/PI3K signalling. Blue titles indicate cells have a mutation in BRCA1/2. Black titles indicate cells don't possess mutations in either pathway.



Appendix Figure 5. Co-treatment with olaparib and FK866 in EOC cell lines

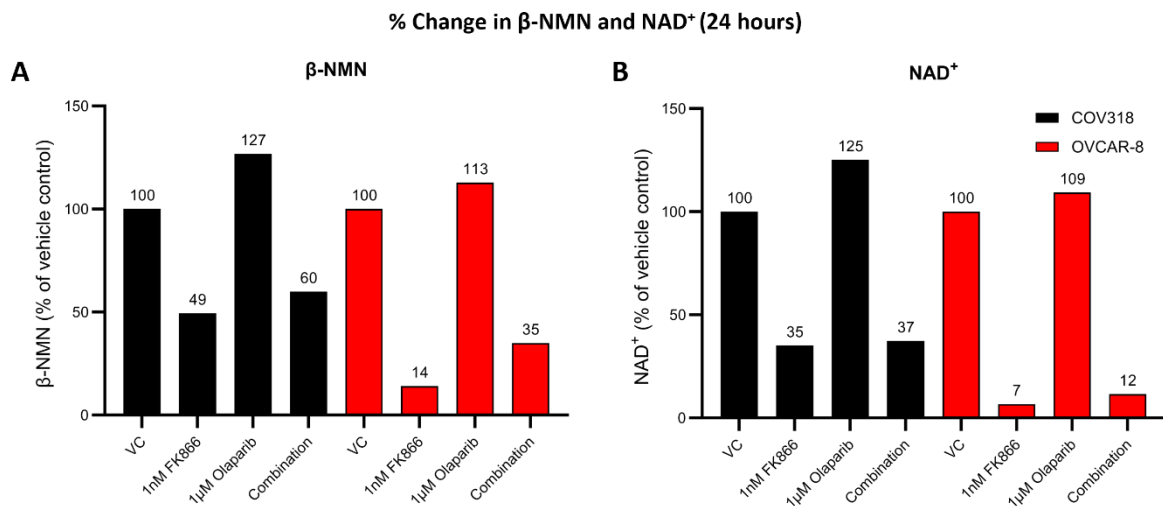
EOC cell lines were treated with olaparib and FK866 for 6-days treatment. Cell proliferation was measured using the SRB assay. Red titles indicate cells have a mutation in KRAS/PI3K signalling. Blue titles indicate cells have a mutation in BRCA1/2. Black titles indicate cells don't possess mutations in either pathway. For A2780, OVCAR-8 and TOV21G graphs +500pM FK866 data was removed as the FK866 monotherapy alone reduced cell proliferation. Data are presented as mean $\pm$ SD from three independent experiments.

3-D spheroid cell viability (6 days)



Appendix Figure 6. FK866 and olaparib reduces the viability of KRAS/PI3K-mutant EOC spheroids

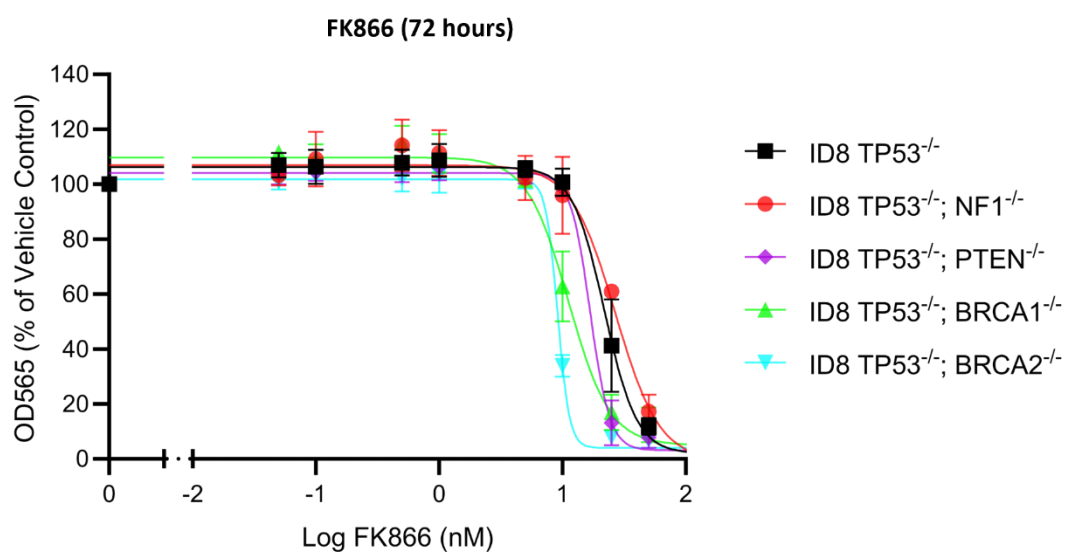
EOC cell lines were co-treated with olaparib (0.001 to 50μM) and FK866 (0.5 to 1nM) to assess their sensitivity to the combination after 6-days treatment. Cell viability was measured using the CellTitre-Glo® 3D assay. Data is the average $\pm$ SD of three independent experiments. Red titles indicate cells have a mutation in KRAS/PI3K signalling. Blue titles indicate cells have a mutation in BRCA1/2. Black titles indicate cells don't possess mutations in either pathway.



Appendix Figure 7. % change in β -NMN/NAD⁺ pools in COV318 and OVCAR-8 EOC cell lines

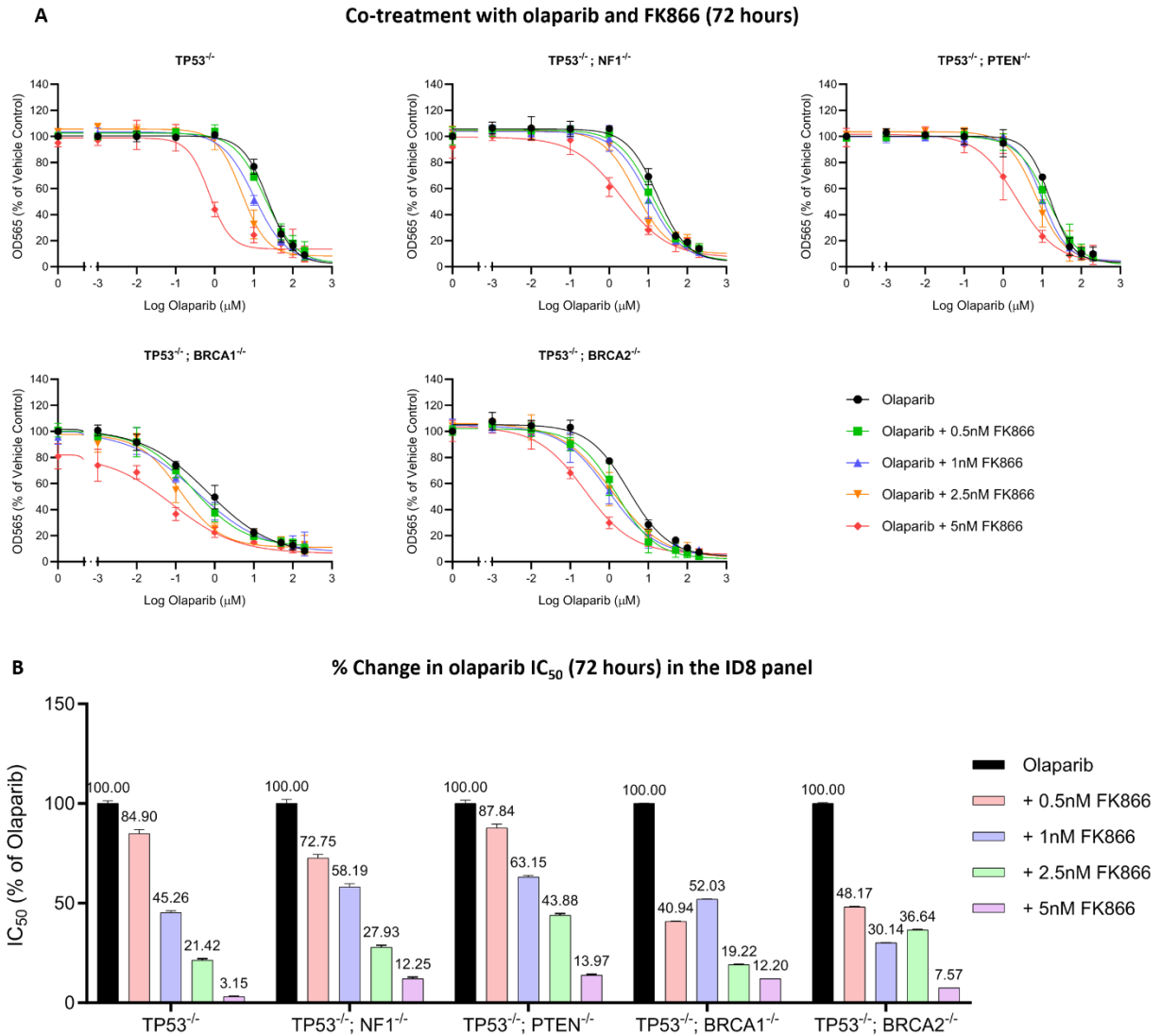
COV318 (KRAS-wild type, black) and OVCAR-8 (KRAS-mutant, red) cells were treated for 24 hours with vehicle, 1μM olaparib, 1nM FK866 or the combination and then extracted for RP UPLC-MS/MS

analysis. % change in **(A)** β -NMN and **(B)** NAD^+ levels was calculated from the average molar concentration/per million cells in each condition. Data is the mean of three independent experiments.



Appendix Figure 8. FK866 sensitivity in ID8 cell lines

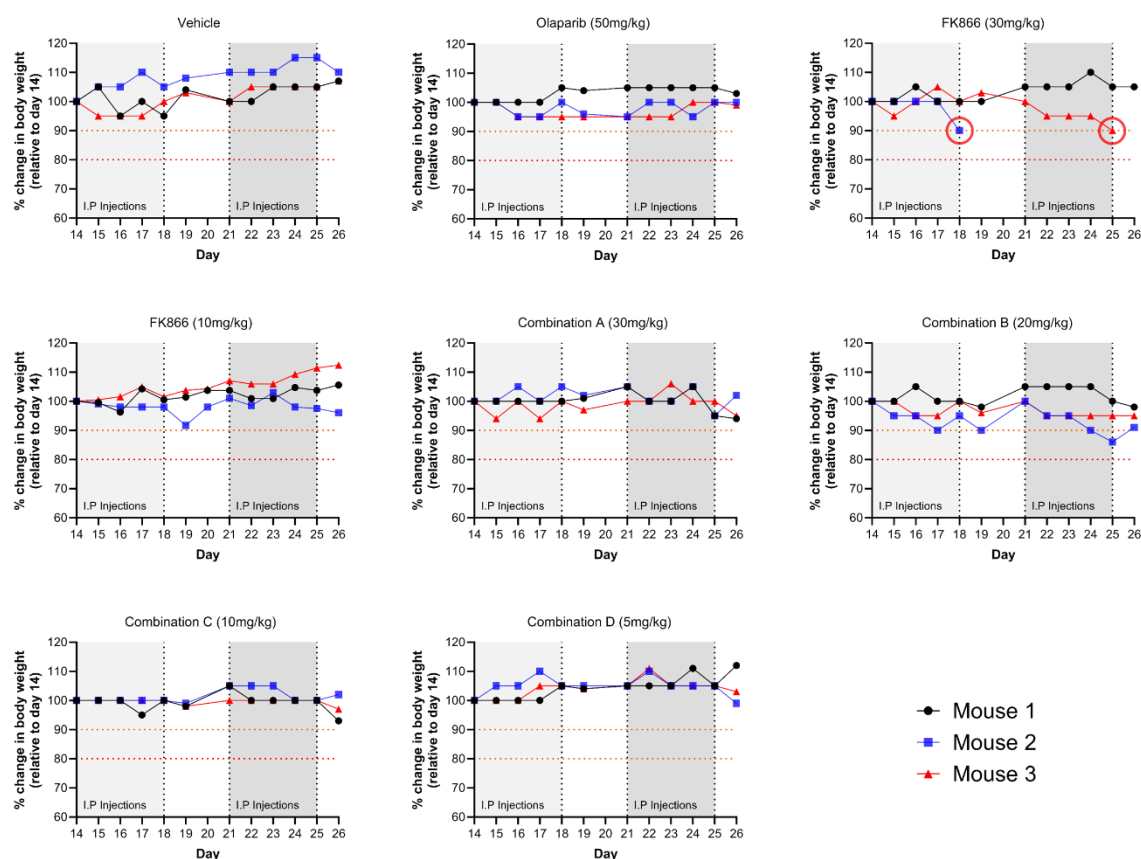
ID8 cell lines FK866 sensitivity after 72 hours treatment. Cell proliferation was measured using the SRB assay. Data are presented as mean $\pm$ SD from three independent experiments.



Appendix Figure 9. Co-treatment with olaparib and FK866 in ID8 panel

(A) ID8 cell lines were treated with olaparib and FK866 for 72 hours. Cell proliferation was measured using the SRB assay. **(B)** % change in olaparib IC_{50} following co-treatment with FK866. Data are presented as mean $\pm$ SD from three independent experiments.

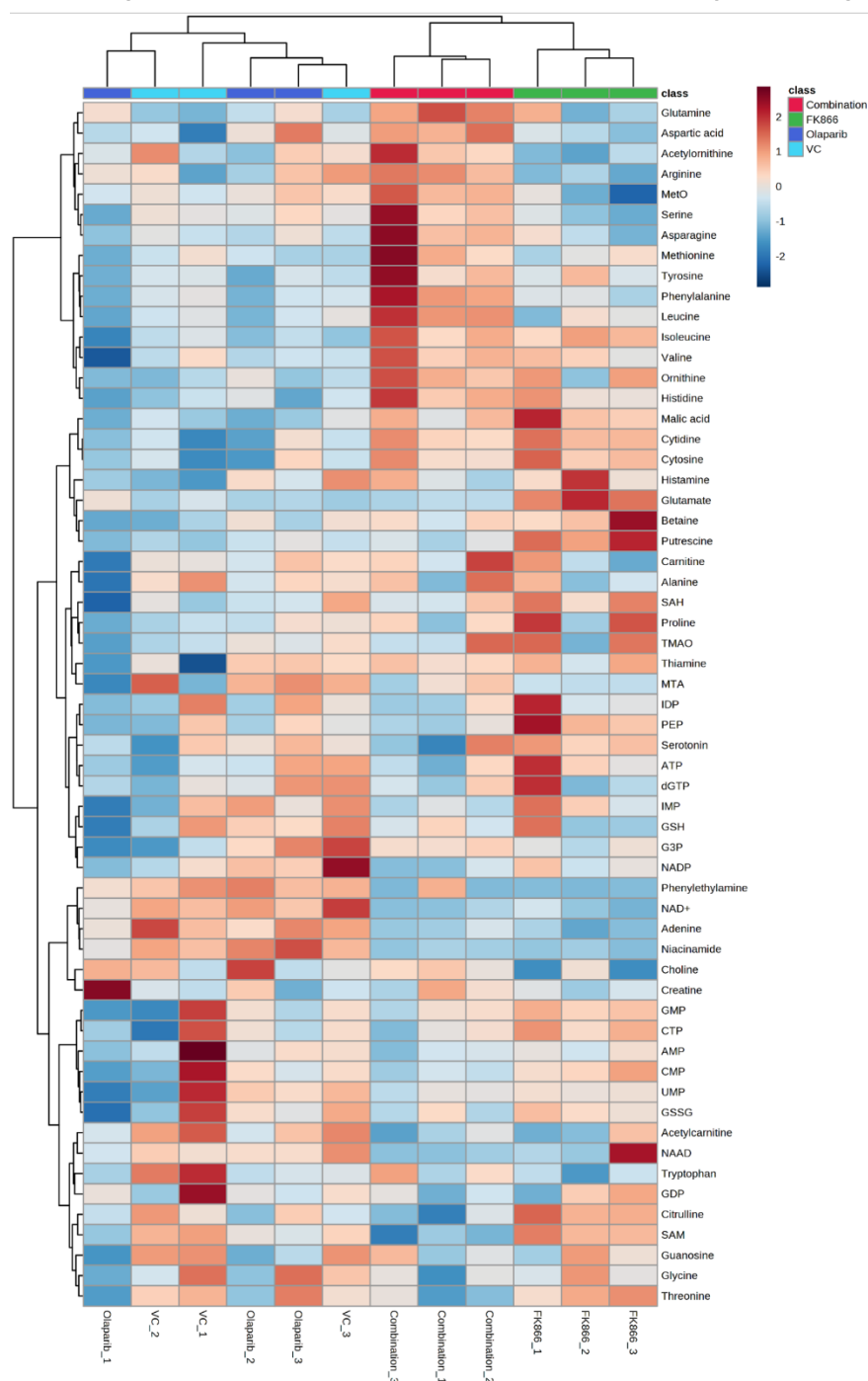
Relative body weight changes of mice during the endpoint (toxicology) *in vivo* study



Appendix Figure 10. Relative body weight changes of mice during the endpoint *in vivo* study

Relative body weight changes in mice were measured during drug administration. FK866 (10mg/kg) data is from an independent experiment. Two mice in the FK866 (30mg/kg) group experienced weight loss (10%), constipation and signs of pain. These mice were culled before the studies completion and the day of culling is highlighted with a red circle. Orange and red dotted lines from the y-axis indicate the threshold for 10% and 20% (maximum limit) drops in body weight, respectively. Excluding FK866 (10mg/kg), mice were weighed on scales that were unable to measure changes in weight to at least one decimal point.

Heatmap of omental tumour metabolomics – *in vivo* (endpoint) study

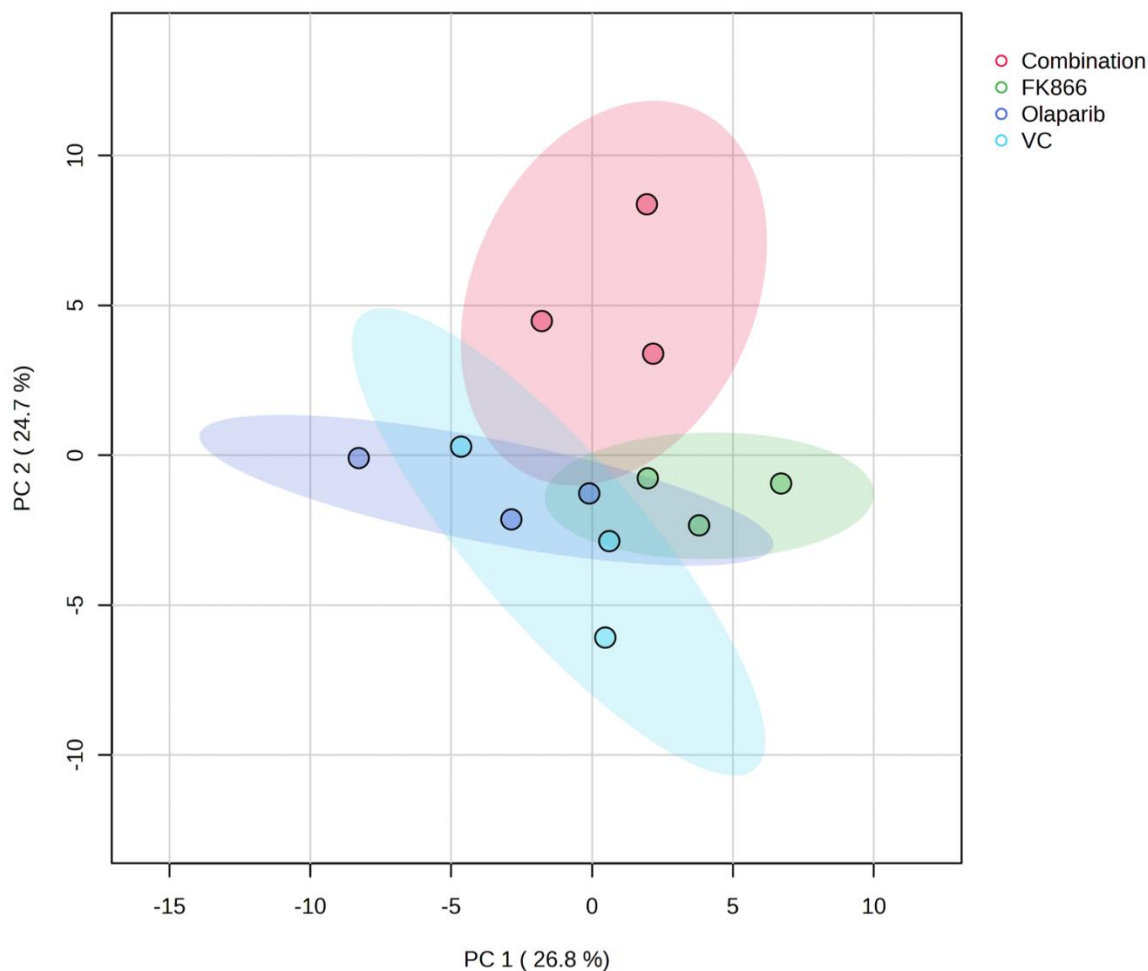


Appendix Figure 11. Heatmap of metabolites that discriminate between treated and untreated omental tumours during the endpoint *in vivo* study

Vehicle, olaparib (50mg/kg), FK866 (10mg/kg) and combination (50mg/kg olaparib and 10mg/kg FK866) treated omental tumours were homogenised and metabolites were extracted (in collaboration with PhD student Yitao Xu). Targeted metabolomics analysis was performed and heatmaps were generated using Metaboanalyst (v.5.0.), using peak abundances of all metabolites. Hierarchical

clustering was performed on individual samples using Pearson distance and Ward clustering; scale is based on Z-score.

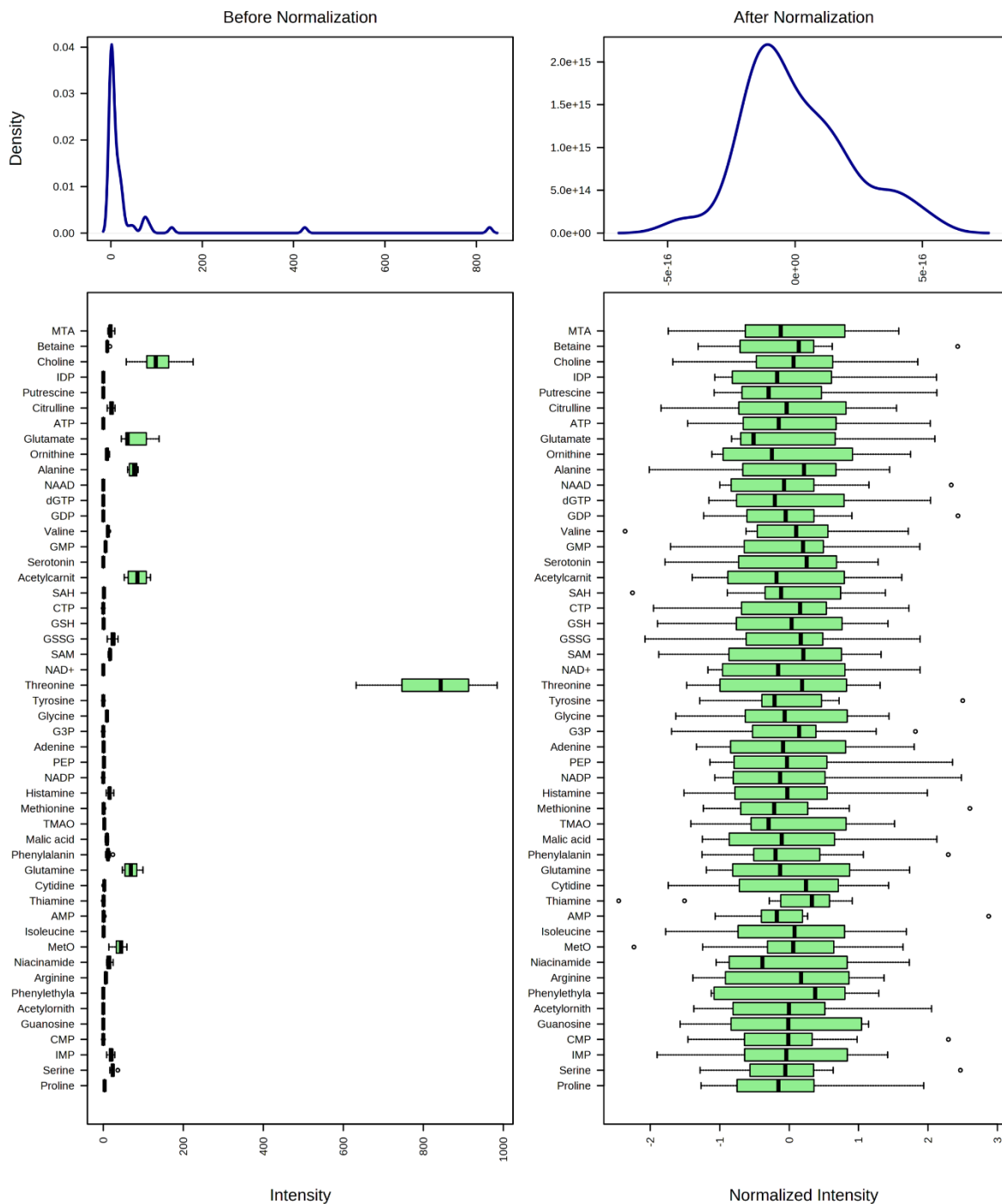
PCA scores plot for omental tumour metabolomics – *in vivo* (endpoint) study



Appendix Figure 12. PCA scores plot based on metabolites from treated and untreated omental tumours during the endpoint *in vivo* study

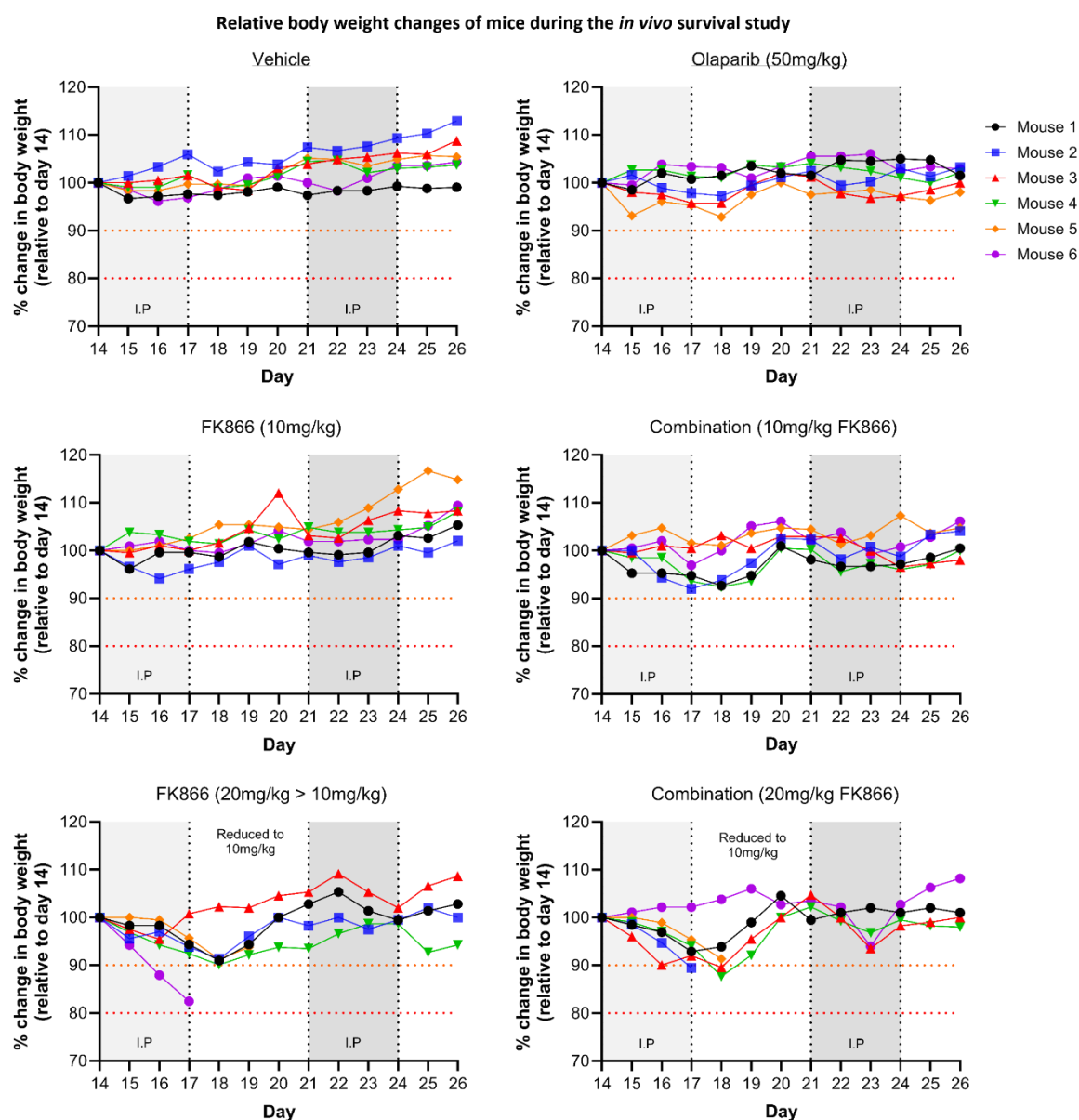
Vehicle, olaparib (50mg/kg), FK866 (10mg/kg) and combination (50mg/kg olaparib and 10mg/kg FK866) treated omental tumours were homogenised and metabolites were extracted (in collaboration with PhD student Yitao Xu). Targeted metabolomics analysis was performed and a PCA score plot was generated using Metaboanalyst (v.5.0.). The two-dimensional PCA scores plot indicates the grouping and differentiation of individual samples, due to variation in their metabolic profiles. Coloured circles represent 95% confidence intervals. Coloured dots represent individual samples.

Data normalisation for omental tumour metabolomics – *in vivo* (endpoint) study



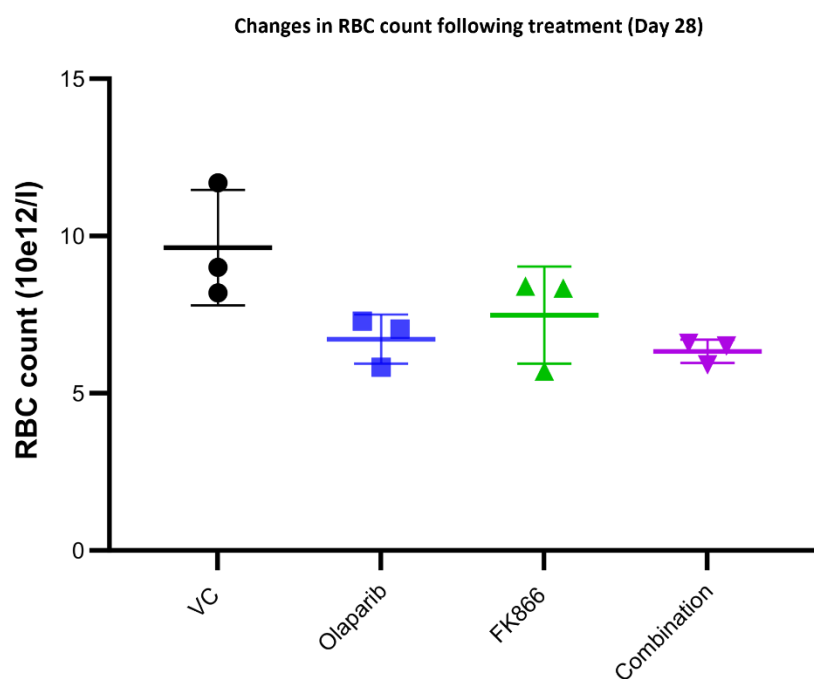
Appendix Figure 13. Data normalisation in MetaboAnalyst for metabolites from omental tumours in the endpoint *in vivo* study

Box plots and kernel density plots are shown before and after normalisation. The boxplots show 50 features at most due to space limit. The density plots are based on all samples. Autoscaling (mean-centred and divided by the SD of each variable) was performed. Data was normalised by the sum.



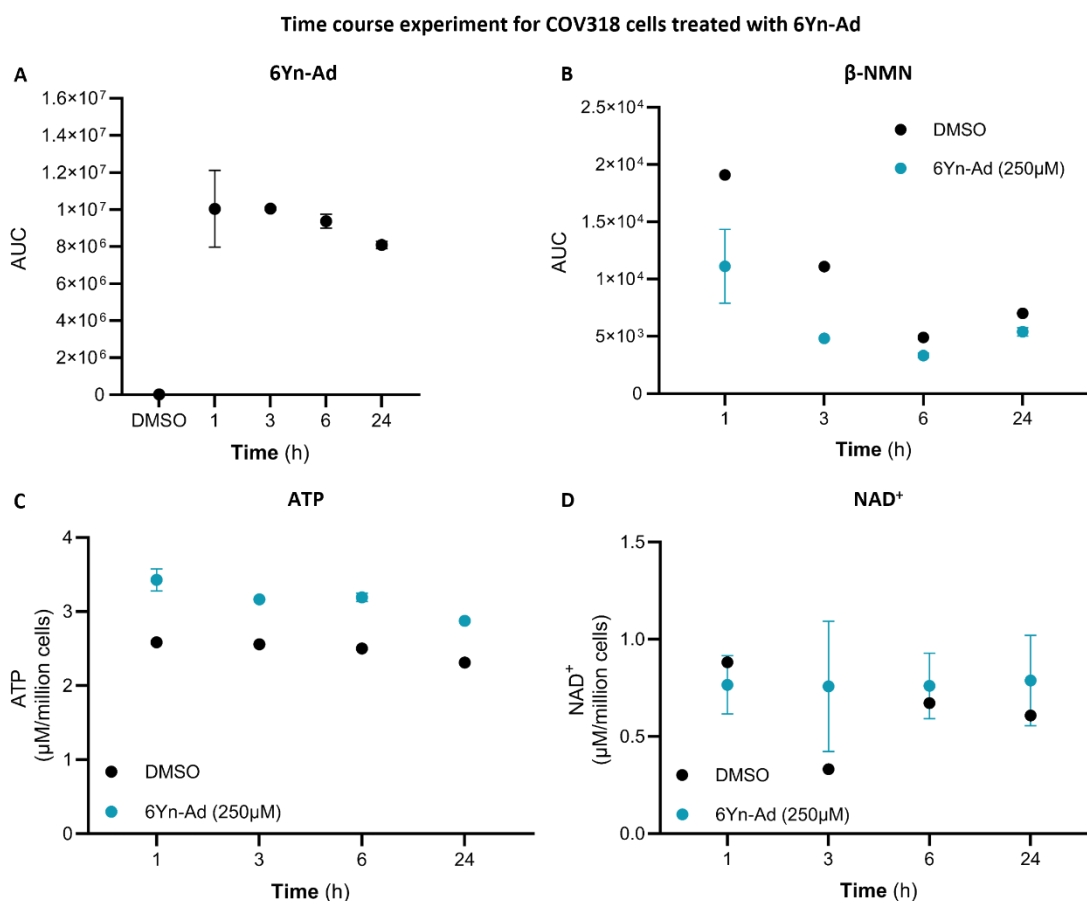
Appendix Figure 14. Relative body weight changes of mice during the survival *in vivo* study

Relative body weight changes in mice were measured during drug administration. FK866 (10mg/kg) data is from an independent experiment. Mice in the FK866 (20mg/kg) and combination (20mg/kg FK866) groups experienced significant weight loss (10%) and signs of pain. These mice were culled once reaching a humane endpoint. Orange and red dotted lines from the y-axis indicate the threshold for 10% and 20% (maximum limit) drops in body weight, respectively.



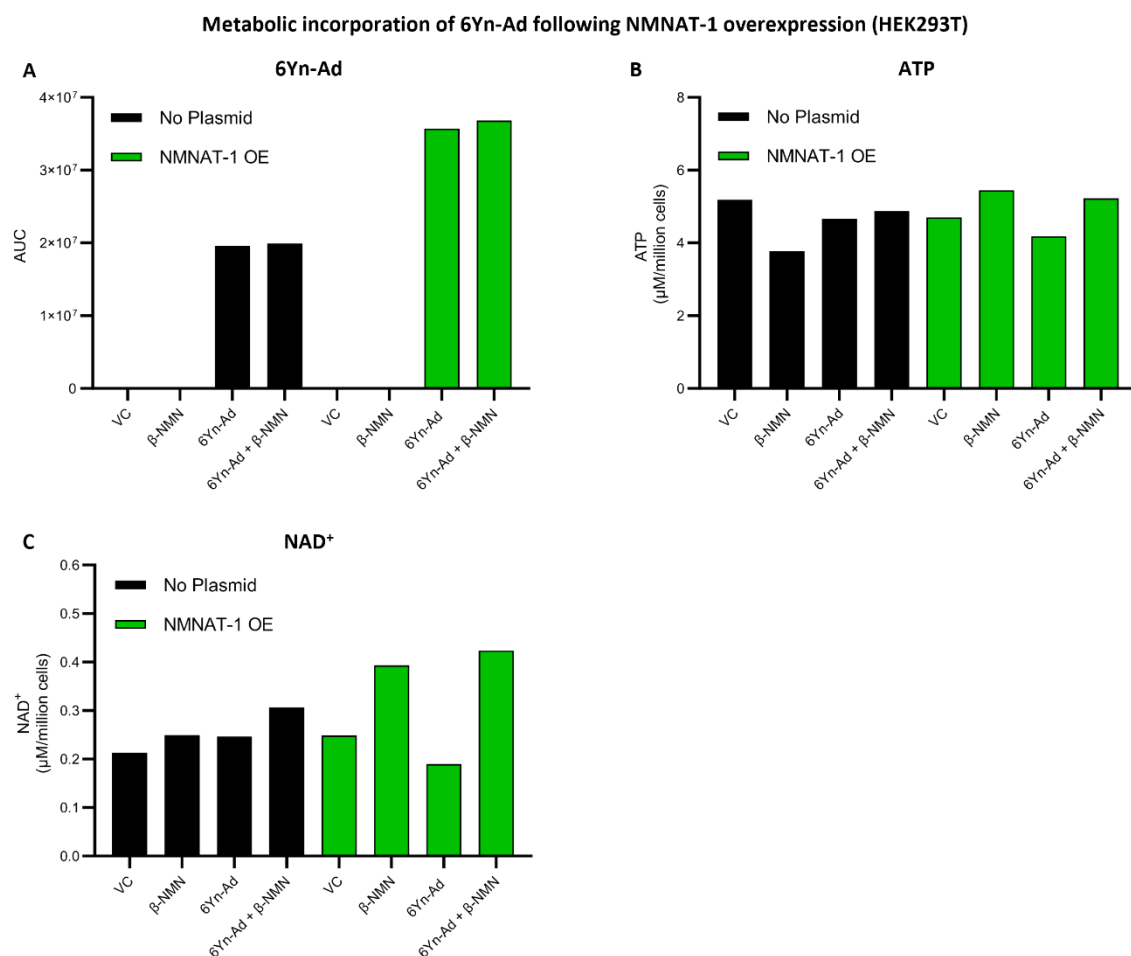
Appendix Figure 15. Red blood cell (RBC) count during the survival *in vivo* study

Mice were treated with vehicle, olaparib (50mg/kg), FK866 (10mg/kg) or the combination. On day 28 blood was extracted from mice via a tail bleed, or when blood pressure was too low for this blood was instead retrieved by a saphenous vein bleed. Blood was then transferred to the Royal Veterinary College's pathology unit for a RBC count test. Statistical significance was determined using one-way ANOVA followed by Šídák's multiple comparisons test (* $p < 0.05$).



Appendix Figure 16. 6Yn-Ad, β-NMN, ATP and NAD⁺ levels during the time course experiment

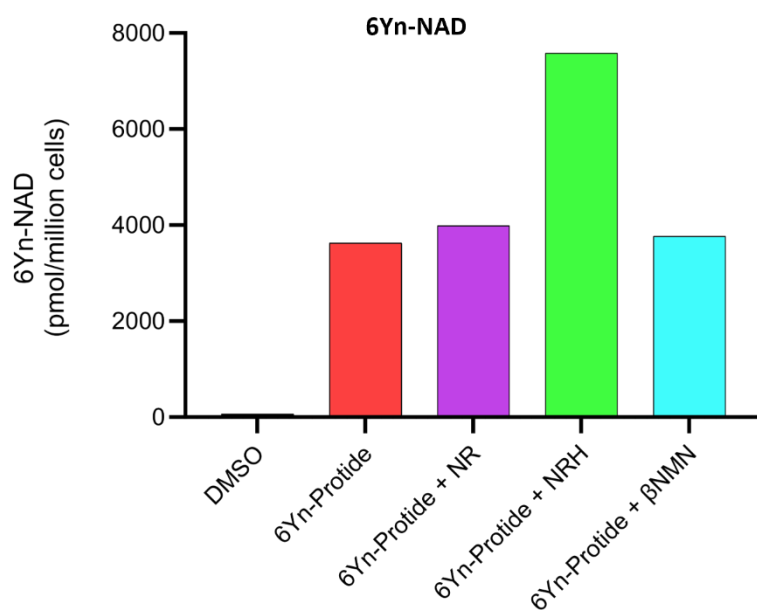
COV318 cells were treated with vehicle or 6Yn-Ad (250 μM) for 1, 3, 6 and 24 hours. Concentrations of **(A)** 6Yn-Ad and **(B)** β-NMN are shown as the area under the curve (AUC). **(C)** ATP and **(D)** NAD⁺ are shown as mol/million cells. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².



Appendix Figure 17. 6Yn-Ad, ATP and NAD⁺ levels during after NMNAT-1 overexpression

HEK293T cells were treated with vehicle, β -NMN and/or 6Yn-Ad ($\pm$ NMNAT-1 OE). **(A)** 6Yn-Ad concentrations are shown as the area under the curve (AUC). **(B)** 6Yn-ATP and **(C)** 6Yn-NAD are shown in mol/million cells. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².

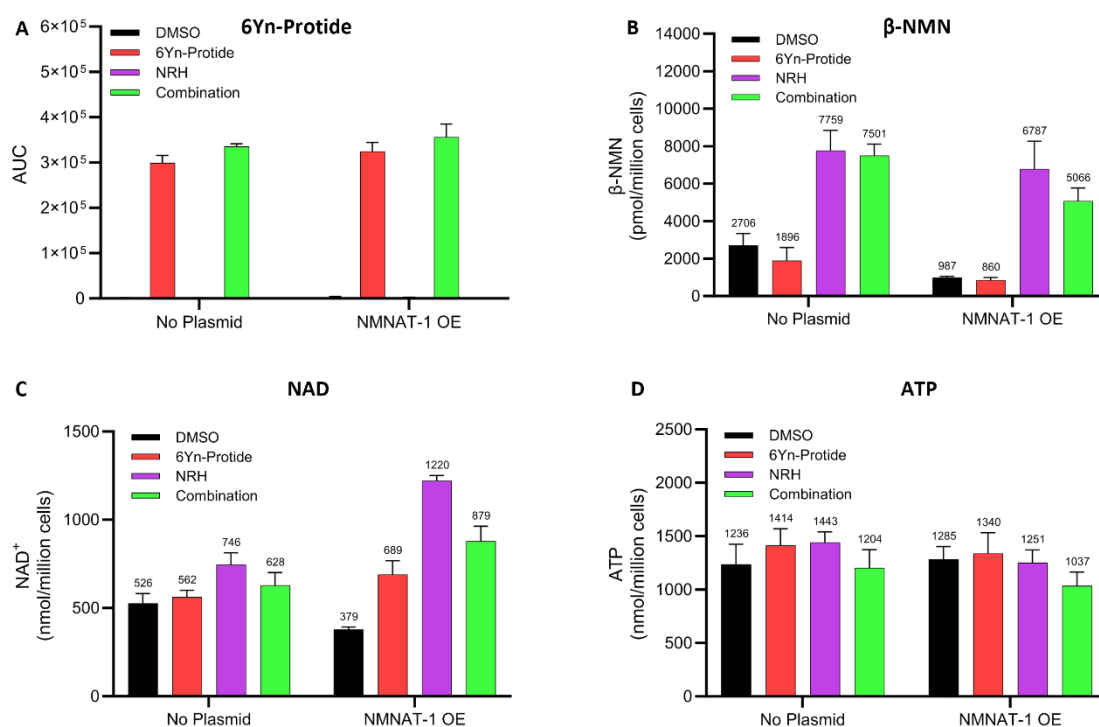
Influence of NR, NRH and β -NMN supplementation on 6Yn-NAD synthesis (HEK293T)



Appendix Figure 18. 6Yn-NAD synthesis is boosted following NRH co-treatment in HEK293T cells

HEK293T cells were treated for 24 hours with vehicle, 6Yn-ProTide (100 μ M) alone or in combination with NR (500 μ M), NRH (500 μ M) or β -NMN (500 μ M). Concentrations of 6Yn-NAD are shown in pmol/million cells. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴². Tissue culture, treatment and metabolite extraction was carried out by Simeon Draganov.

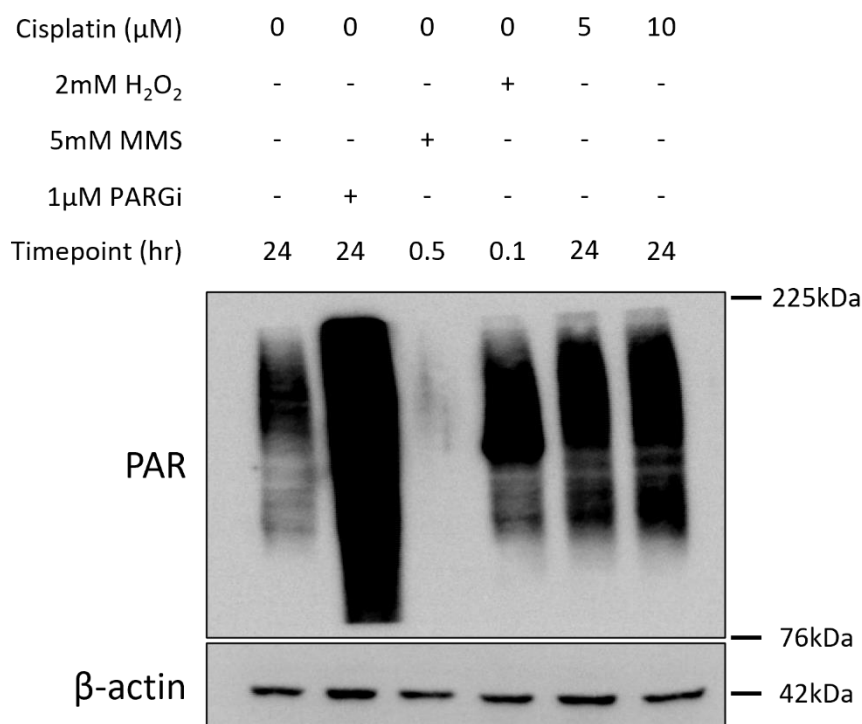
Metabolic incorporation of 6Yn-ProTide following NMNAT-1 overexpression and NRH supplementation (HEK293T)



Appendix Figure 19. Influence of NRH on 6Yn-ProTide, β-NMN NAD⁺ and ATP in HEK293T cells

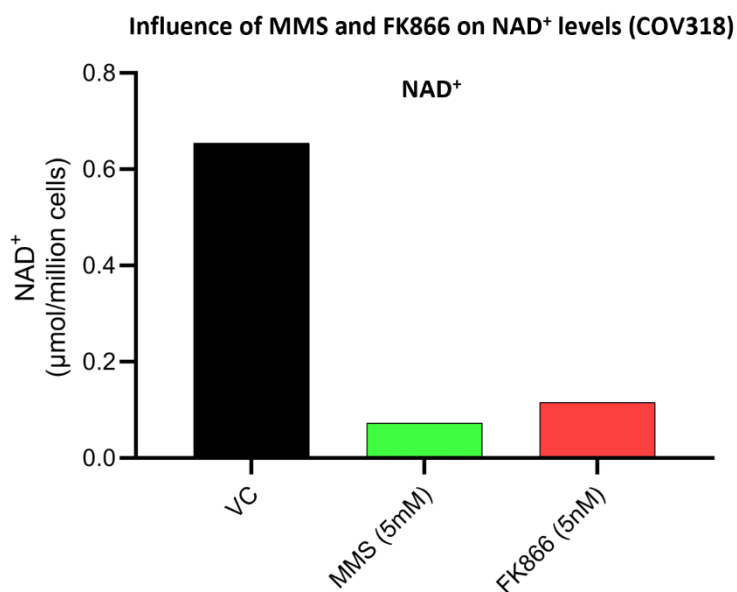
HEK293T cells were treated for 24 hours with vehicle, NRH (500μM) and/or 6Yn-ProTide (100 μM) (where indicated NMNAT-1 was overexpressed 24 hours prior to treatment). Concentrations of **(A)** 6Yn-ProTide are shown as the area under the curve (AUC). **(B)** β-NMN, **(C)** NAD and **(D)** ATP concentrations are shown in mol/million cells. Data is from three independent experiments. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².

Influence of different PARP activators on PARylation (HEK293T)



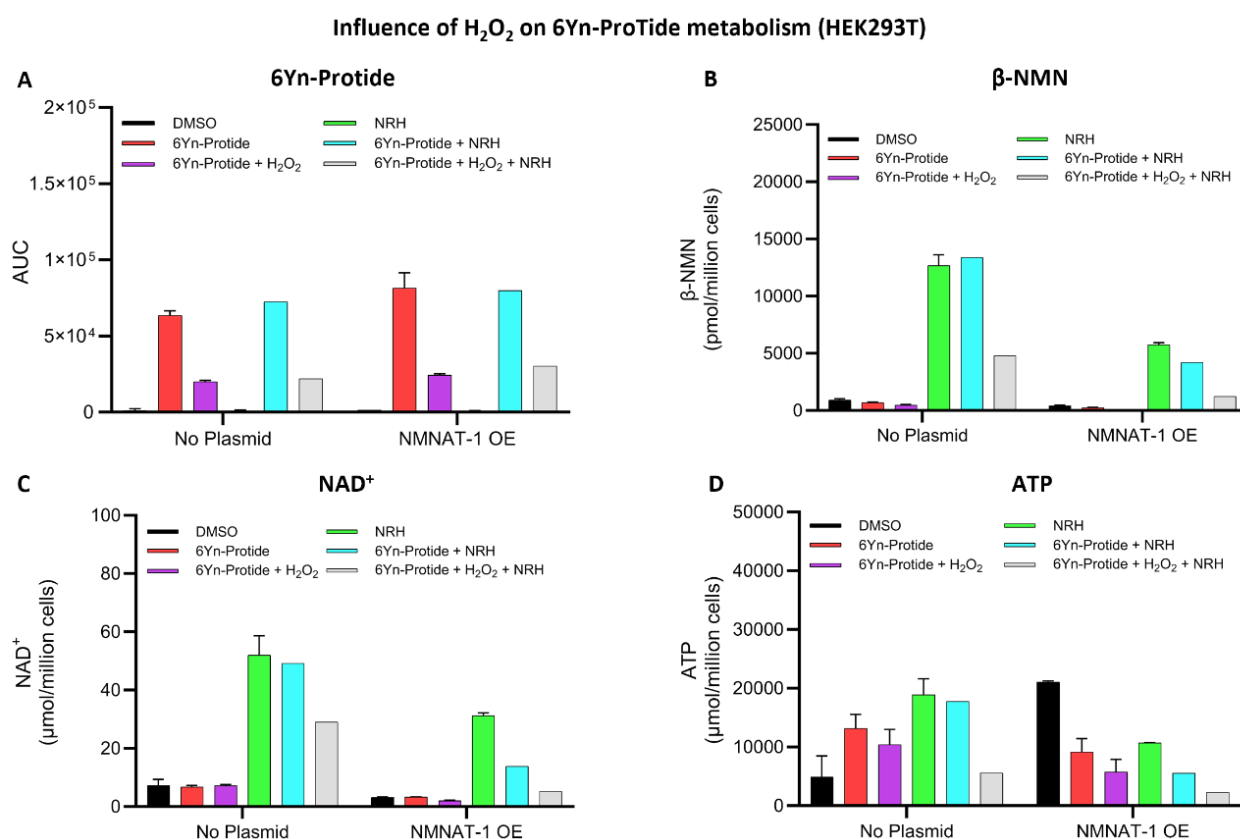
Appendix Figure 20. Influence of PARP activators on PARylation in HEK293T cells

HEK293T cells were treated with vehicle, a PARG inhibitor (PDD00017273), cisplatin, MMS or H_2O_2 . Lysates were extracted and each compounds influence on PARylation assessed by western blotting. Data is from one independent experiments. Part of this image has previously been published in a thesis by Dr Simeon Draganov²⁴². β -actin = loading control.



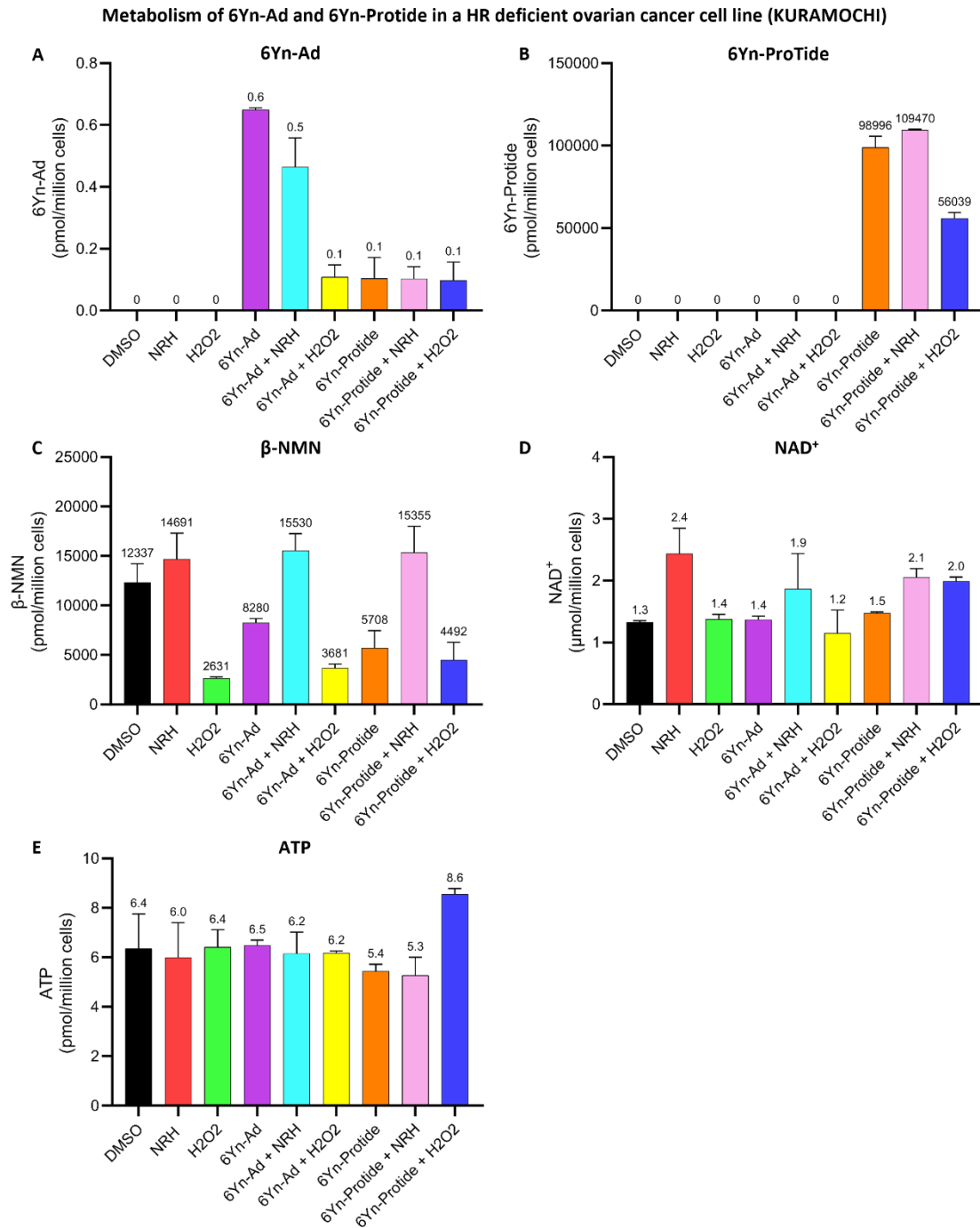
Appendix Figure 21. Influence of MMS and FK866 on NAD^+ levels in COV318 cells

COV318 cells were treated for 24 hours with vehicle or FK866 (5 nM), or with MMS (5 mM) for 30 minutes prior to metabolite extraction. NAD⁺ concentration is shown as nmol/million cells. Data is from one independent experiment.



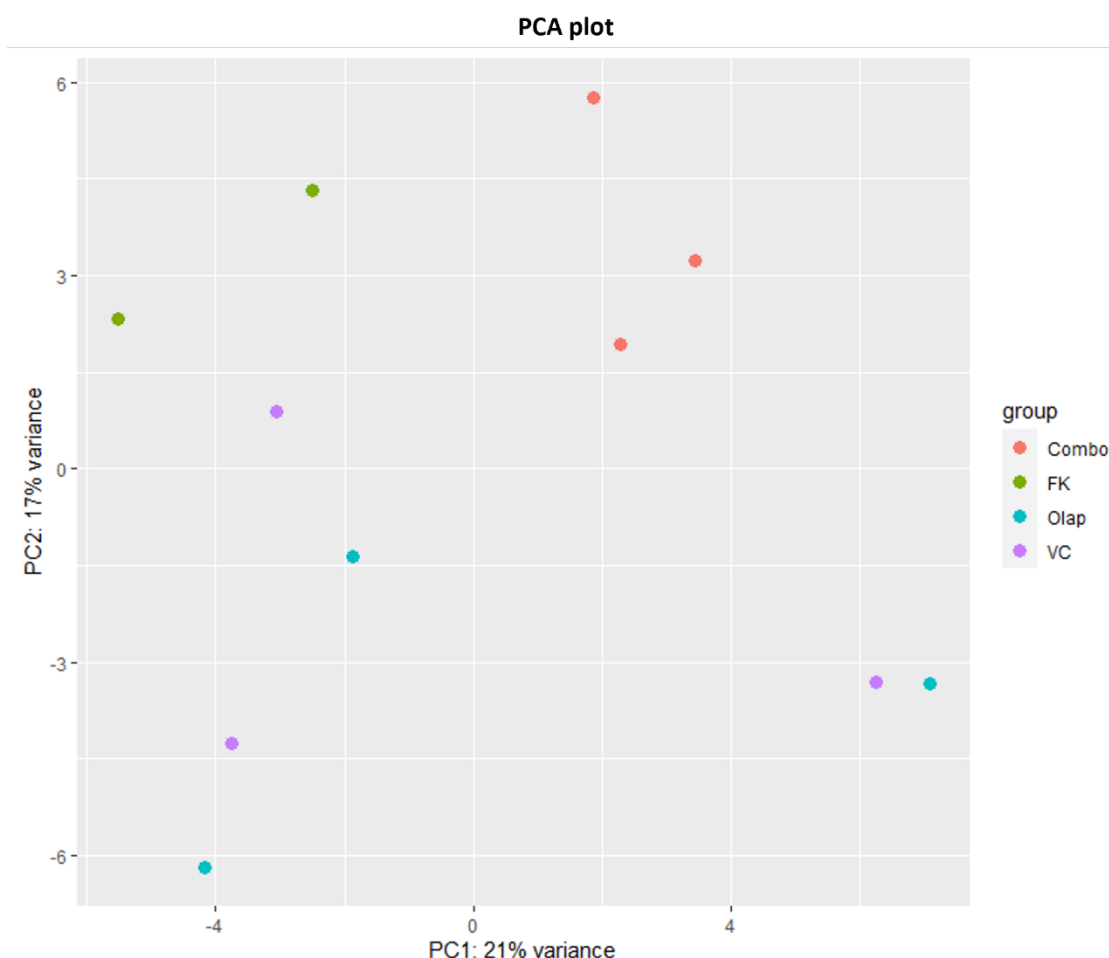
Appendix Figure 22. Influence of H₂O₂ on 6Yn-ProTide, β-NMN, NAD⁺ and ATP metabolism

HEK293T cells were treated for 24 hours with vehicle, NRH (500 μM) and/or 6Yn-ProTide (100 μM) (± NMNAT-1 OE 24 hours prior to treatment). Where indicated, samples were treated with 2mM H₂O₂ in PBS 10 minutes prior to metabolite extraction. **(A)** 6Yn-ProTide concentrations are shown as their area under the curve (AUC) value. Concentrations of **(B)** β-NMN **(C)** NAD⁺ and **(D)** ATP are shown in mol/million cells. Data is from one independent experiment. This experiment has previously been published in a thesis by Dr Simeon Draganov²⁴².



Appendix Figure 23. 6Yn-Ad and 6Yn-ProTide metabolism in KURAMOCHI cells

KURAMOCHI cells were treated for 24 hours with vehicle, 6Yn-Ad (500 μM) or 6Yn-ProTide (100 μM). Where media was supplemented with NRH (500 μM, 24 hr) or cells were treated with 2mM H₂O₂ (in PBS) for 10 minutes prior to metabolite extraction. Concentrations of **(A)** 6Yn-Ad, **(B)** 6Yn-ProTide and **(C)** β-NMN, **(D)** NAD⁺ and **(E)** ATP are shown in mol/million cells. Data is from one independent experiment.



Appendix Figure 24. PCA scores plot based RNA-sequencing differential expression analysis

COV318 cells were treated for 24 hours with vehicle (VC, purple), 1nM FK866 (FK, green), 1 μ M olaparib (Olap, blue) or the combination (Combo, red). RNA-sequencing was performed and differential expression analysis was undertaken using DESeq2. A two-dimensional PCA plot indicates was generated to visualise the grouping and differentiation of individual samples, due to variation in their gene expression profiles. Coloured dots represent individual samples. FK866 replicate 3 was excluded from analysis as a sufficient sequencing depth was not achieved.

Appendix Table 1. ASEs that are known determinants of PARPi sensitivity

Gene name	Full name	Occurrences
DYNC1H1	Dynein Cytoplasmic 1 Heavy Chain 1	Olaparib, FK866, Combination
EP400	E1A Binding Protein P400	Olaparib, FK866, Combination
NEMF	Nuclear Export Mediator Factor	Olaparib, FK866, Combination
RPL12	Ribosomal Protein L12	Olaparib, FK866, Combination
XPO5	Exportin 5	Olaparib, FK866, Combination
AHCTF1	AT-Hook Containing Transcription Factor 1	Olaparib, FK866

BRD4	Bromodomain Containing 4	Olaparib, Combination
BSDL2	BSDL2 Lipid Droplet Biogenesis Associated, Seipin	Olaparib, FK866
C17orf53	Homologous Recombination Factor With OB-Fold	Olaparib, FK866
CAPRIN1	Cell Cycle Associated Protein 1	Olaparib, Combination
CLUH	Clustered Mitochondria Homolog	FK866, Combination
CNOT2	CCR4-NOT Transcription Complex Subunit 2	FK866, Combination
DDX39B	DExD-Box Helicase 39B	FK866, Combination
DIS3L2	DIS3 Like 3'-5' Exoribonuclease 2	Olaparib, Combination
EHMT2	Euchromatic Histone Lysine Methyltransferase 2	FK866, Combination
FIP1L1	Factor Interacting With PAPOLA And CPSF1	FK866, Combination
HDAC10	Histone Deacetylase 10	Olaparib, FK866
HECTD1	HECT Domain E3 Ubiquitin Protein Ligase 1	FK866, Combination
HM13	Histocompatibility Minor 13	FK866, Combination
INTS1	Integrator Complex Subunit 1	FK866, Combination
KIF20A	Kinesin Family Member 20A	FK866, Combination
KMT2C	Lysine Methyltransferase 2C	FK866, Combination
KRT7	Keratin 7	Olaparib, Combination
LDLR	Low Density Lipoprotein Receptor	FK866, Combination
MAP3K6	Mitogen-Activated Protein Kinase Kinase Kinase 6	FK866, Combination
PDE4DIP	Phosphodiesterase 4D Interacting Protein	FK866, Combination
SCNN1D	Sodium Channel Epithelial 1 Subunit Delta	Olaparib, Combination
SEN5	SUMO Specific Peptidase 5	Olaparib, Combination
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4	Olaparib, Combination
TAGLN	Transgelin	FK866, Combination
TONSL	Tonsoku Like, DNA Repair Protein	Olaparib, FK866
TSC2	TSC Complex Subunit 2	Olaparib, Combination
UROD	Uroporphyrinogen Decarboxylase	Olaparib, FK866
USP11	Ubiquitin Specific Peptidase 11	FK866, Combination
USP34	Ubiquitin Specific Peptidase 34	Olaparib, Combination

COV318 cells were treated for 24 hours with vehicle, 1nM FK866, 1μM olaparib or the combination. RNA was extracted for RNA-sequencing analysis and alternative splicing events (ASEs) (compared to vehicle groups) were detected using SpliceFisher³²⁰. Genes with an ASE detected in at least two conditions were included in a table to accompany the Venn diagram analysis (**Figure 46**). Of these genes, those that have previously been identified as determinants of PARPi sensitivity from CRISPR-screens^{36,94,321-323} were included in the above table.