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Oleoylethanolamide and palmitoylethanolamide synergize with IFNβ to potentiate apoptosis and PD-L1 induction in SH-SY5Y neuroblastoma cells

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List of abbreviations

aCGH: Comparative Genomic Hybridization Array

AD: Alzheimer's Disease

AEA: N-arachidonoyl ethanolamide

AEs: Acylethanolamines

AF-1: Activation Function

AHSC: Autologous hematopoietic stem cell

ALK: Anaplastic Lymphoma Kinase

CAFs: Cancer-associated fibroblasts

CBRs: Cannabinoid receptors

CML: Chronic Myeloid Leukemia

COG: Children Oncology Group

DAG: Diacylglycerol

DBD: DNA-binding domain

DHEA: docosahexaenoylethanolamide

DNMT1: DNA methyltransferase 1

eCBs: Endocannabinoids

eCBs-like: Endocannabinoid-like

EPEA: Eicosapentaenoylethanolamide

FA: Fatty acid

FAAH: Fatty acid amide hydrolase

FADD: Fas-associated death domain

FISH: Fluorescent in situ hybridization

GAS: Gamma-activated sequence

GPR55: G-protein-coupled receptor 55

GPR119: G-protein-coupled receptor 119

GWAS: Genome-wide association study

HCC: Hepatocellular carcinoma

HVA: Homovanillic acid

IAPs: Inhibitor of apoptosis proteins

IFN: Interferon

INRGSS: International Neuroblastoma Risk Group Staging System

INSS: International Neuroblastoma Staging System

IRF9: IFN-regulatory factor 9

ISGF3: IFN-stimulated gene factor 3

ISGs: IFN-stimulated genes

ISRE: IFN-stimulated response elements

ITIM: Immunoreceptor tyrosine-based inhibition motif

ITSM: Immunoreceptor tyrosine-based switch motif

JAK: Janus activated kinase

JAK/STAT: Janus kinase/signal transducers and activators of transcription

LBD: Ligand-binding domain

LEA: Linoleoylethanolamide

LPA: Lysophosphatidic acid

LPS: Lipopolysaccharide

LTB4: Leukotriene B4

MAPK: Mitogen-activated protein kinase

MAPKAPK5: MAPK activated protein kinase 5

MEM: Minimum Essential Medium

MGMT: O6-methylguanine DNA methyltransferase

MRI: Magnetic resonance imaging

MS: Multiple Sclerosis

NAAH: N-acyl-ethanolamine-hydrolyzing acid amidase

NAEs: N-acylethanolamides

NAPEs: N-acyl phosphatidylethanolamines

NAPE-PLD: N-acyl-phosphatidylethanolamine-specific phospholipase D

NB: Neuroblastoma

NEAA: Non-essential amino acids

NGF: Nerve growth factor

NGS: Next-generation sequencing

NF-κB: Nuclear factor-κB

OEA: Oleoylethanolamide

OS: Overall survival

Pan-cadh: Pan-cadherin

PARP-1: poly (ADP-ribose) polymerase-1

PD: Parkinson’s disease

PD-L1: Programmed death ligand-1

PDX: Patient-derived xenograft

PE: Phosphatidylethanolamines

PEA: Palmitoylethanolamide

PGE2: Prostaglandin E2

PHOX2B: Paired-like Homeobox 2B

PKR: Protein kinase R

PLGF: Placental growth factor

PPAR: Peroxisome proliferator-activated receptor

PRMT6 : Protein arginine methyltransferase 6

PPREs: PPAR response elements

RXR: Retinoid X receptor

SCI: Spinal cord injury

SDI: Sociodemographic index

SEA: Stearoylethanolamide

TKIs: Tyrosine kinase inhibitors

TME: Tumor microenvironment

TMZ: Temozolomide

TNBC: Triple-negative breast cancer

TRADD: TNF receptor-associated death domain

TRPV1: Transient receptor potential cation channel subfamily V member 1

TYK2: Tyrosine kinase 2

VEGF-A: vascular endothelial growth factor A

VMA: vanillylmandelic acid

VR1: vanilloid receptors type 1

Abstract

Oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) are endogenous bioactive lipids that act as selective agonists of the peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor implicated in lipid metabolism, inflammation, and cancer biology. While their anti-tumour potential has been suggested in various malignancies, their role in human neuroblastoma (NB) remains unexplored. NB, a paediatric solid tumour with poor prognosis in aggressive forms, may benefit from novel therapeutic strategies that exploit molecular vulnerabilities and enhance the efficacy of existing treatments. Type I interferons (IFNs), particularly IFNβ, are cytokines with well-characterized antiviral and anti-proliferative actions and documented capacity to trigger apoptosis in tumour cells, including NB. In this study, we investigated whether OEA and PEA could modulate IFNβ-induced responses in human SH-SY5Y NB cells.

Through a combination of viability assays, scratch wound healing, clonogenic assays, and western blotting, we show that OEA and PEA significantly enhance IFNβ-mediated cytotoxicity. Co-treatment with IFNβ and either lipid potentiated apoptosis, as indicated by increased cleavage of caspase 3 and poly-ADP-ribose polymerase (PARP), together with reduced levels of survivin, a protein involved in anti-apoptotic regulation. Importantly, this effect occurred without amplifying canonical IFNβ signalling through the JAK-STAT pathway or PKR induction, suggesting a parallel and complementary apoptotic mechanism. Furthermore, OEA and PEA enhanced IFNβ-induced PD-L1 expression both in whole-cell lysates and on the plasma membrane. Pharmacological inhibition and the genetic silencing of PPARα attenuated PARP cleavage and PD-L1 upregulation, confirming the receptor's contribution to the observed effects.

Collectively, our findings demonstrate that OEA and PEA potentiate IFNβ-induced apoptosis in SH-SY5Y cells through PPARα-dependent mechanisms that synergize with, but remain

distinct from, classical IFN signalling. This synergism promoting apoptotic cell death while modulating PD-L1 expression, highlights a novel interplay between lipid signalling and cytokine pathways in NB. These results open avenues for further studies aimed at evaluating OEA and PEA as adjuvants in IFN-based therapies and raise the possibility that combining bioactive lipids with immunomodulatory cytokines could improve therapeutic efficacy against NB and other IFN-sensitive malignancies.

Introduction

Part I: Neuroblastoma

1- Neuroblastoma: A general introduction

Neuroblastoma (NB) is the most common extracranial solid malignancy in children, originating from neural crest-derived progenitor cells of the sympathetic nervous system during embryonic development. Primary NB tumours are typically located in the adrenal medulla or along the sympathetic chain, including the abdomen, pelvis, thoracic, and cervical regions. The disease often metastasizes to sites such as the liver, bone, lymph nodes, and, less commonly, the central nervous system (Dubois et al., 1999; Kalaskar and Kalaskar, 2016; Mahapatra and Challagundla, 2023). NB is generally diagnosed at a median age of 17–18 months, with approximately 90% of cases occurring in children under five years of age. It accounts for around 10% of all paediatric cancers and contributes to approximately 15% of childhood cancer-related mortality (London et al., 2005; Park et al., 2010; Althoff et al., 2015).

Globally, the incidence of NB increased by 30.26% between 1990 and 2021. Similarly, both NB-associated mortality and disability-adjusted life years, a parameter used to measure disease burden, have risen by 20.35% and 20.08%, respectively (Nong et al., 2024). However, NB prevalence shows marked geographic and socioeconomic disparities. A decline in incidence and mortality has been observed in countries with high and medium-high sociodemographic index (SDI), likely reflecting improved healthcare infrastructure, clinical awareness, early diagnosis, and access to advanced therapies. Declining birth rates in these regions may also contribute to the trend. In contrast, NB burden continues to rise in low, low-medium, and medium SDI regions, underscoring disparities in healthcare access and disease management (Nong et al., 2024).

2- Symptoms, diagnosis, prognosis, and risk stratification of NB

Apart from the presence of protruding abdominal, thoracic or neck masses in patients (Chu et al., 2011; Alvi et al., 2017; Mahapatra and Challagundla, 2023), common signs of NB manifest as systematic symptoms including fever, anaemia, diarrhoea, weight loss and hypertension.

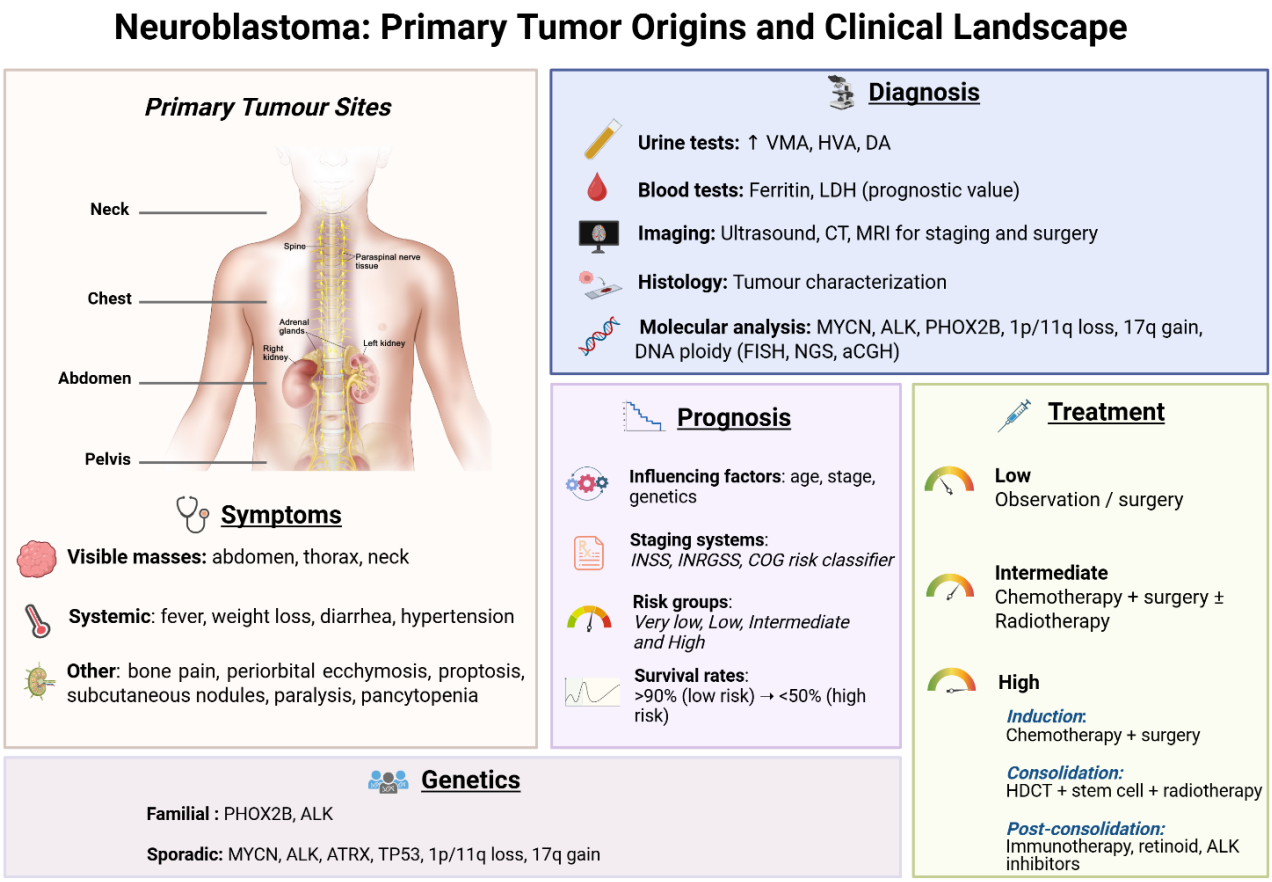

Figure 1: An overview of NB from primary tumour localization to genetic landscape, diagnosis, prognosis, and therapeutic strategies

Other symptoms such as bone pain, proptosis, periorbital ecchymosis, pancytopenia, paralysis and subcutaneous skin nodule can also be observed (Citak et al., 2006; Kalaskar and Kalaskar, 2016; Zhu et al., 2020) (**Figure 1**)

To diagnose NB, several laboratory tests, pathological exams and imaging techniques are used. Because of their neuronal origin, NB cells can excessively release catecholamines and catecholamine metabolites including dopamine, homovanillic acid (HVA) and vanillylmandelic acid (VMA) (Romero et al., 2015; Harding et al., 2021). These molecules are

considered robust and reliable biomarkers for the presence of NB, as elevated urinary levels are observed in approximately 90% of affected paediatric patients (Strenger et al., 2007).

In addition, an overall blood analysis where different parameters, specifically ferritin and lactate dehydrogenase, is performed as a basic and standard routine test for NB diagnosis (Hann et al., 1980; Shuster et al., 1992; Tolbert et al., 2018) (**Figure 1**). This is mainly due to the strong association that has been discovered between these parameters and survival in NB.

Furthermore, imaging techniques including ultrasound, cross-sectional computed tomography imaging and magnetic resonance imaging (MRI) are essential for primary tumour characterization, precise tumour monitoring and staging, as well as visualization for surgical interventions and screening for any possible NB-related metastatic formations (Kembhavi et al., 2015; Littooij and de Keizer, 2023). NB biopsies are also performed, particularly for prognosis, and are used in histological and molecular testing (Paraboschi et al., 2021). The histological subtype of the NB tumour, along with the patient's age at diagnosis, can help in risk stratification (Tolbert and Matthay, 2018). Additionally, molecular testing such as fluorescent in situ hybridization (FISH), flow cytometry, next-generation sequencing (NGS), and array comparative genomic hybridization (aCGH) are used to detect key prognostic markers in NB, including MYCN gene amplification, cell ploidy status, somatic and germline mutations (e.g., mutations in ALK, PHOX2B), and segmental chromosomal alterations such as gains of 17q and losses of 1p and 11q, all of which contribute to risk stratification and therapeutic decision-making (Ambros et al., 2009; Bosse et al., 2016; Tolbert and Matthay, 2018; Szymansky et al., 2021) (**Figure 1**).

The clinical behaviour of NB is highly heterogeneous, ranging from cases of spontaneous regression to those exhibiting aggressive metastatic spread (Matthay et al., 2016). This variability is largely influenced by a combination of genetic and molecular characteristics,

patient age at diagnosis, and disease stage (Tolbert and Matthay, 2018). To better predict clinical outcomes and guide therapeutic decisions, several risk stratification systems have been established to categorize NB into prognostic groups and tailor treatment intensity accordingly (Tolbert and Matthay, 2018; Sokol and Desai, 2019; Pediatric Treatment Editorial Board, 2002). Over time, staging and risk assessment criteria have evolved to incorporate both anatomical and biological parameters. The International Neuroblastoma Staging System (INSS), developed in the early 1990s, is primarily based on surgical findings (Brodeur et al., 1993), whereas the International Neuroblastoma Risk Group Staging System (INRGSS) classifies tumours according to pre-treatment imaging features and specific biomarkers (Monclair et al., 2009). In parallel, the Children's Oncology Group (COG) Risk Classifier integrates clinical, histological, and genetic factors such as MYCN amplification, chromosomal aberrations, and ploidy status to refine patient stratification and optimize treatment strategies (London et al., 2005) (**Table 1**).

3- Origin and pathophysiology of NB

During embryogenesis, the ventrolateral neural crest cells, endowed with multipotent differentiation potential, migrate away from the neural tube to give rise to the sympathetic ganglion cells and adrenal chromaffin cells (Cheung and Dyer 2013, Johnsen et al., 2019). These multipotent precursors can be predisposed to alterations in differentiation, development or in migration, leading to malignant transformation (Takita 2021; Louis and Shohet 2015). Several regulatory mechanisms influence this process including genetic, epigenetic and environmental factors (Jung et al., 2024). Although the exact causes of NB are not fully elucidated, a great deal of interest has been given to the genetic component underlying this disease.

Table 1: A comparison between the different neuroblastoma staging and risk stratification systems

System	Stages	Determining criteria	References
International Neuroblastoma Staging System (INSS) Used in North America	Stage 1, 2A/ 2B, 3, 4, 4S:	**Surgical-based findings** Tumour location, resection margin status, metastasis, lymph node involvement…	Brodeur et al., 1993
International Neuroblastoma Risk Group Staging System (INRGSS) International standard	L1, L2, M, MS	**Pre-treatment imaging, image-defined risk factors** Age, histology, MYCN status, 11q loss, DNA ploidy	Monclair et al., 2009
Children Oncology Group Risk Classifier (COG) Used in the United States of America	Low risk, intermediate risk and high Risk	Combination of INSS stage, age, MYCN amplification, histology, DNA ploidy, 11q status	London et al., 2005

NB is a sporadic cancer with only 1-2% of cases being attributed to familial autosomal dominant inheritance (Ritenour et al., 2018). Nevertheless, the genetic component in NB pathogenesis is very important. Thanks to genome-wide association study (GWAS), more insights have been provided on the genetic basis of spontaneous NB (Ritenour et al., 2018). The underlying genetic contribution to familial NB oncogenesis is attributed to two key germline mutations in the paired-like Homeobox 2B (PHOX2B) gene and the anaplastic lymphoma kinase (ALK) gene (Trochet et al., 2004; Mossé et al., 2008).

PHOX2B, a transcription factor that is crucial for neural crest differentiation into noradrenergic neurons, is found to be mutated in 10% of familial cases and 2% of spontaneous NB cases (Van limpt et al., 2004; Serra et al., 2008). ALK, on the other hand, a receptor tyrosine kinase involved in neurogenesis and neuronal differentiation, is the most mutated gene in both familial and sporadic cases (O'Donohue et al., 2021; Wulf et al., 2021). Mutations in R1275, F1174, and F1245 account for around 85% of ALK mutations in NB. While R1275Q is the most common mutation in familial cases, mutations in F1174 and F1245 are found in sporadic NB (Janoueix-Lerosey et al., 2008; Maris, 2010). Gene amplification that leads to an enhancement in ALK expression and its constitutive activation is also a mechanism by which ALK is involved in NB oncogenesis (Aktaş et al., 2023), in addition to other genetic alterations that result in the truncation of this receptor, both of which are less frequently found in comparison to point mutations (Trigg and Turner 2018). Alternatively, the overexpression of ALK without any underlying genetic mutation or amplification is also observed in some NB tumours, leading to a hyperactivation of its downstream signalling pathways (Passoni et al., 2009).

Sporadic NB, accounting for over 98% of cases, is believed to arise from somatic genetic alterations during neural crest cell development (Yadwad et al., 2024). With few recurrent driver mutations, key somatic changes are implicated in genes such as *MYCN* and *ALK*. MYCN, a member of the MYC oncogene family, is one of the first genetic markers discovered and characterized for its involvement in NB tumorigenesis (Kohl et al., 1983; Weiss et al., 1997). MYCN is a transcription factor involved in cellular survival, growth and proliferation (Müller et al., 2024). In approximately 20-30% of NB cases, MYCN is found to be amplified. In this case, tumours are directly classified into the high-risk class characterized by poor prognostic outcomes and an overall survival rate that is inferior to 50% (Huang and Weiss 2013; Otte et al., 2021; Müller et al., 2024).

Somatic activating ALK mutations, similar to those found in familial cases, are found in 9% of NB tumours and 14% of high-risk cases (Janoueix-Lerosey et al., 2008; Trigg and Turner 2018). Other somatic alterations include inactivating mutations in ATRX, p53 pathway disruptions, and segmental chromosomal aberrations such as deletions of 1p and 11q or gain of 17q, which likely target tumour suppressors and oncogenes that are important in neural crest cell growth (van Gerven et al., 2022; Tweddle et al., 2003; Mlakar et al., 2024; Guan et al., 2021).

4- Treatment of NB

NB treatment is risk-adapted. Low-risk patients, with a five-year overall survival (OS) >90%, often require only observation or surgery due to the potential for spontaneous regression (PDQ Pediatric Treatment Editorial Board 2025). Intermediate-risk NB is managed with biology-driven chemotherapy, typically followed by surgery and, in some cases, radiotherapy (Luo et al., 2018; Twist et al., 2018). High-risk NB, associated with a five-year OS <60%, requires an aggressive multimodal strategy including induction chemotherapy and tumour resection, consolidation with high-dose myeloablative chemotherapy and autologous hematopoietic stem cell transplantation (AHSC), frequently combined with radiotherapy, and post-consolidation immunotherapy and differentiation therapy with 13-cis-retinoic acid (Luo et al., 2018; Twist et al., 2018; Casey et al., 2018; Makimoto et al., 2024). Targeted therapies such as ALK inhibitors, HDAC inhibitors, and antibody conjugates have recently been introduced to improve specificity and reduce toxicity (Witt et al., 2009; Buongervino et al., 2021; Pastorino et al., 2023; Balla et al., 2025).

Despite these advances, relapse due to therapy-resistant residual disease remains frequent and often fatal (Cole et al., 2012; Moreno et al., 2017). Treatment intensification is limited by severe toxicity, while acquired resistance, tumour heterogeneity, variable target expression,

and high costs further compromise efficacy (Zhou et al., 2023; Lundberg et al., 2022; Benchia et al., 2025). These challenges underscore the need for more effective, safer, and accessible therapeutic strategies for paediatric NB.

5- In vitro research in cancer and NB

In vitro models are essential tools in cancer research, enabling studies of tumour biology and systematic evaluation of anti-cancer compounds under highly controlled conditions (Katt et al., 2016; Antunes et al., 2020). Both two and three-dimensional cancer cell cultures are widely used to investigate intracellular signalling pathways, characterize oncogenic drivers, and assess drug efficacy across defined doses and time courses. By recapitulating key cellular processes such as proliferation, migration, and apoptosis, in vitro systems provide a robust foundation for hypothesis testing prior to more complex in vivo studies (Sajjad et al., 2021; Zhang et al., 2024).

These models offer high reproducibility, experimental flexibility, cost-effectiveness, and rapid throughput compared to animal models, making them indispensable for cancer research and drug development. However, their inability to fully reproduce the tumour microenvironment (TME) and complex cell-to-cell interactions limits accurate modelling of processes such as metastasis and immune evasion, potentially affecting translational relevance (Katt et al., 2016; Antunes et al., 2020).

NB research relies on a broad panel of well-characterized human and murine cell lines that reflect the molecular heterogeneity and clinical diversity of the disease (Ornell and Coburn, 2019). Commonly used human NB cell lines include SH-SY5Y, SK-N-BE (2), SK-N-AS, IMR-32, Kelly, LAN-1, and LAN-5, alongside murine lines such as Neuro-2a and N1E-115 (Cogo et al., 2020; Krawczyk and Kitlińska, 2023). These cell lines recapitulate key genetic alterations observed in patients. MYCN amplification, present in approximately 20% of NB cases and associated with aggressive disease, is represented in lines such as IMR-32, BE (2)-C, and Kelly

(Upton et al., 2020), while activating mutations of the ALK receptor tyrosine kinase, including the F1174L hotspot mutation, are found in SH-SY5Y, Kelly, and LAN-1 cells, while LAN-5 harbours distinct ALK variants (George et al., 2008).

6- SH-SY5Y cells an in vitro model of NB

SH-SY5Y is a human NB cell line that is widely used as an in vitro model for neuronal cells in biomedical research. It is a thrice-subcloned subline of the parental SK-N-SH NB cells. These derive from a bone marrow biopsy established in the early 1970s from a 4-year-old female patient with NB (Biedler et al., 1973).

SH-SY5Y cells have garnered attention thanks to their neuronal characteristics despite their tumoral origin. They display a characteristic morphology with elongated neuritic processes and possess the biochemical machinery necessary for neurotransmitter synthesis (Kovalevich and Langford, 2016). The expression of enzymes such as tyrosine hydroxylase and dopamine-β-hydroxylase enables the production of dopamine and norepinephrine, conferring them with a distinct catecholaminergic phenotype (Kume et al., 2008; Kovalevich and Langford 2016). Additionally, they express cholinergic markers such as choline acetyltransferase, indicating a degree of neurotransmitter versatility (de Medeiros et al., 2019; Ducray et al., 2020). They also express a broad range of neurotransmitter receptors including adrenergic, dopaminergic, glutamatergic, and cholinergic receptors (Adem et al., 1986; Lopes et al., 2010). These characteristics and features further emphasize their adrenergic nature, like that of sympathetic neurons, a common origin for the rise of primary NB tumours.

SH-SY5Y cell line represents the most used cell line for the investigation and the study of NB (Cogo et al., 2020). Because of its human origin, stable growth properties, and neural lineage features, SH-SY5Y continues to serve as a fundamental translational model for understanding NB biology and screening potential therapeutical compounds (Gheeya et al., 2009). SH-SY5Y cells harbour an activating ALK F1174L mutation and carry a MYCN gain, which is a moderate

increase in the number of MYCN copies that has limited clinical impact (Lodrini et al., 2017). Therefore, these cells can recapitulate intermediate-risk tumour behaviour in comparison to other NB cell lines such as IMR-32, Kelly and LAN-1 that model high-risk MYCN-amplified NB subtypes (Upton et al., 2020).

Beyond its oncologic properties, SH-SY5Y is one of the most used human NB cell-line in neuroscience research as well. Its ability to proliferate in a tumoral-like manner and its capacity to differentiate into a more neuronal-like phenotype upon exposure to certain treatments such as retinoic acid, combined with its human origin that allows expression of neuron-specific proteins that are absent in animal models, makes it an optimal and practical alternative to primary neurons, particularly for studying neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Forster et al., 2016; Xicoy et al., 2017; de Medeiros et al., 2019).

Part II: Key tumoral mechanisms and signalling pathways: Apoptosis, immune evasion and p38 MAPK

Apoptosis and immune evasion are two critical biological processes that play central roles in cancer development and progression. While apoptosis ensures the controlled elimination of damaged or unwanted cells, immune checkpoints regulate immune responses to maintain self-tolerance and prevent autoimmunity (Igney and Krammer 2002; Elmore 2007; Galassi et al., 2024).

1- Intrinsic and extrinsic apoptosis pathways

Apoptosis is a regulated and programmed form of cell death that is essential for normal biological functioning and for the preservation of tissue homeostasis (Afford and Randhawa 2000). It has two main pathways: the extrinsic (death receptor-mediated) and the intrinsic (mitochondrial) pathway, both of which ultimately lead to the activation of caspases, the effector proteins of apoptosis (Mustafa et al., 2024).

In general, the extrinsic pathway is triggered by the binding of death ligands such as FasL or TNF-α to their receptors Fas and TNFR, also referred to as death receptors. Once activated, the intracellular domain of these receptors recruit what is known as adapter proteins including TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD). This is followed by a cascade of reactions that initiates the assembly and activation of pro-caspase 8 which then directly leads to the activation of caspase-3 (Ashkenazi et al., 1998; Li et al., 1998) **(Figure 2)**.

The intrinsic pathway, on the other hand, is activated by intracellular stress induced by oxidative stress or DNA damage via exposure to radiation and chemicals. When looking at the core molecular events of intrinsic apoptosis, this pathway involves the permeabilization of the mitochondrial outer membrane through the dysregulation of the BCL2 family, a class of protein

that includes both anti-apoptotic MCL-1 and BCL2 as well as pro-apoptotic Bax and Bak. The latter collectively control mitochondrial integrity and play a regulatory role in apoptosis (Brunelle and letai 2005; Kalkavan and Green, 2018). The permeabilization of the mitochondrial membrane leads to the release of cytochrome c, Smac, DIABLO and HtrA2. These represent a group of apoptogenic proteins that reside within the intermembrane space of the mitochondria (Cory and Adams, 2002; Wang and Youle, 2016). Once in the cytoplasm, the formation of apoptosomes with APAF1 and caspase-9 takes place, followed by downstream caspase-3 activation (Zou et al., 1999; Green and Llambi, 2015).

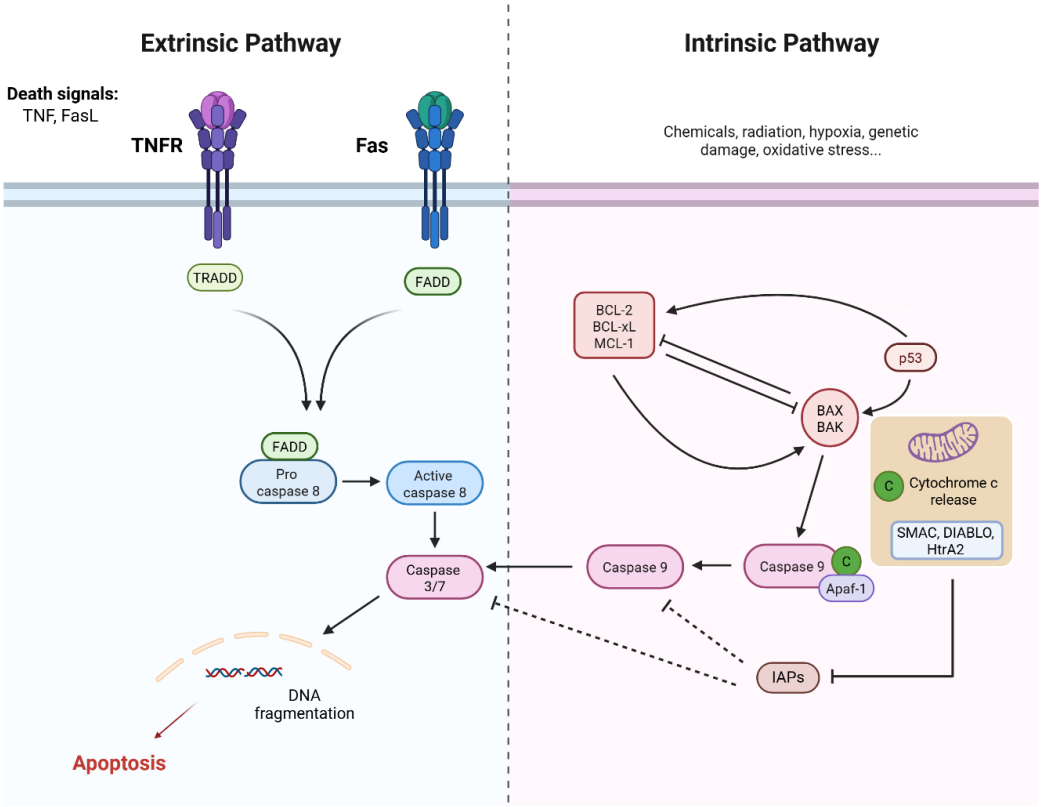

Figure 2: General schematic representation of the extrinsic and intrinsic apoptotic pathways

A simplistic illustration of the main proteins involved in the signalling cascades of the extrinsic intrinsic apoptosis pathway. Extrinc apoptosis (left) is triggered by death receptors (TNFR and Fas), leads to caspase-8 activation and eventual cell death. Intrinsic apoptosis pathway (Right) is initiated by cellular stress signals and involves as a hallmark the mitochondrial cytochrome c release and caspase-9 activation.

Caspase-3, as an executer of apoptosis, further cleaves poly (ADP-ribose) polymerase-1 (PARP-1) a DNA repair protein, thereby promoting and guarantying DNA fragmentation and cellular death (Soldani and Scovassi 2002). Furthermore, inhibitor of apoptosis proteins (IAPs) such as XIAP and survivin, are suppressed by the release of the mitochondrial apoptogenic proteins in the cytosol, thus allowing caspase activation to proceed and ensuring the effective execution of apoptosis (Altieri 2011) **(Figure 2)**.

2- Immune checkpoints and PD-L1 in tumours

a) Introduction on immune checkpoints

Tumours can escape immune surveillance by utilizing immune checkpoint pathways. Immune checkpoints are crucial regulatory pathways that modulate the magnitude and specificity of immune responses, maintaining self-tolerance and preventing excessive immune activation that could result in damage to healthy tissue (Taefehshokr et al., 2020; He and Xu 2020; Tufail et al., 2025).

The most renowned and studied key checkpoints are CTLA-4 and the programmed death-1 (PD-1/PD-L1) axis that act as inhibitory regulators on T cells. While tumours upregulate the expression and recruitment of CTLA-4–bearing regulatory T cells to suppress anti-tumour T cell responses (Buchbinder and Desai 2016; Lin et al., 2024), the PD-1/PD-L1 checkpoint axis functions differently within the TME.

PD-1 is an immune-inhibitory receptor expressed mostly on activated T cells while its ligand PD-L1 is expressed on the surface of tumour and tumour-infiltrating immune cells (Simon and Labarriere 2017). By upregulating PD-L1 expression on their surface, tumour cells allow the binding of this receptor to PD-1 on tumour-specific T cells, thereby effectively neutralizing their cytotoxic function (Buchbinder and Desai 2016; Lin et al., 2024). In this way, cancer cells can ‘hijack’ the immune checkpoint system and effectively evade immune surveillance,

contributing to the formation of an immunosuppressive microenvironment that impairs effector lymphocytes and enables tumour growth despite an active immune response.

b) The mechanism of action of the PD-1/PD-L1 pathway

Upon PD-L1 binding, two signal transducer motifs found in the cytoplasmic tail of PD-1, the immunoreceptor tyrosine-based inhibition motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM), become phosphorylated and lead to the recruitment of the phosphatase SHP-2 (Bardhan et al., 2016; Marasco et al., 2020). SHP-2 subsequently dephosphorylates critical intermediates in the signalling of TCR/CD28, which normally mediates antigen recognition and co-stimulatory signals that activate downstream kinase cascades essential for T-cell proliferation and effector function (Esensten et al., 2016; Hui et al., 2017). Consequently, effector T cells fail to effectively proliferate or induce their cytotoxic activity against PD-L1–expressing tumour cells, shifting the immune balance toward suppression (Lin et al. 2024). PD-L1 is overexpressed in a wide range of malignancies including non–small cell lung cancer (NSCLC), melanoma, NB, glioblastoma, renal cell carcinoma, and breast cancer (Dondero et al., 2015; Mandalà et al., 2016; Qian et al., 2018; Lamberti et al., 2020; Sobhani et al., 2023). This overexpression clinically correlates with poor prognosis, consistent with its role in suppressing anti-tumour immunity.

c) Regulation of PD-L1 expression in tumours: IFNs role

PD-L1 expression in the TME is dynamically regulated by both intrinsic oncogenic (genetic/epigenetic) pathways and extrinsic adaptive inflammatory signals (cytokine-driven) (Asgarova et al., 2018). While tumour-intrinsic genetic alterations such as MYC activation, epidermal growth factor receptor mutations and chromosomal 9p24.1 amplification can constitutively upregulate PD-L1 via PI3K–AKT, MAPK, STAT3 and nuclear factor-κB (NF-κB) signalling pathways (Barrett et al., 2015; Tang et al., 2015; Kim et al., 2017), another important adaptive resistance mechanism is the upregulation of PD-L1 in response to

interferons (IFNs) and other cytokines produced by activated T cells (Wang et al., 2017). Several studies have highlighted how IFNs can lead to the upregulation of PD-L1 in different cancers including ovarian cancer, gastric cancer and NB (Abiko et al., 2015; Dondero et al., 2015; Mimura et al., 2017; Padmanabhan et al., 2022).

Infiltrating CD8+ T cells secreting IFN-γ can induce robust PD-L1 and participate in the creation of what is referred to as adaptive immune resistance, a feedback loop that enables immune attack on malignant cells (Cui et al., 2024). Through the activation of the JAK/STAT pathways, IFN-γ induces PD-L1 transcription as a means for tumours to counteract T cell cytotoxicity. Upon exposure to IFN-γ, tumour cells rapidly activate STAT1 which leads to the recruitment of the transcription factor IRF1 to the PD-L1 promoter, resulting in an upregulation of PD-L1 mRNA, which drives transcription of PD-L1, ultimately resulting in increased PD-L1 surface expression (Garcia-Diaz et al., 2017). It is also noteworthy to mention that type I IFNs have been shown to similarly upregulate PD-L1, nevertheless, the magnitude and the intensity of the response is less than that observed with IFN-γ (Garcia-Diaz et al., 2017; Bazhin et al., 2018; Morimoto et al., 2018).

d) PD-L1 therapeutic targeting

With PD-L1 being expressed on tumour cells, tumour-infiltrating immune cells, and antigen-presenting cells across multiple cancer types, studies have investigated the clinical relevance and advantages of targeting the PD-1/PD-L1 axis with therapeutic antibodies through checkpoint blockade therapy. Anti-PD-1 and anti-PD-L1 monoclonal antibodies such as nivolumab, pembrolizumab, atezolizumab… etc, are now approved for therapeutic use against numerous cancers (Topalian et al., 2012; Jiang et al., 2019). By blocking PD-1/PD-L1 signal, the anti-tumour immune function is restored, leading to enhanced T-cell proliferation, cytokine production, and cytotoxic activity (Jenkins et al., 2018; Jiang et al., 2019).

Melanoma and NSCLC were among the first solid tumours to respond positively and maintain a durable remission after anti-PD-1 therapy (Topalian et al., 2014; Punekar et al., 2022). Several trials have reported positive response rates ranging from 20–30% in advanced melanoma, NSCLC, and RCC after treatment with PD-1 antibodies, with many responses lasting beyond one year (Topalian et al., 2012; Robert et al., 2018). It is important to note that the effectiveness of immunotherapy utilizing anti-PD1 antibodies is tied to the level of PD-L1 expression. In addition, the level of this expression can also be used as a predictive biomarker for immunotherapy success. This has been seen in studies with NSCLC and melanoma where high tumour PD-L1 leads to substantial anti-PD1 response and improved overall survival (Topalian et al., 2012; Reck et al., 2016).

Interestingly, other cancers that are classically less responsive to immunotherapy have also shown that in the case of PD-L1 expression, checkpoint blockade can significantly improve outcomes. In a large recent clinical trial, the use of anti-PD-L1 antibody atezolizumab in combination with bevacizumab, an antibody targeting the vascular endothelial growth factor A (VEGF-A), as well as the chemotherapeutic agent paclitaxel significantly prolonged progression-free survival in patients with metastatic triple-negative breast cancer (TNBC) (Gion et al., 2025), an aggressive subtype known to be refractory to several standard treatment underscoring how checkpoint inhibition via PD-1/PD-L1 axis targeting can be utilized to render resistant cancers more susceptible.

e) PD-L1 expression, function and therapeutic implication in NB

PD-L1 is expressed on NB cells, although reports vary widely with immunohistochemical studies finding PD-L1 positivity in anywhere from 15% to more than 70% of NB tumours (Wienke et al., 2021). Several studies have linked higher PD-L1 expression in NB to more advanced stage, inferior survival, and general poor outcomes (Chowdhury et al., 2015; Saletta et al., 2017; Zuo et al., 2020). Nevertheless, other controversial findings have reported that

lower PD-L1 was found in aggressive MYCN-amplified and high-risk NB tumours leaving the prognostic significance of PD-L1 to be uncertain in NB (Wienke et al., 2021).

When it comes to its expression, NB cells show both constitutive and inducible PD-L1. Nearly all tested NB cell lines display baseline PD-L1 on the surface (Dondero et al., 2015). In addition, and as previously mentioned, PD-L1 is further upregulated by inflammatory mechanisms such as IFN-γ exposure that has been proven to drive robust PD-L1 induction in several NB cell lines and xenografted tumours (Dondero et al., 2015; Nallasamy et al., 2018).

Furthermore, the presence of a functional PD-1/PD-L1 pathway in NB has led to its investigation as a target for checkpoint blockade therapies. The blockade of PD-1/PD-L1 using the novel compound SF-9-2 successfully decreased NB cell proliferation and migration while also restoring T cell activity through reactivating its proliferative and cytokine productive actions in in vitro co-culture systems (Wang et al., 2025). Moreover, a NB mouse model treated with SF-9-2 also exhibited a strong tumoral growth inhibition, confirming the efficacy of PD-1/PD-L1 pathway targeting (Wang et al., 2025).

Early-phase clinical trials of PD-1/PD-L1 inhibitors in several paediatric solid tumours including NB have shown some success as reported in a study where anti-PD1 prolongs survival of patients with recurrent and metastatic NB (Hoshi et al., 2024). Nevertheless, its action has been proven to be limited, likely due to low immunogenicity in NB in comparison to other cancers such as melanoma or NSCLC (Kennedy et al., 2023). Therefore, combination therapy has been actively explored to render these tumoral cells more vulnerable.

In line with this, a study has indeed reported that PD-1/PD-L1 blockade alone was ineffective against disseminated NB, but when used in combination with an anti-CD4 monoclonal antibody induced strong CD8+ T cell–mediated responses and provided long-term protection and improved survival in mice bearing NB tumours (Rigo et al., 2017).

Similarly, combined immune checkpoint blockade with anti-PD-1 and anti-CTLA-4 antibodies has been reported to be more effective than single-agent therapy in suppressing established NB tumours (Shirinbak et al., 2021). Moreover, the administration of chemotherapy prior to these immunotherapeutic options further improved survival, highlighting multimodal approaches as a promising treatment for NB (Shirinbak et al., 2021). A clinical study has also documented two cases of refractory NB in which treatment with the PD-1 inhibitor nivolumab in combination with the anti-GD2 antibody dinutuximab beta resulted in the remission of both NB patients (Ehlert et al., 2020). These findings suggest that dual targeting of PD-1 and GD2 may represent a rational strategy to overcome resistance in high-risk NB and warrant further clinical investigation.

3- p38MAPK signalling pathway in cancer

The p38 mitogen-activated protein kinase (p38 MAPK) pathway is a highly conserved kinase cascade that serves as a central mediator of cellular responses to stress signals (Zarubin and Han 2005; Canovas and Nebreda, 2021). In mammals, there are four p38 isoforms (p38α/β/γ/δ, encoded by MAPK14, MAPK11, MAPK12, MAPK13 respectively) that share activation mechanisms but have distinct expression patterns and functions (Cuadrado and Nebreda, 2010).

p38α is the most ubiquitously expressed and was originally identified as a 38-kDa protein rapidly phosphorylated in cells exposed to endotoxin and hyperosmotic shock (Han et al., 1994). All p38 isoforms are threonine/tyrosine kinases activated by dual phosphorylation on a conserved Thr-Gly-Tyr motif in their activation loop. This phosphorylation is carried out by the upstream MAPK kinases MKK3 and MKK6, which themselves are activated by a variety of MAPK kinases in response to stress (Enslen et al., 1998). Notably, p38 responds to a wide range of stressors including inflammatory cytokines, UV irradiation, oxidative stress, DNA damage, and osmotic shock (Zarubin and Han, 2005; Kyriakis and Avruch, 2012; Canovas and Nebreda, 2021).

Once activated, p38 MAPKs phosphorylate a broad array of substrates both in the cytoplasm and nucleus, including transcription factors such as ATF2 and p53, cell-cycle regulators, and downstream kinases like MK2/3 that modulate mRNA stability and translation, thereby orchestrating complex cellular programs (Zarubin and Han, 2005). Interestingly, the outcome of p38 signalling on the cell is closely dictated by its amplitude and duration; strong or sustained p38 activation drives apoptosis, senescence or differentiation, while transient, low-level p38 activity promotes cell survival (Puri et al., 2000; Haq et al., 2002).

a) The dual role of p38MAPK in cancer

Given its functions in growth arrest, apoptosis, and differentiation, p38 has been implicated in tumour suppression and was initially characterized as a tumour-suppressor kinase for its ability to antagonize oncogenic signals (Martínez-Limón et al., 2020). One mechanism of this anti-cancer effect is via enforcing oncogene-induced senescence through inducing an irreversible cell-cycle arrest accompanied by senescence markers (Haq et al., 2002). In addition, it has been demonstrated that the inhibition of p38 signalling allows cells to bypass senescence and undergo transformation (Hui et al., 2007). Moreover, mice lacking p38α or lacking the p38-activated MAP kinase-activated protein kinase 5 (MAPKAPK5) show accelerated cancer development, supporting that the p38 pathway normally restrains tumorigenesis in mammary and skin cancer (Bulavin et al., 2004; Sun et al., 2007). Conversely, removing negative regulators of p38 such as Wip1 has been proven to reduce mammary tumour formation in mice, confirming its anti-cancer effects (Bulavin et al., 2002).

On the other hand, and oppositely to what was reported above, several studies have conversely showed that p38 MAPK can also function as a tumour promoter in certain contexts (Gupta et al., 2014; Kudaravalli et al., 2022). The latter has been found to be hyperactivated in certain tumours enhancing their survival, invasiveness, and contributing to therapy resistance. For instance, head & neck squamous cell carcinoma as well as lung carcinoma cells frequently

show elevated active p38 compared to normal cells (Greenberg et al., 2002; Leelahavanichkul et al., 2014). In breast cancer, p38α was found to be required for efficient tumour growth since the ablation of p38α caused excessive replication stress, chromosome instability and consequently limited the tumour's progression (Cánovas et al., 2018). Similarly, p38 signalling was also linked to improved invasion and metastasis in bladder cancer cells (Kumar et al., 2010). Furthermore, in KRAS-driven lung cancer, p38α switches from a tumour-suppressive role to a tumour-promoting role by sustaining growth signals. Blocking p38α reduces lung tumour cell proliferation and slows their progression (Vitos-Faleato et al., 2020).

This dual action of p38 between tumour-suppressive and tumour-promoting roles can be, as mentioned before, reconciled by considering context and timing. Early in tumour development, when oncogenic stress first arises, p38 activation tends to trigger anti-proliferative responses (DNA repair, apoptosis or senescence) that suppress tumour initiation. However, in established tumours, especially under harsh microenvironmental conditions such as elevated inflammation or during treatment initiation with chemotherapy for example, cancer cells that maintain or reactivate p38 signalling gain a survival advantage (Martínez-Limón et al., 2020).

b) p38MAPK signalling in NB

In NB, reports indicate that p38 signalling mediates an aggressive phenotype of this cancer. Exposure to chemotherapeutic agents such as etoposide has been shown to strongly activate p38 in NB cells, which in turn induces drug-efflux genes such as MDR1, thereby endowing these cells with chemoresistance (Marengo et al., 2013). Moreover, pharmacologic inhibition of p38α/β sensitizes NB cells to etoposide, further confirming its involvement in treatment resistance. This is achieved through promoting angiogenesis and invasive markers like VEGF expression and MMP-9 activity in NB cells, driving its progression and metastasis (Marengo et al., 2013).

In addition, treatment of SH-SY5Y NB cells with IFNβ was shown to activate p38 MAPK (Dedoni et al., 2014), most importantly, this signalling pathway was found to counteract IFNβ-induced apoptosis, showing that p38 functions as a pro-survival mediator in the IFN response. Conversely, p38 signalling has been oppositely linked to the induction of apoptosis by mediating reactive oxygen species induced NB cell death after treatment with the anti-cancer agent Fenretinide (Osone et al., 2004). This underscores the dual and context-dependent role of p38 MAPK in cancer, particularly in NB, acting either as a pro-survival factor that promotes tumour progression, or as a pro-apoptotic mediator in response to specific stress signals.

Part III: Interferons

1- IFN classes, subtypes and receptors: A comprehensive overview

Interferons (IFNs), mainly named upon their discovery in 1957 for their ability to 'interfere' with viral infection, are a family of cytokines that are endowed with several immunomodulatory, anti-proliferative and anti-tumoral properties (Isaacs and Lindenmann 1957; Negishi et al., 2018). IFNs are class II alpha-helical cytokines and are generally categorized in humans into three subclasses, type I, type II, and type III, each of which uses a well-defined and distinct receptor complex (**Table 2**) (Pestka et al., 2004; de Weerd and Nguyen 2012; Negishi et al., 2018).

Table 2: Comparison of type I, II and III interferons

IFN Type	Subtypes	Receptor Complex	Expression Pattern	Key Features
Type I IFNs	IFNα (13 subtypes), IFNβ, IFN-ε, IFN-κ, IFN-ω	IFNAR heterodimer: IFNAR1: low affinity IFNAR2: high affinity	Widely expressed	Strong antiviral activity, first line of defence and has a broad activity due to its wide expression
Type II IFN	IFN-γ	IFNGR: IFNGR1 and IFNGR2	Broadly expressed: Mostly in immune cells	Immunoregulatory role: Bridges innate and adaptive immunity
Type III IFNs	IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B)	IFNLR1 (IL-28RA) and IL-10R2 (shared with IL-10 and IL-22)	Mostly epithelial cells and restricted tissue distribution	Tissue specific antiviral activity: important for mucosal immunity

Type I IFNs include IFNα, IFNβ, and several other less known subtypes which are IFN-ε, IFN-κ and IFN-ω. On the other hand, Type II IFN is represented by a single member, IFN-γ. Later

in 2003, a genome analysis study identified a new IFN subtype, type III IFNs, also referred to as IFN-λ (Sheppard et al., 2003; Kotenko et al., 2003).

2- IFNs cellular signalling pathway

Type I and type II IFNs signal through IFNAR and IFNGR, respectively. Both receptors are heterodimeric single pass transmembrane proteins (Platanias, 2005). IFNAR is composed of IFNAR1 (low-affinity) and IFNAR2 (high-affinity) subunits while IFNGR is comprising IFNGR1 and IFNGR2 chains. The subunits of both receptors interact directly with a member of the Janus activated kinase (JAK) family, an important component of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway through which IFNs signal mainly. IFNAR subunits are associated with JAK1 and tyrosine kinase 2 (TYK2), while those composing IFNGR interact with JAK1 and JAK2 (Platanias, 2005; de Weerd and Nguyen 2012).

Unlike type I and type II IFNs, type III IFNs signal through a receptor complex composed of a unique subunit, IFNLR1, and a shared subunit, IL10RB, which is also utilized by IL-10, IL-22, and IL-26 (Kotenko et al., 2003; Mendoza et al., 2017). IFN-λ first binds to IFNLR1 and subsequently recruits IL10RB to form the functional signalling complex. Similar to type I IFNs, the kinases associated with the IFNLR subunits are JAK1 (for IFNLR1) and TYK2 (for IL10RB) (Kotenko et al., 2003; Mendoza et al., 2017).

Once the IFN binds to its receptor, the associated JAKs are rapidly activated through autophosphorylation and will then themselves activate STAT proteins. In the case of type I IFNs, the STAT1 and STAT2 will dimerize and further associate IFN-regulatory factor 9 (IRF9) to form the IFN-stimulated gene factor 3 (ISGF3) complex (Platanias 2005). The latter will then translocate to the nucleus and bind to IFN-stimulated response elements (ISRE) in gene promotors to consequently induce the transcription of what is known as IFN-stimulated genes (ISGs). These genes give rise to several proteins, also referred to as IFN-induced proteins, that

reinforce IFN response through transducing different signalling pathways (Schneider et al., 2014; Lukhele et al., 2019) **(Figure 3).**

As for IFN-γ, homodimers of STAT1 bind to gamma-activated sequence (GAS) elements in target gene promoters, inducing a distinct set of genes (Hu and Ivashkiv, 2009) (**Figure 3**). Aside from the classic JAK-STAT route, IFNs activate other non-canonical signalling pathways that contribute to the full spectrum of IFN responses including MAP kinase, PI3K/AKT, and NF-κB (Mazewski et al., 2020).

3- IFNs therapeutic application: Between viral infections and cancer

As previously mentioned, IFNs derive their name from their ability to interfere with viral infections. Thanks to recombinant DNA technology, IFNs were FDA approved and used for

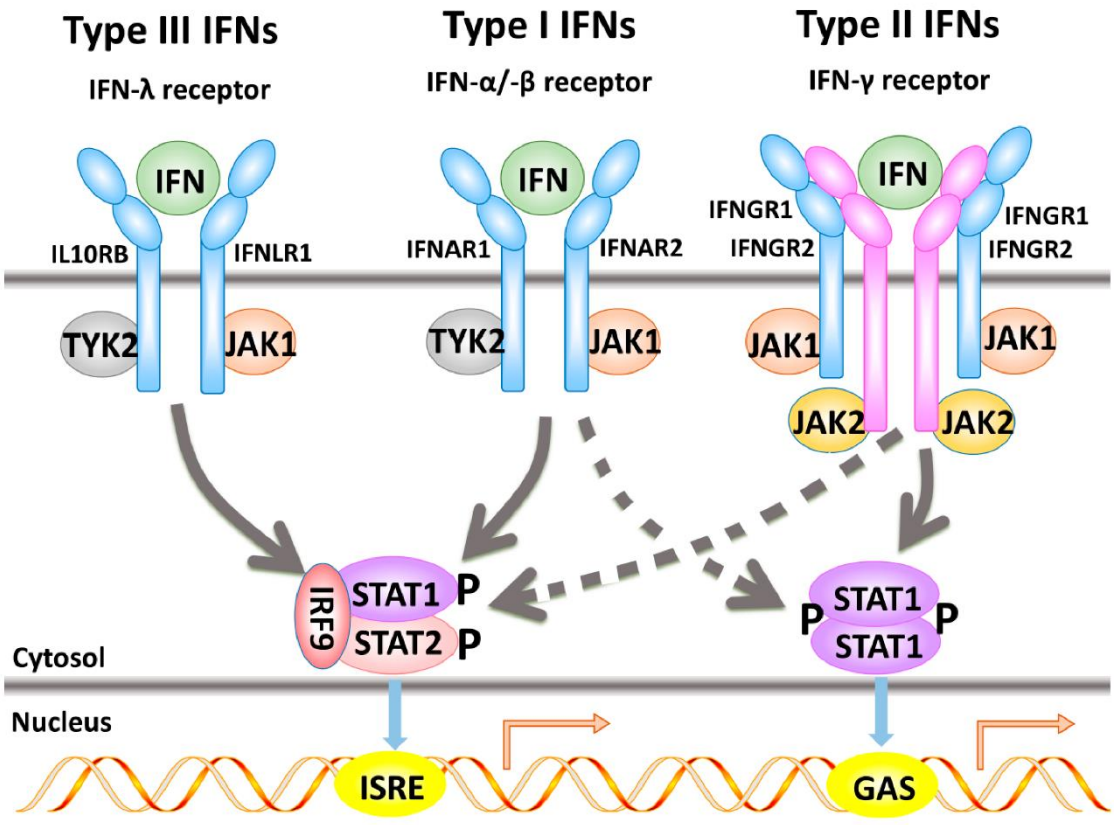

Figure 3: Signalling pathways activated by type I, II, and III interferons *The diagram illustrates the distinct receptor complexes and downstream signalling cascades of type I (IFNα/β), type II (IFN-γ), and type III (IFN-λ) interferons. Activation of JAK/STAT pathways leads to the formation of transcriptional complexes that translocate into the nucleus and bind specific DNA elements: ISRE for type I and III IFNs, and GAS for type II IFN.*

several viral diseases including hepatitis B and C (Borden et al., 2007; Chen and Yu 2010). By 1986, recombinant IFNα2 became the first IFN approved by the U.S. FDA for the treatment of hairy cell leukaemia (Quesada et al., 1986). As years went by, IFNs were used for multiple other cancers including myelogenous leukaemia, low-grade lymphomas, AIDS-related Kaposi's sarcoma, malignant melanoma and renal cell carcinoma (Murren and Buzaid 1989; Mitsuyasu 1991; Fosså 2000; Di Trolio et al., 2015; Healy et al., 2021). In 1993, IFNβ was approved as the first disease-modifying therapy for multiple sclerosis (MS), thanks to its ability to balance and reduce autoimmune T cell activity in the central nervous system (Paty and Li 1993; Nakatsuji et al., 2007)

Several mechanisms endow IFNs with these antiviral and anti-tumoral properties. Upon infection, virus-infected cells release type I IFNs, which trigger an antiviral state in both infected and neighbouring cells through the activation of the JAK-STAT pathway, which induces the expression of ISGs that directly inhibit viral replication and promote apoptosis in infected cells, thus limiting viral spread (Stetson and Medzhitov, 2006). In addition, in cancer, IFNs signalling can upregulate the expression of genes that cause cell-cycle arrest, induce apoptosis, and inhibit tumour cell survival and proliferation (Chawla-Sarkar et al., 2003; Sato et al., 2006; Parker et al., 2016).

IFNs can also exert important immunomodulatory functions. Type I IFNs can enhance MHC class I expression across almost all cell types (Seliger et al., 2020), while IFN-γ induces MHC class II on both immune cells such as macrophages, dendritic cells, as well as non-immune cells such as fibroblasts (Butticè et al., 2006; Bian et al., 2023). By increasing the presentation of viral or tumour-derived antigens on MHC I and II molecules, IFNs facilitate the recognition and elimination of infected or malignant cells by cytotoxic T lymphocytes ($CD8^+$ T cells) and helper T cells ($CD4^+$).

While IFN-λ shares signalling mechanisms with type I IFNs through the JAK-STAT pathway, its restricted receptor distribution enables it to act predominantly on mucosal cells found in the respiratory and gastrointestinal epithelia where it functions as a first-line defence against viral replication in these tissues during respiratory and enteric infections (Lasfar et al., 2016).

4- IFNβ in NB

IFNβ has been proven to successfully modulate NB tumour vasculature and perfusion, enhancing blood flow and delivery of adjuvant therapy (Sims et al., 2009). A similar outcome was found in a xenografted-NB mouse model where systemic delivery of human IFNβ via adeno-associated virus vectors combined with the chemotherapeutic agent cyclophosphamide, significantly restricted tumour growth and reduced tumour blood vessel density leading tumour regression and longer survival (Steck et al., 2005). Furthermore, in vitro and in vivo treatment of NB with a combination of IFNβ and temozolomide (TMZ) successfully decreased cellular proliferation and tumour burden through the attenuation of the DNA repair enzyme O^6-methylguanine DNA methyltransferase (MGMT) expression (Rosati et al., 2009). In human SH-SY5Y NB cells, IFNβ has been shown to induce intrinsic apoptotic cell death (*Refer to intrinsic apoptosis pathway earlier in the text in section; Figure 2*) through the activation of the JAK-STAT signalling cascade (Dedoni et al., 2010). Specifically, IFNβ led to an increased phosphorylation and nuclear translocation of STAT1, resulting in protein kinase R (PKR) induction that plays a major role in regulating cellular processes such as growth, differentiation, and apoptosis (Samuel et al., 1997). In SH-SY5Y cells, PKR ultimately leads to the upregulation of pro-apoptotic proteins such as caspase-3, resulting in apoptosis (Dedoni et al., 2010) (**Figure 4**).

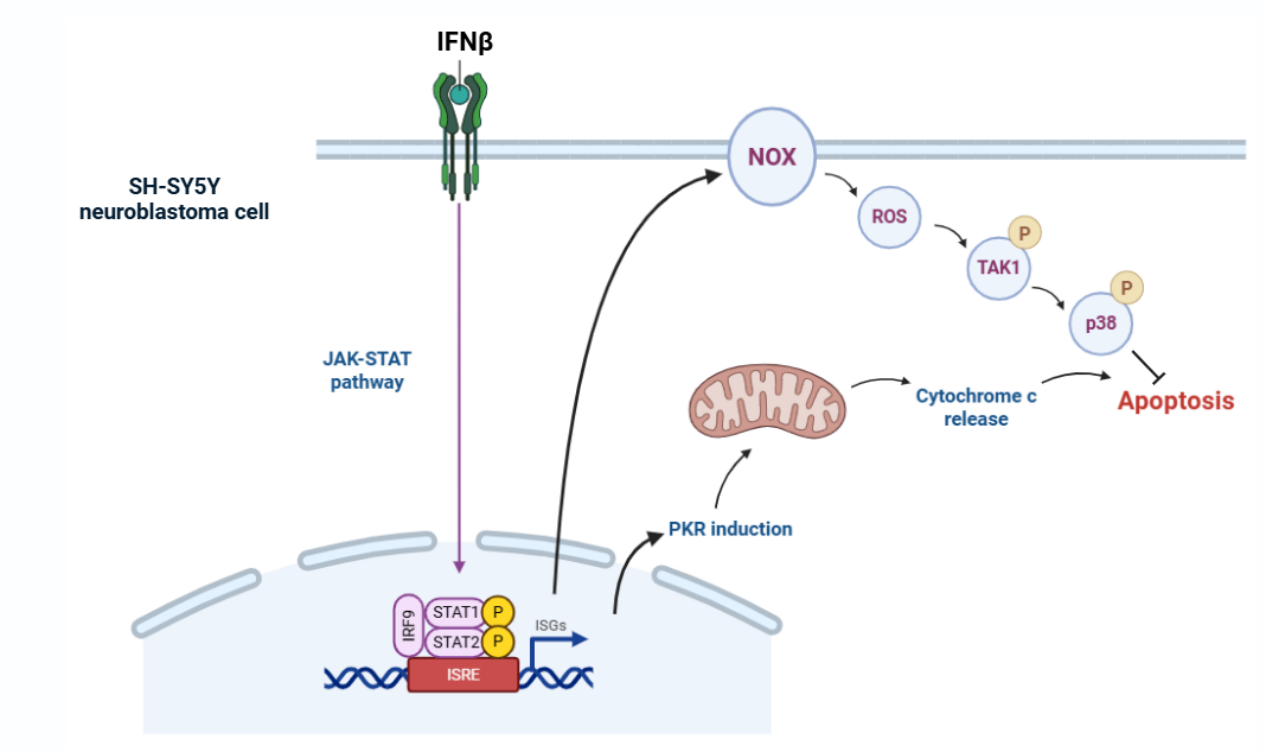

Figure 4: IFNβ–mediated apoptotic and counter-regulatory p38 MAPK signalling in SH-SY5Y neuroblastoma cells
IFNβ activates JAK-STAT pathway in SH-SY5Y cells, leading to the induction of PKR, enhanced expression of NADPH oxidase subunit and ROS production. These events stimulate TAK1 phosphorylation and trigger a delayed, sustained activation of p38 MAPK pathway, a counteractive mechanism to the intrinsic apoptosis initiated by PKR induction

Additionally, IFNβ treatment led to the downregulation of the PI3K/Akt pathway marked by a decrease in the phosphorylation of Akt and GSK-3β, diminishing cell survival and further enhancing apoptosis within these cells (Dedoni et al., 2010). As a continuation to this study, IFNβ was further investigated in this cell line, and was shown to activate p38 MAPK pathway in SH-SY5Y cells (Dedoni et al., 2014). The latter, as mentioned previously in the text, are serine/threonine-specific protein kinases that play a dual role in cancer either by being tumour suppressors or tumour promotors (Martínez-Limón et al., 2020). The activation of p38 MAPK by IFNβ in SH-SY5Y cells was found to serve as a counter-regulatory mechanism through which this cytokine modulates the JAK-STAT-mediated apoptosis it initially induces (Dedoni et al., 2014) (**Figure 4**).

5- Limitations and side effects of IFNs in treatment

Although efficacious, type I IFNs therapy has been associated with significant toxicity, contributing to its decline in popularity. IFNα use was shown to cause “flu-like” symptoms such as fever, chills, malaise, headaches, myalgias, and fatigue that patients experienced frequently, sometimes after each dose (Malik and Wadler 2001; Gold et al., 2005; Weber et al., 2015). Other common side effects include anorexia, weight loss, nausea, and liver enzyme elevations. Additionally, IFNs can cause hematologic suppression (leukopenia, thrombocytopenia) and autoimmune phenomena (thyroiditis, hepatitis…) (Quesada et al., 1986; Raanani and Ben-Bassat 2002; Weber et al., 2015). These toxicities often lead to dose reductions or early discontinuation in clinical trials and treatment, limiting the tolerability of long-term IFN therapy as well as its efficacy (Raison et al., 2005; Bregman et al., 2011). Furthermore, IFN can also cause neuropsychiatric effects where up to 30–45% of patients develop clinically significant depression during prolonged therapy (Miyaoka et al., 1999; Musselman et al., 2001). Cognitive impairment, mood disturbances, and even suicidal ideation have been reported, mainly as a result of the high and prolonged doses required to achieve therapeutic efficacy (Musselman et al., 2001; Capuron et al., 2002; Raison et al., 2005).

Part IV: Lipids as structural and functional mediators

1- Lipids

Lipids are a group of hydrophobic or amphipathic molecules that play essential roles as structural components and functional signalling mediators in biological systems (Fahy et al., 2005; Wymann and Schneiter, 2008; Sunshine and Iruela-Arispe, 2017). They are classified into eight major categories depending on distinct chemical composition and origin of biosynthesis. These classes are: Fatty acids, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, saccharolipids, prenol lipids, and polyketides (Fahy et al., 2011).

- ***Fatty acids*** (FA) serve as fundamental building blocks and energy-rich fuel. These lipids can be enzymatically converted into potent bioactive lipids such as eicosanoids and acylethanolamines (AEs) that regulate inflammation and different other signalling pathways (de Carvalho and Caramujo, 2018; Cisa-Wieczorek and Hernández-Alvarez, 2020; Mock et al., 2023).
- ***Glycerolipids,*** on the other hand, represent mainly the principal lipidic energy storage within cells (Voelker, 2013). Nevertheless, some glycerolipids such as diacylglycerol (DAG) can carry the role of second messengers that activate protein kinase C and other signalling cascades, creating a bridge between lipid metabolism and essential downstream processes (Bell, 1986; Spiegel and Kolesnick, 1996).
- ***Glycerophospholipids*** are the predominant structural constituents of the membrane lipidic bilayer, and their cleavage can also yield key signalling molecules such as DAG and inositol trisphosphate (Hishikawa et al., 2014; Kolczynska et al., 2020).
- ***Sphingolipids*** likewise are also integral membrane components, especially in nerve and lipid raft microdomains, and they are also well-characterized signalling elements that modulate apoptosis, proliferation and stress responses (Posse de Chaves, 2006; Patwardhan et al., 2016).

- ***Sterol lipids*** such as cholesterol and its derivatives can stabilize cellular membranes while also maintaining the role of steroid hormones' precursors (Urich, 1994). The latter can initiate several signalling cascades that can regulate development, metabolism, and homeostasis (Mani et al., 2012).
- ***Prenol lipids*** also known as isoprenoids, are derived from isoprene units. These include the coenzyme Q, an essential electron carrier in the mitochondrial electron transport chain (Guerra and Pagliarini, 2023). This class also includes farnesyl and geranyl groups that attach to certain proteins and anchor them to cellular membranes (Holstein and Hohl, 2004).
- ***Saccharolipids*** are lipid–carbohydrate conjugates, meaning that they are composed of a FA that is linked to a sugar backbone (Park et al., 2021). The most renowned saccharolipid is bacterial lipopolysaccharide (LPS) which is expressed on the outer membranes of gram-negative bacteria. Its role is to provide structural integrity while also acting as a powerful endotoxin that triggers host innate inflammatory responses (Rhee, 2014; Park et al., 2021).
- ***Polyketides*** represent a vast class of secondary metabolites that are often produced by microbes and plants. They are known for their diverse biological activities and their pharmacological importance. Notably, they comprise various anticancer and antifungal agents including antibiotics, immunomodulators and parasiticides (Gomes et al., 2014; Barbosa et al., 2020).

Among endogenous lipid mediators, FA derivatives include the sub-class of N-acylethanolamides (NAEs). NAEs group is composed of endocannabinoids (eCBs) and endocannabinoid-like molecules (eCBs-like). Both groups have demonstrated significant anti-cancer potential by inducing apoptosis, suppressing proliferation and angiogenesis in different cancers including prostate cancer, colorectal cancer, ovarian carcinoma, glioblastoma and

breast cancer (Picardi et al., 2014; Pagano et al., 2021; Romano et al., 2022; Lin et al., 2023; Costas-Insua and Guzmán, 2023).

2- PEA and OEA

a) Classification and overview

NAEs are a group of naturally occurring signalling lipids that are found in mammals, invertebrates and plants (Borrelli and Izzo, 2009). The family of NAEs include two distinct classes of lipids: eCBs and eCBs-like molecules. N-arachidonoyl ethanolamide (AEA) belongs to the eCBs class as it is an endogenous ligand of cannabinoid receptors (CBRs) (Fezza et al., 2014; Wellner et al., 2013; Mechoulam and Parker 2012). On the other hand, other saturated and monounsaturated NAEs including oleoylethanolamide (OEA), palmitoylethanolamide (PEA), stearoylethanolamide (SEA), linoleoylethanolamide (LEA), docosahexaenoylethanolamide (DHEA) and eicosapentaenoylethanolamide (EPEA) are described as eCBs-like compounds mostly since they are structurally related to eCBs but have limited to absent affinity to CBRs (Borrelli and Izzo, 2009; Fezza et al., 2014).

These lipid mediators contribute to the enhancement and prolongation of eCBs signalling through the so-called "entourage effect." This occurs either by inhibiting the enzymatic hydrolysis of eCBs, thereby increasing their bioavailability, or by allosterically modulating their interaction with cannabinoid receptors (Fezza et al., 2014). Beyond their modulatory role in eCBs signalling, these compounds also act as ligands for various receptors and mediate multiple biological processes. Among the most extensively studied NAEs are OEA and PEA, with PEA being the most common (Rankin and Fowler, 2020) and the first NAE to ever be discovered (Kuehl et al., 1957).

b) PEA and OEA structure and distribution

As members of the same family, OEA and PEA composition is that of a typical eCBs-like lipid. While the main structure of these molecules is commonly formed by a FA linked to an

ethanolamine, the primary difference between these two bioactive lipids lies in the nature of the FA chain they are derived from. PEA is derived from palmitic acid (C16:0), a saturated fatty acid, while in contrast, OEA is formed by oleic acid (C18:1, n-9), a monosaturated fatty acid (Lo Verme et al., 2005; Hesselink et al., 2014). These small structural variations heavily influence the three-dimensional conformation of these molecules and as a consequence, differentially determines their functional specificity.

Additionally, these molecules exhibit distinct patterns of occurrence in both dietary sources and endogenous biosynthesis across various tissues. PEA was initially discovered as a food component in egg yolk, peanut meal, and soybean (Lo Verme et al., 2005). Later, endogenously synthesized PEA was isolated from mammalian tissue (Bachur et al., 1965) and was found to be synthesized in the nervous system, the immune system as well as peripheral tissues such as intestines and muscles where it exerts a plethora of different effects (Mattace Raso et al., 2014; Hesselink et al., 2014). Similarly, OEA can be detected in traces in different dietary sources including oatmeal, soybean and cocoa beans (Bowen et al., 2017). Endogenously, the latter is mainly synthesized in the small intestines and can also be found in adipocytes, brain, muscles, heart, lung, spleen etc… (Bowen et al., 2017).

c) PEA and OEA metabolism

Generally, NAEs share a similar metabolism involving the formation of a N-acylethanolamine phospholipids, called N-acyl phosphatidylethanolamines (NAPEs). These NAPES are formed through a reaction catalyzed by a calcium- and cyclic AMP-regulated N-acyltransferase, and it consists of the binding of a FA deriving from membrane phospholipids to the amine group of phosphatidylethanolamines (PE). NAPEs are then finally hydrolyzed by N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) to give the final NAE. What differentiates one NAE from the other is the nature of the FA bound to PE. In the case of OEA and PEA, the NAPE species from which they emerge consist respectively of oleic acid

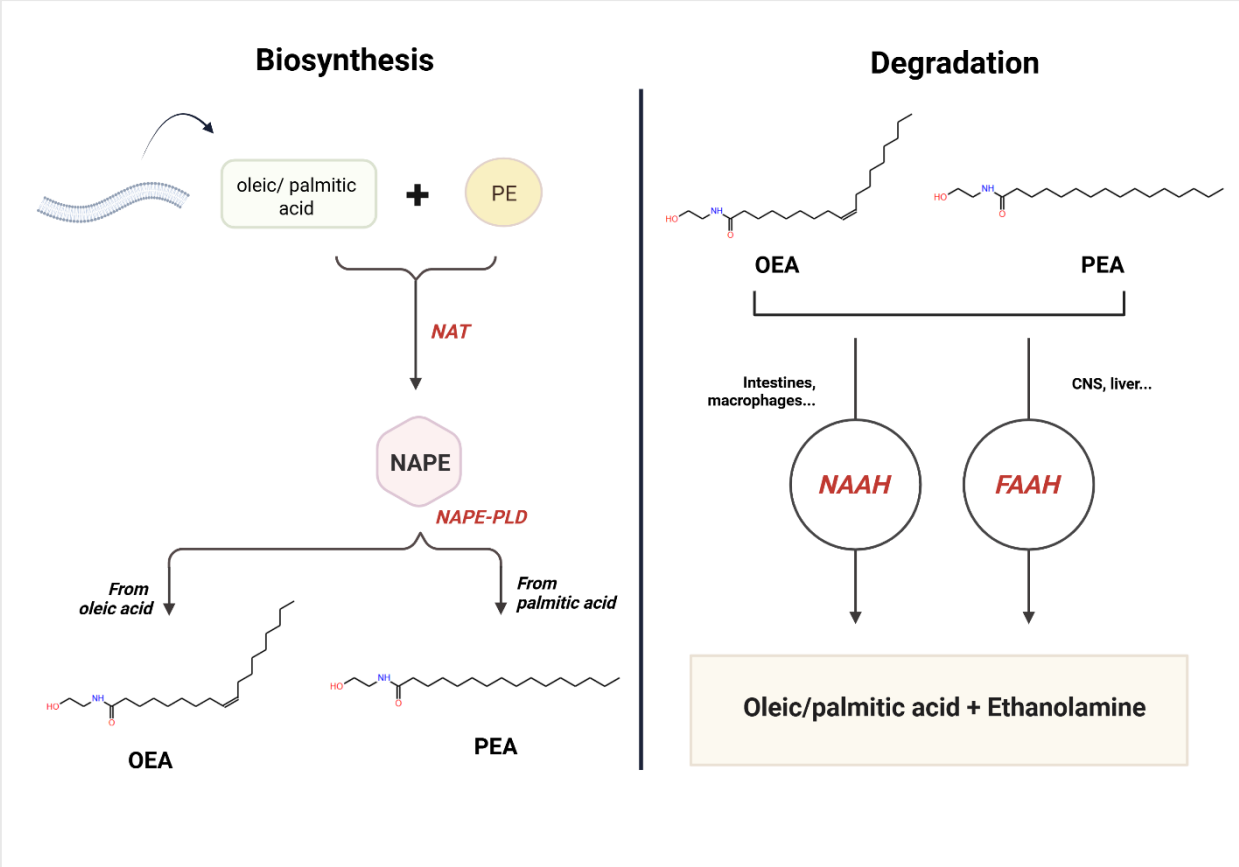

Figure 5: Biosynthesis and degradation pathways of OEA and PEA *The biosynthetic pathway of oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) from oleic and palmitic acids via NAPE intermediates (left). The enzymatic degradation by NAAH and FAAH into oleic/palmitic acid and ethanolamine (right).*

and palmitic acid. When it comes to degradation, the hydrolysis of both these lipid mediators is ensured by two amidase enzymes: N-acyl-ethanolamine-hydrolyzing acid amidase (NAAH) and fatty acid amide hydrolase (FAAH). Both enzymes contribute to the degradation of NAEs to FA and ethanolamine. While the NAAH is a lysosomal cysteine hydrolase and FAAH is a membrane-bound serine hydrolase, the expression, activity and therefore the involvement of each enzyme are tissue-dependent. FAAH is predominantly found in the CNS and liver, while NAAH is mostly abundant in the intestine and macrophages. However, it is worth noting that some cross-species variability in the expression and activity of these enzymes is recorded (Di Marzo and Petrosino 2007; Thabuis et al., 2008; Sihag and Jones, 2018; Sá and Castor, 2023) **(Figure 5).**

d) PEA and OEA targets, roles and effects

As mentioned previously, and unlike the eCBs anandamide, OEA and PEA do not activate classical cannabinoid CB1 or CB2 receptors. Instead, their biological effects are mediated through a variety of non-cannabinoid targets, enabling a wide range of physiological and therapeutic actions (Fezza et al., 2014).

After identifying PEA in different dietary sources in the 1950s and attributing the anti-allergic properties of certain foods such as egg yolks and peanut oil to it (Long et al., 1951; Coburn et al., 1954; Kuehl et al., 1957), PEA was further studied for its role as an immune-boosting and anti-inflammatory molecule. PEA can bind to different receptors including the nuclear receptor peroxisome proliferator-activated alpha (PPARα) as well as PPARγ, to a much lower affinity, transient receptor potential cation channel subfamily V member 1 (TRPV1), G protein-coupled receptor 55 (GPR55) and G protein-coupled receptor 119 (GPR119) (Table 1). This multi-target signalling endows PEA with its pleiotropic effect (Kleberg et al., 2014; Fezza et al., 2014) **(Figure 6).**

During pathological states, PEA is synthesized on demand and is highly upregulated (Franklin et al., 2003; Clayton et al., 2021) enabling it to activate different pathways and mediate different effects. Its anti-inflammatory and immunomodulatory properties are attributed to its binding to PPARα receptor on different immune cells. For instance, PEA can reduce inflammation through decreasing mast cells degranulation and macrophage activation (Aloe et al., 1993; Mazzari et al., 1996; Hesselink, 2013; Facci et al., 1995). In addition, PEA is an effective analgesic molecule that has long been used for the management of pain thanks to its affinity to TRPV1 channels and PPARα receptors (Gabrielsson et al., 2016; Varrassi et al., 2025; Lang-Illievich et al., 2023; D'Amico et al., 2020). GPR55, a newly identified target of PEA (Ryberg et al., 2007), has also been shown to mediate PEA's anti-inflammatory and anti-nociceptive

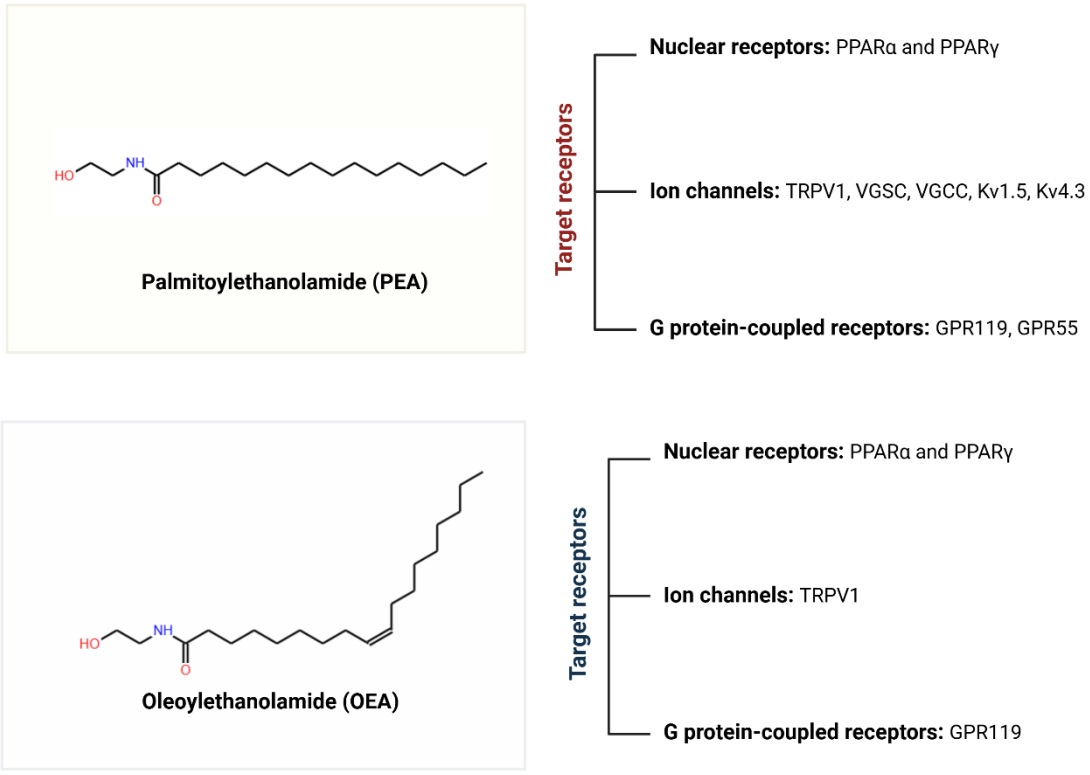

Figure 6: Summary of target receptors of PEA and OEA

activity (Naderi et al., 2012). PEA has also been shown to exert anti-epileptic effects (Sheerin et al., 2004; Post et al., 2018; Bortoletto et al., 2022) and neuroprotective effects against AD (Beggiato et al., 2019), Parkinson's disease (PD) (Esposito et al., 2012) and spinal cord injury (SCI) (Genovese et al., 2008).

When it comes to OEA, changes in its levels are generally governed by food intake. Several studies have reported that food deprivation leads to a decrease in intestinal levels of OEA, an effect that is reversed upon re-feeding (Fu et al., 2007; Peterson et al., 2006; Igarashi et al., 2015). Specifically, mucosal cells within the duodenum and jejunum synthesize and release OEA upon feeding (Fu et al., 2008). These OEA level changes have been proven to underlie satiety and feeding behaviour by acting through different receptors, including TRPV1 (Wang et al., 2005) and the PPARα receptor (Fu et al., 2003), which are known to be locally expressed within the gut and on vagal afferents. This is further corroborated by the fact that OEA levels within the brain do not go through any fluctuations during starvation or feeding (Izzo et al.,

2010; Bowen et al., 2017), further supporting the possibility of an influential peripheral modulation on feeding behaviour. In addition, OEA has been involved in weight control, lipid metabolism, insulin sensitivity and glycemic control (Fu et al., 2003; Sears and Perry, 2015; Pouryousefi et al., 2022). These effects are ensured by the PPARα receptor as well as the GPR119 receptor.

OEA can alleviate inflammatory reactions and suppress pro-inflammatory cytokines (Yang et al., 2016; Lama et al., 2020). It can also participate in the modulation of helper T-cells profile and enhance symptoms related to allergic asthma and atopic dermatitis all by acting through GPR119 (Lee and Im, 2025). OEA can also exert anti-nociceptive effects by signalling through both PPARα and TRPV1 (Ho et al., 2008; Ambrosino et al., 2014). Furthermore, the neuroprotective action of OEA was also reported in different models of neurodegenerative diseases (Gonzalez-Aparicio et al., 2014; Quentin et al., 2023) and ischemic brain injury (Sabahi et al., 2022).

e) Anti-tumoral effects of OEA and PEA

Thanks to their anti-inflammatory effects and the strong record supporting the anti-tumoral activity of cannabinoids and vanilloids (Hinz and Ramer, 2018; Kafali et al., 2024), an interest in the tumour-suppressing potential of eCB-like molecules, particularly OEA and PEA, has grown over the years. The nature of these bioactive lipids and their role as entourage compounds that enhance cannabinoids' action has led to their investigation first within this context. A study by Di Marzo and colleagues showed that PEA had no anti-tumoral effect on the human breast cancer MCF-7 cell line (Di Marzo et al., 2001). Nonetheless, when used concomitantly with AEA, PEA significantly enhanced the latter's anti-proliferative action (Di Marzo et al., 2001). This was shown both in basal conditions and in MCF-7 cells that were stimulated by the nerve growth factor (NGF). This effect was explained by a suppression of the expression of the hydrolysis enzyme FAAH, which consequently can lead to a decrease in AEA

degradation and guarantee a prolonged effect (Di Marzo et al., 2001). A similar finding regarding the entourage effect of PEA in enhancing anti-proliferative activity in human breast cancer cells was also reported, where PEA enhanced the effects of vanilloid receptors type 1 (VR1) agonists (De Petrocellis et al., 2002). Moreover, the inhibition of the enzyme NAAA in TNBC lead to an increase in endogenous PEA levels, which consequently inhibited TNBC cell migration in vitro, reduced inflammatory cytokines IL-6 and IL-8, decreased angiogenic factors including the vascular endothelial growth factor A (VEGF-A) and the placental growth factor (PLGF), lowered the volume and number of tumour masses in vivo, and eventually lead to an increased survival rate in a mouse model of TNBC (Benchama et al., 2022). Similarly, the use of PEA along with a FAAH inhibitor decreased B16 melanoma cell line viability in vitro and decreased tumour progression in a mouse model of melanoma (Hamtiaux et al., 2012). The effectiveness of PEA was also investigated in colon cancer where it suppressed tumour cell proliferation and cell migration triggered G2/M phase cell cycle arrest and DNA fragmentation all through PPARα and GPR55 receptors (Pagano et al., 2021). Additionally, it significantly reduced azoxymethane-induced preneoplastic lesions and total number of tumours in vivo (Pagano et al., 2021). Moreover, its positive effect on the survival rate of a mouse model of leukaemia that was undergoing chemotherapy highlighted its great potential as a molecule of choice for combination therapy in cancer (Keppel Hesselink, 2013).

The direct effect of OEA on cancer hasn't been extensively studied like PEA has. This is probably due to its strict characterization as a molecule involved in feeding behaviour and lipid metabolism. Nonetheless, oleic acid, the precursor of OEA, was investigated for its anti-tumoral effects in lung cancer, where it was shown to suppress tumoral growth and metastasis (Kimura, 2002; Piegari et al., 2017). It was also investigated in oesophageal cancer (Moon et al., 2014) as well as squamous cell carcinoma (Jiang et al., 2017). Whereas OEA itself was investigated in co-treatment with IFN-γ in human lung carcinoma cells and was shown to

induce its anti-cancer effect through enhancing STAT phosphorylation, suppressing ISGs expression such as PD-L1 and increasing the expression of pro-apoptotic markers such as caspase 3 and Bax (Yamagata et al., 2021).

The use of these two bioactive lipids alone as a therapeutic option specifically for cancer is probably avoided due to its poor pharmacokinetics (Benchama et al., 2022). It is then more convenient to use them as adjunct molecules to enhance the action of conventional therapies.

Part V: PPAR receptors

1- Overview

PPARs are ligand-activated transcription factors in the nuclear receptor superfamily that regulate genes involved in metabolism, differentiation, and inflammation (Chinetti and Staels 2000; Tyagi et al., 2011; Christofides et al., 2020).

In mammals, three PPAR isoforms exist; PPARα, PPARβ/δ, and PPARγ, which are classified in subfamily 1 of nuclear hormone receptors (NR1C1–C3) (Nuclear Receptors Nomenclature Committee, 1999). Each PPAR isoform has distinct tissue distribution, physiological roles, and ligand preferences (Grygiel-Górniak, 2014). In addition, they are encoded by a distinct gene that exists on human chromosomes 22, 6, and 3, respectively and they all share a conserved modular structure with four major domains: an N-terminal A/B domain containing a ligand-independent activation function (AF-1), a central DNA-binding domain (DBD) with two zinc-finger motifs, a hinge region (D domain) for cofactor docking, and a C-terminal ligand-binding domain (LBD) harbouring the ligand-dependent activation function AF-2 (Kota et al., 2005; Christofides et al., 2020).

Several endogenous and exogenous ligands can bind to the PPAR LBD domain, including FA (particularly acylethanolamides) and eicosanoids (Xu et al., 1999; Michalik et al., 2006). Upon binding, PPARs heterodimerize with the retinoid X receptor (RXR). This complex then binds to specific DNA sequences called PPAR response elements (PPREs) in target gene promoters, and recruit coactivator complexes to modulate transcription (Berger and Moller, 2002). When not activated by a ligand, corepressors can bind to PPAR/RXR. Nevertheless, in the presence of ligands, AF-2 helix is stabilized, and a conformational change takes place releasing the corepressors and facilitating coactivator binding (Decara et al., 2020; Lin et al., 2022).

In addition, PPARs can also exert trans-repression of other signalling pathways. For instance, PPAR activation can suppress NF-κB- or AP-1-mediated inflammatory gene expression without directly binding DNA (Delerive et al., 1999).

As mentioned before, both endogenous and synthetic ligands can activate PPARs. PPARα is activated by endogenous lipids, including FA such as OEA and PEA as well as certain eicosanoids like leukotriene B4 (Devechand et al., 1996; Grygiel-Górniak, 2014). Clinically, synthetic fibrate agonists of PPARα like fenofibrate and clofibrate are used as hypolipidemic agents to treat dyslipidemia (Forman et al., 1997; Qiu et al., 2023). PPARβ/δ also binds numerous endogenous ligands derived from FA, and these count metabolites of linoleic and arachidonic acid, including various eicosanoids (Grygiel-Górniak, 2014). Nevertheless, and although synthetic PPARβ/δ-specific agonists such as GW501516 and GW0742 have been successfully developed, none of them are yet approved for clinical use (Giordano Attianese and Desvergne, 2015). Lastly, PPARγ is targeted by endogenous oxidized fatty acid metabolites and cyclopentenone prostaglandins (Itoh et al., 2008). In contrast, synthetic thiazolidinedione drugs such as rosiglitazone and pioglitazone act as high-affinity PPARγ agonists and are widely used as insulin-sensitizing agents in type 2 diabetes (Grygiel-Górniak 2014; Kroker and Bruning 2015).

2- PPAR in cancer

With their ability as nuclear receptors to regulate genes governing lipid metabolism, cell proliferation and inflammation, PPAR can profoundly, and differentially, influence cancer cell proliferation, survival, apoptosis, and even angiogenesis (Sun et al., 2023; Psilopatis et al., 2023; Asgharzadeh et al., 2024; Wang et al., 2025).

PPARγ is regarded as a tumour-suppressive receptor since its activation leads to anti-proliferative and pro-apoptotic effects in several cancers. Both endogenous and synthetic PPARγ ligands were shown to trigger apoptosis in bladder cancer, gastric cancer, lung cancer

and multiple myeloma (Guan et al., 1999; Sato et al., 2000; Tsubouchi et al., 2000; Eucker et al., 2004). Other mechanisms that underlie the anti-tumoral effects of PPARγ include transcriptional modulation of cell-cycle regulators such as c-Myc, the downregulation of inflammatory mediators, and the inhibition of angiogenesis via the downregulation of VEGF and COX-2 (Akinyeke and Stewart, 2011; Scoditti et al., 2010).

In addition, PPARγ activation through its ligands was also studied in the context of combination therapy, most prominently, for its effect in increasing chemosensitivity (Girnun et al., 2008; Lian et al., 2020; Skelhorne-Gross et al., 2012). PPARγ ligands were also used in combination with tyrosine kinase inhibitors (TKIs) for the treatment of chronic myeloid leukemia (CML) (Glodkowska-Mrowka et al., 2016). In addition, it was used in combination with radiotherapy and other molecules such as retinoid X receptor agonists and leukotriene B4 (LTB4) receptor antagonist for the treatment of different cancers (Huang et al., 2018; Duvic et al., 2001; Baetz et al., 2006).

Conversely, studies investigating PPARβ/δ in cancer have reported different and controversial findings. PPARβ/δ activation can stimulate pro-angiogenic factors like IL-8 and VEGF (Du et al., 2020; Wagner and Wagner, 2020) and can upregulate pro-inflammatory eicosanoids such as COX-2 and PGE2, creating an environment that is in favour of tumoral growth and metastasis (Wang et al., 2014; Wang et al., 2025). In line with these findings, elevated PPARβ/δ levels were found to correlate negatively with breast cancer patients' survival (Kittler et al., 2013) and the overexpression of PPARβ/δ has been linked to colon cancer progression (Wang et al., 2014; Wang et al., 2025). On the other hand, a study has reported that the genetic knock out of PPARδ can accelerate colon cancer progression (Harmann et al., 2004). Due to these discrepancies in efficacy, no PPARβ/δ-specific drugs have been promoted to clinical use.

3- PPARα: A focus

PPARα plays a central role at the crossroads of cancer metabolism and inflammation. It predominantly governs fatty acid β-oxidation and lipid catabolism, and can regulate different inflammatory pathways (Pyper et al., 2010; Li et al., 2024).

PPARα expression is downregulated in hepatocellular carcinoma (HCC). Interestingly, HCC patients who showcased high tumoral PPARα levels have significantly less aggressive tumours and better survival outcomes (Xiao et al., 2018; Pan et al., 2024). A similar decrease was also found in prostate cancer tissue in comparison to benign prostatic hyperplasia tissues (Kim et al., 2006). Moreover, with PPARα representing an interconnection between metabolism and cancer, a study investigating HCC exacerbated by a high-fat diet found that PPARα activation suppressed HCC growth by attenuating inflammatory pathways that promote the release of neutrophil extracellular traps which prevents immune cell exclusion within the TME and leads to tumoral regression (Pan et al., 2025).

The use of PPARα agonists has been shown to modulate tumoral cells' metabolism by modulating fatty-acid oxidation and depleting cells from their lipidic reservoir needed for rapid growth, thereby inhibiting tumour cell proliferation in cancer (Chandran et al., 2015; Dutta and Sharma-Walia, 2019; Grabacka and Reiss, 2008). In addition to that, PPARα activation can lead to an antiproliferative effect through several mechanisms. It can lead to cell cycle arrest and a decrease in ERK signalling and NF-κB activity in breast cancer and glioma cells (Liang et al., 2014; Binello et al., 2014).

NF-κB is a family of transcription factors that regulate genes involved in inflammation, immune responses, and cell survival. During homeostasis, NF-κB dimers are held inactive in the cytoplasm by inhibitor of κB proteins (IκB) such as IκBα (Gosh et al., 1998). Upon stimulation by pro-inflammatory cytokines including IFNs, the IκB kinase complex (IKK) phosphorylates IκBα, marking it for ubiquitination and proteasomal degradation (Pfeffer, 2011;

Park and Hong, 2016). Freed from IκBα, NF-κB translocate to the nucleus and induces target genes that drive inflammatory responses. Activation of PPARα has been shown to exert anti-inflammatory effects through modulation of the NF-κB pathway. PEA binding to PPARα leads to the repression of NF-κB–driven genes and upregulation of IκBα, the natural NF-κB inhibitor (Wang et al., 2025). Moreover, in TNBC cells, exogenous PEA was reported to significantly reduce NF-κB pathway activity, further supporting its anti-inflammatory and anti-proliferative potential (Benchama et al., 2022).

Similarly, OEA has been shown to inhibit NF-κB activation and stabilize IκBα levels under inflammatory conditions. In human THP-1 macrophage-like cells, OEA treatment following LPS stimulation attenuated NF-κB activity and preserved IκBα expression. This effect was reversed by the blockade of PPARα (Yang et al., 2016).

Aberrant or constitutive activation of NF-κB is a hallmark of many cancers, where it drives tumorigenesis by promoting proliferation, inhibiting apoptosis, and fostering angiogenesis and metastasis (Ma et al., 2024). Conversely, PPARα activation has been associated with pro-apoptotic effects in certain cancer types, including breast cancer, through suppression of NF-κB signalling (Chandran et al., 2015).

Parallelly, PPARα can also decrease the proliferation of head and neck paragangliomas by inhibiting PI3K/GSK3β/β-catenin pathway (Florio et al., 2017). Its activation has also been proven to induce apoptosis and oxidative stress in hepatocarcinoma cells and colon cancer (Maggiora et al., 2010, Jiao et al., 2002; Gao et al., 2015). It was also proven that this receptor exerts its anti-cancer effects through epigenetic modulation, as seen in colon cancer, where DNA methyltransferase 1 (DNMT1) and protein arginine methyltransferase 6 (PRMT6) are upregulated after activation of PPARα (Luo et al., 2019). It can also affect angiogenesis by

inhibiting neovascularization needed for tumour growth via the reduction of pro-angiogenic COX-2 and VEGF (Grau et al., 2008).

Conversely, some studies have reported that the activation of PPARα can have pro-tumoral outcomes depending on the concentration and the duration of the ligand used. For instance, a low dose of fibrates leads to an increase in the proliferation of the MCF-7 cancer cells. However, the same study reported that high doses lead to suppressed proliferation (Tauber et al., 2020). Further to that, chronic activation of PPARα can induce hepatocellular carcinoma in mice (Tanaka et al., 2008). This highlights the dual role of PPARα in cancer, with both protective and potentially harmful effects depending on context and treatment parameters.

Study rationale and objectives

NB is the most common extracranial solid tumour in children and accounts for a disproportionate number of paediatric cancer-related deaths. Its inherent heterogeneity and resistance to therapy present significant clinical challenges, with long-term survival rates remaining poor despite the use of intensive, multimodal treatment regimens. Consequently, identifying novel therapeutic strategies that sensitize tumour cells to apoptosis is of critical importance.

Although classical therapies demonstrate initial efficacy, their prolonged use is often limited by the emergence of resistance and cumulative toxicity. Type I IFNs are pleiotropic cytokines with established roles in antiviral defence, immunomodulation, and anti-proliferative activity. IFNβ has demonstrated anti-tumoral effects in several malignancies, including NB, primarily via the activation of the JAK/STAT pathway and the subsequent induction of ISGs, notably PKR, leading to apoptosis in SH-SY5Y cells, a NB cell line that recapitulates an intermediate-risk phenotype of NB. However, these effects have been predominantly investigated at a high dose (30 ng/mL for 24 h), knowing that the JAK-STAT pathway and apoptosis within SH-SY5Y cells are effectively activated in vitro by doses as low as 5ng/mL. This highlights the need to explore adjuvant agents capable of enhancing IFNβ efficacy against NB at lower concentrations to overcome cellular resistance mechanisms and potential adverse effects.

OEA and PEA are endogenous bioactive lipids that act as ligands of PPARα, a nuclear transcription factor implicated in lipid metabolism, inflammation, and cell survival. While they are well known for their anti-inflammatory and neuroprotective properties, emerging research suggests their potential to modulate tumour cell behaviour. Specifically, PEA has been shown to reduce proliferation and migration in various cancer cell lines through PPARα-dependent pathways, particularly when combined with other therapeutic agents. However, the capacity of OEA and PEA to influence IFNβ-induced apoptosis in NB cells remains largely unexplored. In

addition, IFNs have been reported to upregulate the immune checkpoint molecule PD-L1, which can mediate immune evasion in tumour cells and on the other hand, represent a potential therapeutic target. Whether OEA and PEA can influence PD-L1 expression or signalling in the context of IFNβ treatment is similarly not yet elucidated.

Given the increasing interest in combination therapy involving endogenous lipid mediators and cytokines to enhance anti-tumour efficacy, this study aims to investigate the role of OEA and PEA in modulating IFNβ-mediated apoptosis and PD-L1 induction in SH-SY5Y NB cells. More specifically, the use of a lower dose of IFNβ without losing its apoptotic and immunomodulatory prowess is the main goal of this study. The SH-SY5Y cell line provides a well-established in vitro model for delineating relevant signalling cascades including the JAK/STAT pathway and p38 MAPK, as well as the involvement of PPARα and other downstream effectors like caspase 3, PARP, survivin, Mcl-1, and IκBα, which together orchestrate the balance between cell survival and apoptosis. This model therefore provides an ideal platform to investigate, on a molecular level, how this co-treatment may act synergistically to influence both apoptotic and immunomodulatory responses in SH-SY5Y cells.

Material and methods

1. Cell culture and treatment

The human NB cell line SH-SY5Y was purchased from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK), with vendor-provided authentication. Cells were cultured in a 1:1 mixture of Ham's F12 and Minimum Essential Medium (MEM) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 2 mM L-glutamine, 1% non-essential amino acids (NEAA), and maintained at 37 °C in a humidified 5% CO_2 incubator. Cells were passaged every 72 hours using 0.25% trypsin-EDTA and were not used beyond 15 passages. Mycoplasma contamination was routinely assessed using the MycoFluor™ Mycoplasma Detection Kit (Invitrogen-Life Technologies, Monza, Italy).

2. Treatment conditions and cell lysis

SH-SY5Y cells were serum-starved before treatment and were exposed to each treatment agent under standard incubation conditions. Each treatment is described in detail for each assay. For protein extraction, cells were washed with PBS and lysed in ice-cold RIPA buffer containing 1 mM PMSF, 0.5% phosphatase inhibitor cocktail 3, and 1% protease inhibitor cocktail (Sigma-Aldrich). Lysates were sonicated (5 s), and protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

3. MTT viability assay

The MTT assay was conducted in 96-well plates following 24 h treatment of SH-SY5Y cells with vehicle, OEA, or PEA at various concentrations (0.3, 1, 3, 10 and 30 uM). MTT reagent was added, and, after incubation, the resulting formazan was solubilized using 10% SDS in 10 mM HCl. Absorbance was measured with a Wallac Victor microplate reader (PerkinElmer).

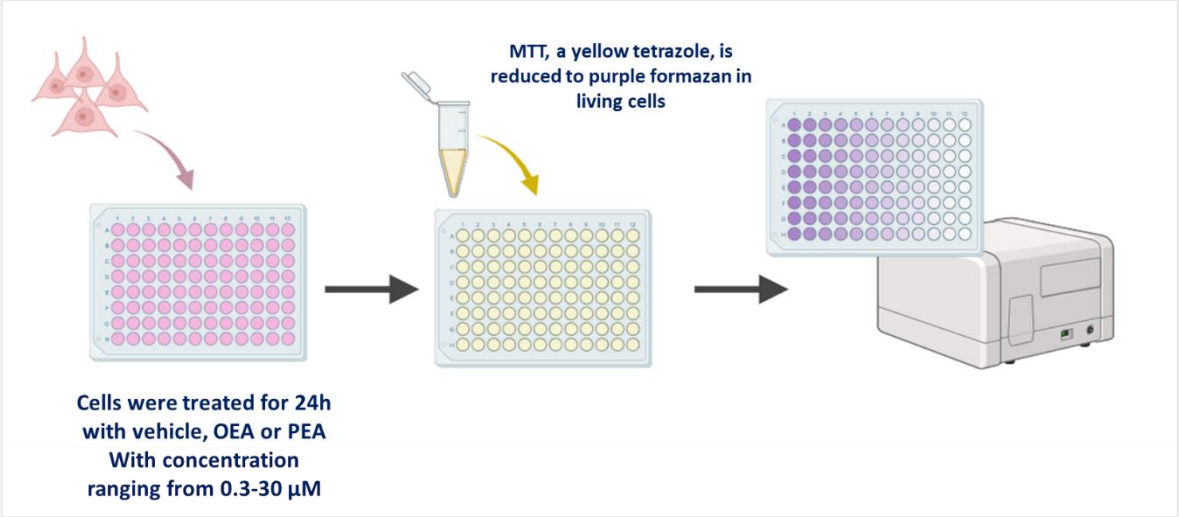

Figure 7: Illustration of the cell treatment and principle of the MTT assay

4. Cell count and viability

Cell count and viability were assessed using the Muse™ Cell Analyzer and Muse™ Viability assay kit (Millipore, Merck Life Sciences). Cells were pre-treated with OEA or PEA at 3 µM for 6 hours, followed by stimulation with IFNβ at 5 ng/mL for 24 hours. After treatment, cells were detached, centrifuged (300×g, 5 min), and resuspended in complete medium. A 20 µL aliquot was mixed with 380 µL of the viability reagent and incubated at room temperature for 5 min before analysis with the Muse™ Cell Analyzer.

5. Scratch wound healing assay

Confluent SH-SY5Y monolayers in 24-well plates were scratched with a sterile pipette tip. After washing with PBS, cells were incubated with serum-free medium containing the designated compounds; Cells were treated with 3 µM OEA or PEA in combination with 5 ng/mL IFNβ. Images were acquired from the same field at 0 and 48 h post-scratch using phase-contrast microscopy (Olympus IX51), and wound closure was quantified using ImageJ.

6. Clonogenic survival assay

Single-cell suspensions (200 cells/well) were plated in 6-well plates and cultured for 10 days with medium changes every 2 days. cells were treated as before; 3 µM OEA or PEA, with or

without 5 ng/mL IFNβ, for a total of 10 days. Colonies were fixed in ethanol and stained with 0.5% crystal violet. Colonies containing ≥50 cells were counted using ImageJ.

7. Western blot analysis

Cells were pre-treated with OEA or PEA at 3 μM for 6 hours, then stimulated with IFNβ at 5 ng/mL for 24 hours. After protein extraction and quantification, these were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked and probed overnight at 4 °C with primary antibodies against PD-L1 (cat. no. 13684, Cell Signaling Technology, Danvers, MA, USA) (1:1000); IKBα (cat. no. 4814, Cell Signaling Technology) (1:1000); (Asp214) (cat. no. 5625, Cell Signaling Technology) (1:1000); PARP (cat. no. 9542, Cell Signaling Technology) (1:1000); cleaved caspase-3 (Asp175) (cat. no. 9664, Cell Signaling Technology) (1:1000); total caspase 3 (cat no. 9665, Cell Signaling Technology) (1:1000); phospho-Tyr701-STAT1 (1:1000) (cat no. ST1P-11A5, Thermo Fisher Scientific, Rockford, IL, USA); anti-STAT1 (1:500) (cat no. sc-592, Santa Cruz Biotechnology, Paso Robles, CA, USA); survivin (cat. no. 2808, Cell Signaling Technology); Mcl-1 (1:1000) (sc-819, Santa Cruz Biotechnology); PKR (1:1000) (cat no. 3072, Cell Signaling Technology); PPARα (1:500) (cat no. sc-592, Santa Cruz Biotechnology, Paso Robles, CA, USA), HDAC1 (1:1000) (cat. no. 2062, Cell Signaling Technology); pan cadherin (1:2000) (cat. no. 4073, Cell Signaling Technology); GAPDH (1:5000) (cat no. 247-002, Synaptic Systems, Gottingen, Germany); actin (1:3000) (cat no. A2066, Sigma-Aldrich). Following incubation with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), detection was performed using Clarity™ Western ECL substrate (Bio-Rad) and visualized on an ImageQuant LAS-4000 system (GE Healthcare). Band intensities were quantified using NIH ImageJ software. Signal normalization was performed using either a corresponding total non-cleaved protein or actin.

8. Surface protein biotinylation

Surface proteins were biotinylated using sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) as described in **Figure 8**. Following 24h treatment with OEA/PEA and IFNβ (as described above for the western blot analysis), cells were incubated with the biotinylation reagent for 1 h at 4 °C. Excess reagent was quenched with 20 mM glycine in PBS. Cells were lysed in RIPA buffer with 1% Triton X-100, and supernatants were incubated overnight at 4 °C with streptavidin-conjugated agarose beads. Proteins were eluted by boiling in sample buffer and analysed via western blotting.

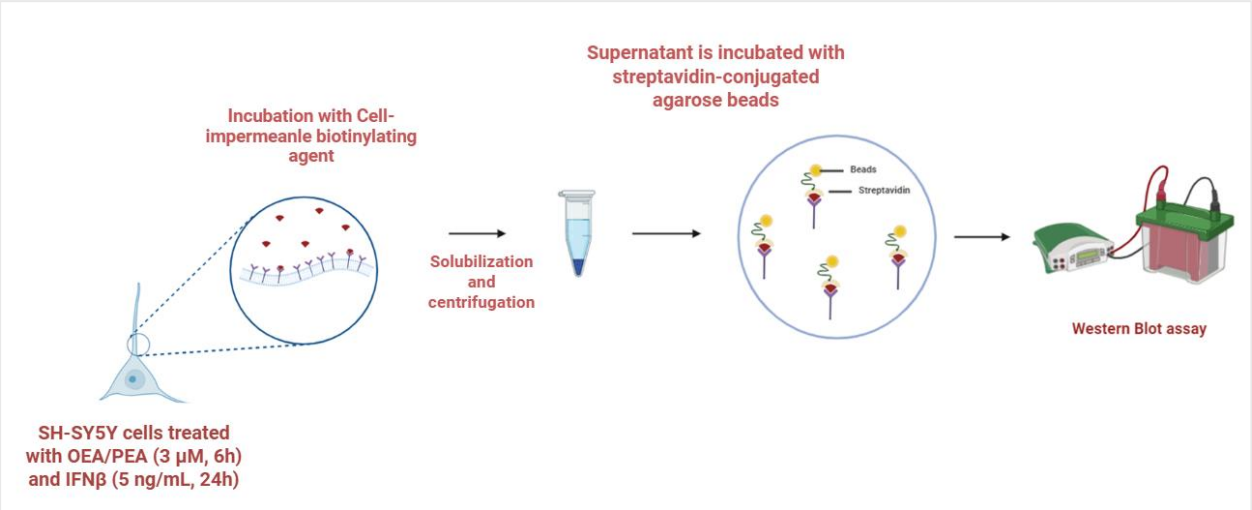

Figure 8: Illustration of the cell treatment and principle of cell surface biotinylation

9. Nuclear protein extraction

Post-treatment, cells were washed with PBS and lysed on ice. Lysates were centrifuged at 3000×g for 10 min at 4 °C. Supernatants were further centrifuged at 24,000×g for 20 min to yield cytosolic fractions. Pelleted nuclei were washed and purified by centrifugation over a 1M sucrose cushion. Nuclear proteins were extracted by incubation in nuclear extraction buffer for 30 min and centrifuged at 24,000×g. Extracts were denatured in sample buffer at 100 °C.

10. siRNA-mediated gene silencing

SH-SY5Y cells were transfected with either control siRNA (SR30004) or PPARA-specific siRNA (SR303653) using Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific). Cells

were seeded in 6-well plates and incubated for 24h in antibiotic-free medium. Transfection was carried out for 4–5 h, followed by replacement with complete medium. Analyses were performed 48 h post-transfection.

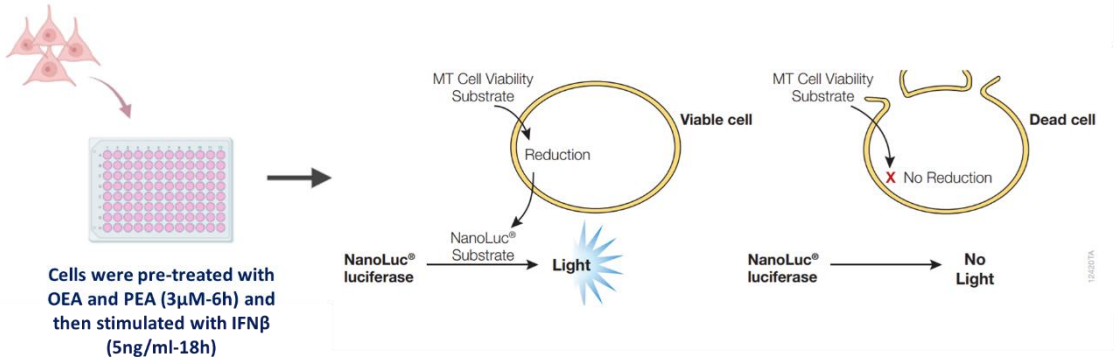

Figure 9: Illustration of the cell treatment and principle of RealTime-Glo™ Cell viability assay

11. RealTime-Glo™ cell viability assay

SH-SY5Y cells plated in 96-well ViewPlates (PerkinElmer) were pre-treated with OEA or PEA at 3 µM for 6 hours, followed by stimulation with IFNβ at 5 ng/mL for 24 hours and then incubated with the RealTime-Glo™ MT assay reagents (Promega) following the manufacturer's instructions. Luminescence was measured using a Wallac Victor III reader. All experiments were performed in triplicate.

12. Caspase 3/7 activity assay

Caspase activity was quantified using the Caspase-Glo® 3/7 assay kit (Promega). Following treatment, SH-SY5Y cells in 96-well plates were incubated with the kit reagents, and luminescence was recorded using a Wallac Victor III plate reader. Assays were run in triplicate.

13. Statistical Analysis

All results are expressed as mean ± SEM. Data were analysed using GraphPad Prism (San Diego, CA, USA). For comparative analysis, unpaired Student’s t-tests or one-way ANOVA followed by Tukey’s post hoc test were employed, as appropriate. Statistical significance was

defined as $p < 0.05$. Control or IFNβ-treated groups were used as reference (set to 100% or 1), and variance was calculated relative to the mean of each experimental set.

Results

To investigate the potential of OEA and PEA in modulating the effects of IFNβ on SH-SY5Y NB cells, we first identified their effect when present alone across a range of concentrations for better insight. Next, we assessed how these lipids influence IFNβ-induced responses, be it proliferative, apoptotic, and immune-related, while exploring some of the underlying signalling pathways mediating these effects.

1. OEA and PEA at different concentrations do not affect SH-SY5Y cell viability

SH-SY5Y cells were exposed to OEA and PEA at concentrations ranging from 0.3 to 30 µM for 24 h. This concentration range has been previously documented to be efficacious and to have anti-cancer effects in in vitro studies (Hamtiaux et al., 2011; Pagano et al., 2021). As shown in **Figure 10**, neither compound reduced cell viability at any of the tested doses, confirming that they do not affect SH-SY5Y cells regardless of the designated concentration and temporal frame. For the follow-up combinatory treatment with IFNβ, we went with the working dose of 3 µM as it was previously used in the treatment of NB murine cells (Hamtiaux et al., 2011).

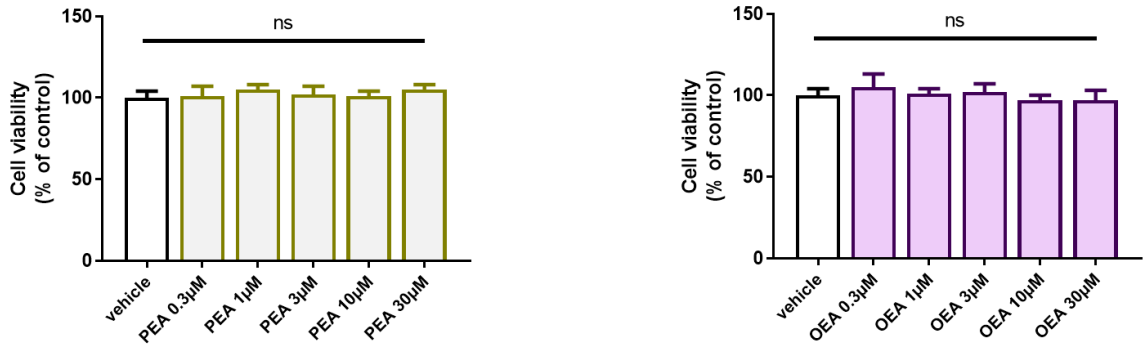

Figure10: Effects of PEA and OEA at different concentrations on SH-SY5Y cell viability

Cells were exposed for 24 hours to vehicle, OEA, or PEA at the indicated concentrations after which cell viability was evaluated using the MTT assay. The values are expressed as percentage of control (referred to here as the vehicle). Data are presented as mean ± SEM from four independent experiments. (ns= non-significant)

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2. OEA or PEA in combination with IFNβ potentiate cell death in SH-SY5Y cells

To examine the combined effects of OEA and PEA with IFNβ, and considering the nuclear localization of PPARα, cells were pre-exposed to OEA or PEA (3 µM) for 6 h to facilitate receptor engagement before stimulation with IFNβ (5 ng/mL, 24 h). The first parameter to be assessed was cell viability, and it was quantified using the Muse™ Cell Analyzer (Millipore, Merck Life Sciences, Darmstadt, Germany). Just as seen in the MTT assay, neither OEA nor PEA alone affected viability in comparison to vehicle-treated controls (**Figure 11**).

However, co-treatment with OEA + IFNβ or PEA + IFNβ significantly enhanced cell death compared to IFNβ alone ($p < 0.05$).

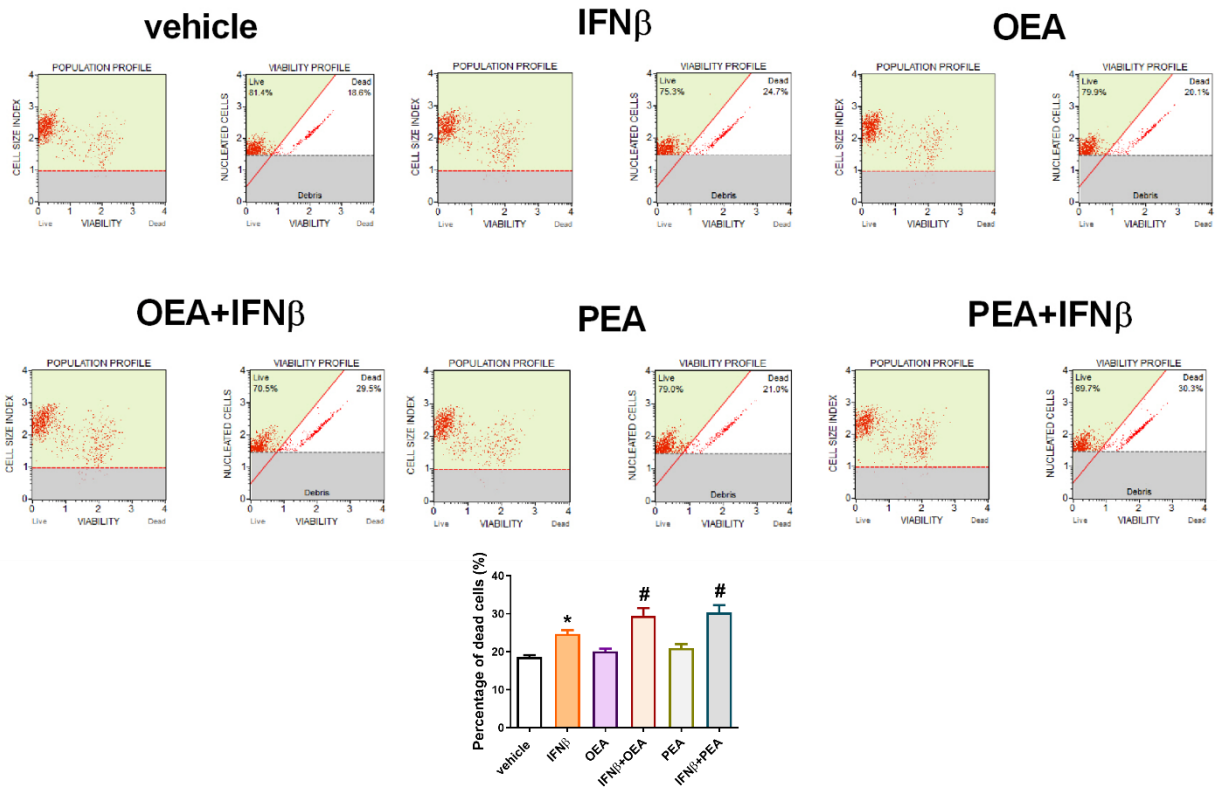

Figure 11: OEA and PEA potentiate IFNβ-induced reduction in SH-SY5Y cell viability

Cells were pretreated with OEA and PEA at a concentration of 3 µM 6 hours prior to stimulation with IFNβ (5 ng/mL, 24 h). Cell viability was assessed using the Muse® Viability Kit. The values are expressed as mean ± SEM from four independent experiments *(*p < 0.05 versus vehicle; # p < 0.05 versus IFNβ)*

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To corroborate these findings, viability was further assessed using the RealTime-Glo™ MT assay based on engineered luciferase. Consistent with the Muse™ data, both OEA + IFNβ and PEA + IFNβ reduced cell viability compared to IFNβ treatment alone ($p < 0.05$; **Figure 12**).

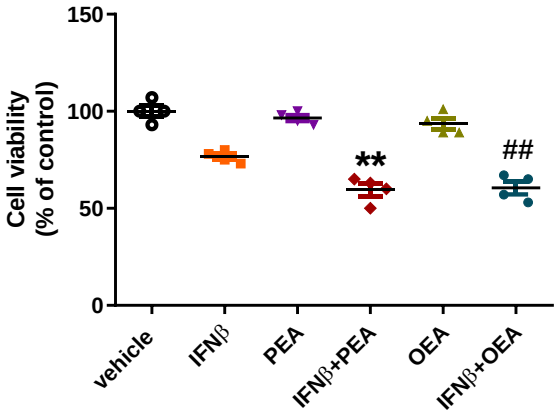

Figure 12: OEA and PEA potentiate IFNβ-induced reduction in SH-SY5Y cell viability

Cells were pretreated with OEA or PEA (3 μM for 6h) and IFNβ (5 ng/mL, 24 h). A luminescence-based assay was used to further assess cell viability. Data are expressed as the percentage relative to vehicle-treated controls and presented as mean ± SEM from four independent experiments. (*$p < 0.05$ versus vehicle; ## $p < 0.01$ versus IFNβ).

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3. Combined treatment with OEA or PEA and IFNβ reduces SH-SY5Y proliferative capacity

Based on the observed reduction in cell viability with combined treatment, we next investigated the impact on cell proliferation using the scratch wound healing assay. Wound closure analysis revealed that OEA or PEA alone did not alter the proliferative capacity of SH-SY5Y cells compared to the controls (**Figure 13**). In contrast, co-treatment with IFNβ and either OEA or PEA significantly impaired wound closure, indicating a further reduction in proliferative potential relative to what is seen in IFNβ alone ($p < 0.05$).

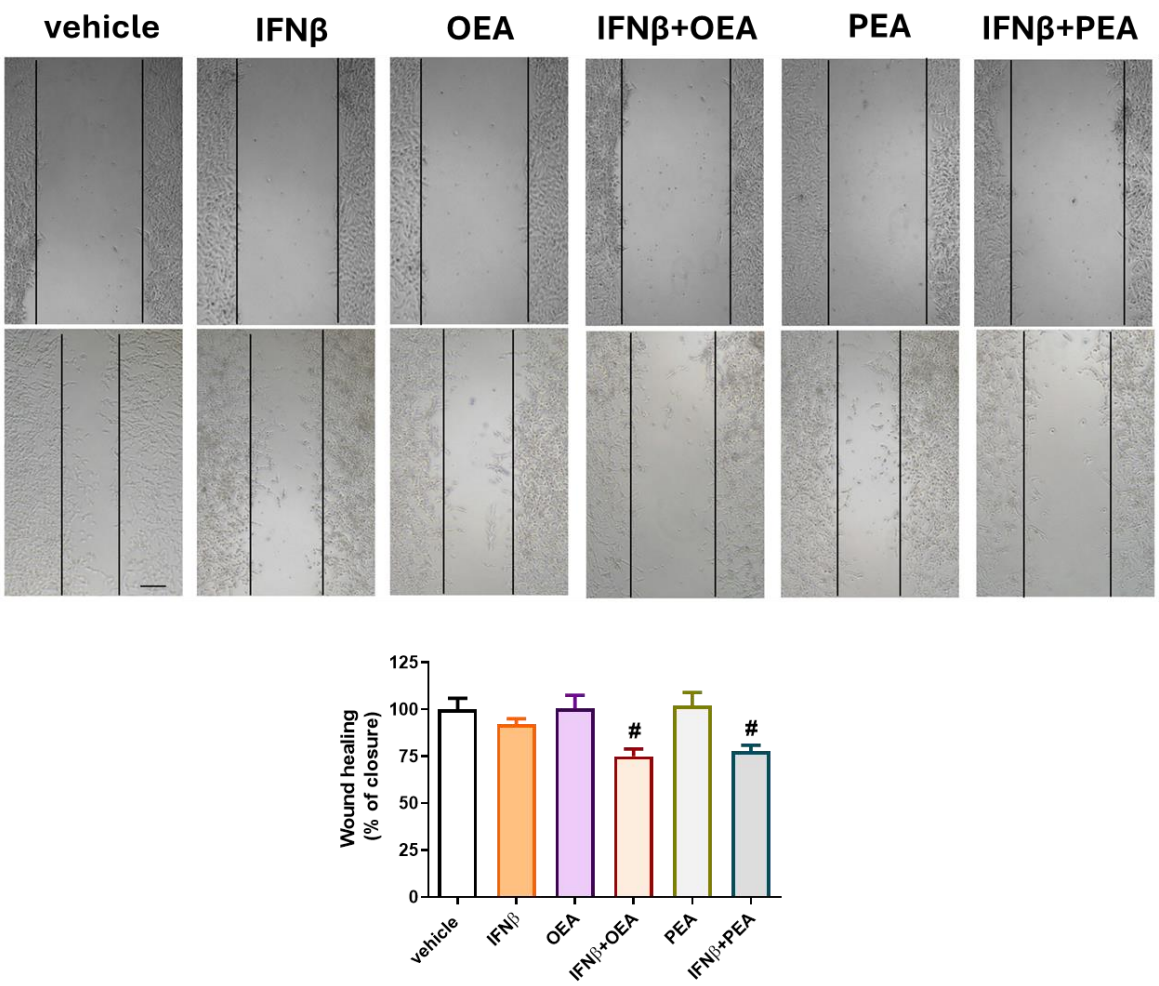

Figure 13: Combined treatment with OEA or PEA and IFNβ reduces SH-SY5Y cell proliferation in scratch wound healing assay

SH-SY5Y cells were treated for 48 h with vehicle, OEA or PEA (3 μM), and IFNβ (5 ng/mL), alone or in combination. Scratch wound healing assay was performed, and representative images of wound closure were acquired at 10x magnification. The percentage of wound closure is expressed as mean ± SEM from four independent experiments. #p < 0.05 versus IFNβ.

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4. OEA and PEA alone and in combination with IFNβ impair SH-SY5Y colony formation

To complement the wound healing findings, we further assessed the clonogenicity of these cells after treatment. Cells were seeded at low density in six-well plates and cultured for 10

days. As illustrated in **Figure 14A**, treatment with OEA or PEA alone significantly reduced colony formation efficiency by 28% ($p < 0.01$) and 53% ($p < 0.001$), respectively, compared to the control. When combined with IFNβ, both lipids exerted an additional inhibitory effect, decreasing colony numbers by 20% ($p < 0.05$, OEA + IFNβ) and 24% ($p < 0.01$, PEA + IFNβ) in respect to IFNβ alone. These results were corroborated by cell quantification (**Fig. 14B**), which confirmed a significant reduction in proliferative potential upon co-treatment with OEA + IFNβ and PEA + IFNβ ($p < 0.01$).

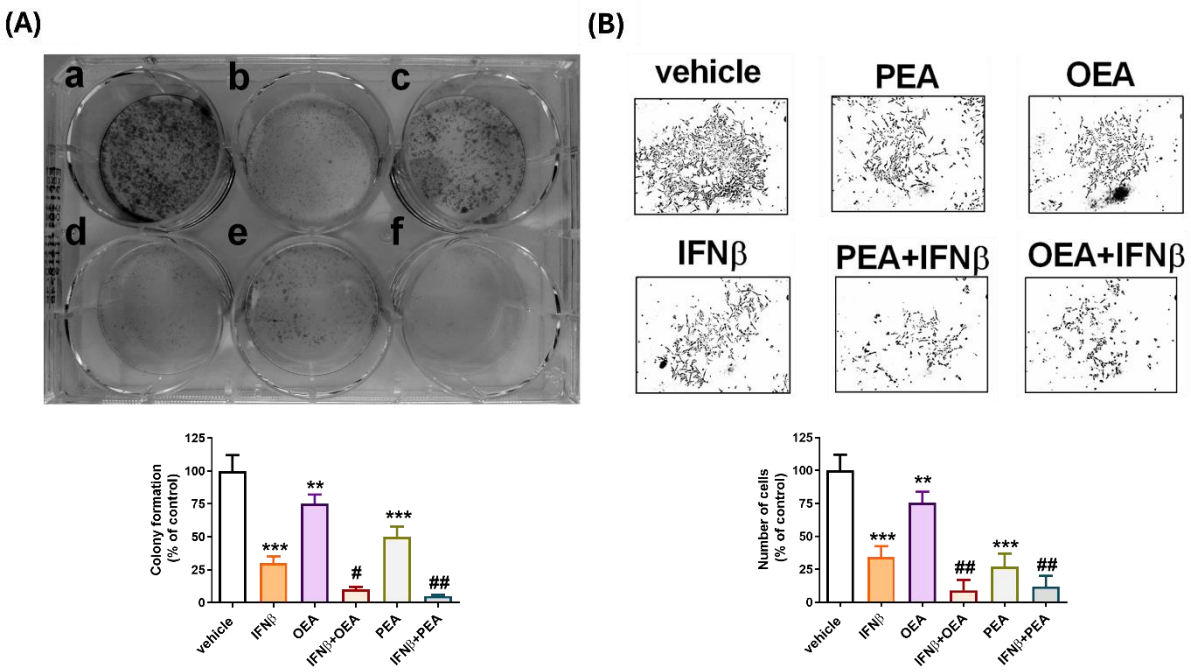

Figure 14: OEA and PEA co-treatment with IFNβ Inhibit SH-SY5Y cell proliferation and clonogenicity

Cells were seeded at a density of 200 cells/well in six-well plates and treated with (a) vehicle, (b) IFNβ (5 ng/mL), (c) OEA (3 μM), (d) OEA + IFNβ, (e) PEA (3 μM), or (f) PEA + IFNβ. After treatment, colonies consisting of ≥50 cells were fixed and stained with crystal violet. Representative images of colony formation are shown (A). Additionally, total cell counts per well within each well was quantified (B). Results are expressed as a percentage relative to vehicle-treated controls and data is represented as the mean ± SEM of four independent experiments. **p < 0.01 and ***p < 0.001 versus vehicle; #p < 0.05 and ##p < 0.01 versus IFNβ.

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5. OEA and PEA amplify IFNβ-mediated apoptosis

As we mentioned previously, IFNβ decreases SH-SY5Y cells viability by activating the intrinsic apoptotic pathway (Dedoni et al., 2010). Since this study used a high dose of IFNβ at 30 ng/mL for 24 h, and after confirming the efficacy of IFNβ at 5ng/mL for 24 h in decreasing cell viability and proliferation, we were intrigued in testing if the induction of the intrinsic apoptotic pathway can be triggered after a shorter exposure.

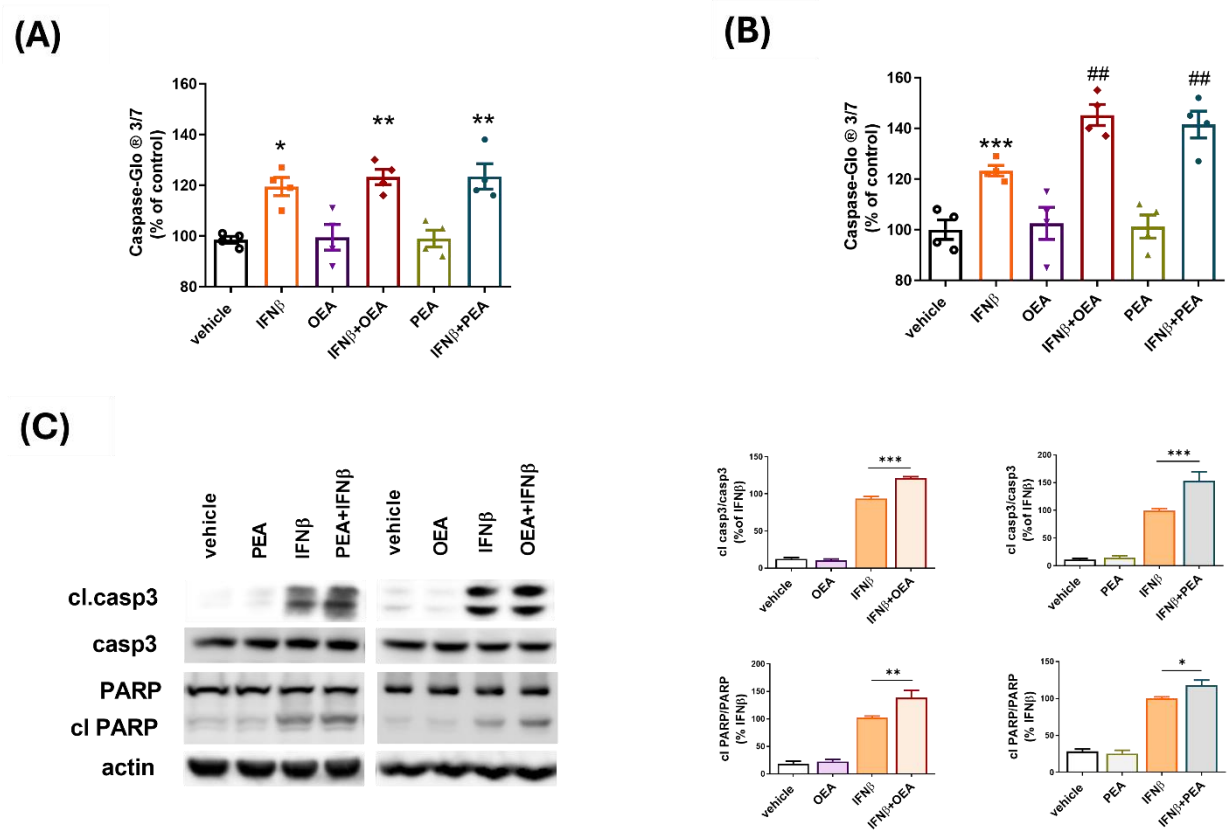

Figure 15: combined treatment with OEA, PEA increases caspase activation and PARP cleavage in SH-SY5Y cells only after a 24-hour stimulation with IFNβ

SH-SY5Y cells were pretreated with OEA (3 Mm, 6h) or PEA (3 Mm, 6h), followed by IFNβ (5 ng/ml) for a period of 12 h. A luminescent assay was used to measure caspase 3/7 activity. Values are expressed as percentage of vehicle-treated control. *p < 0.05, **p < 0.01 versus vehicle (A). Cells were pretreated with OEA or PEA (3 μM, 6 h) and subsequently stimulated with IFNβ (5 ng/mL, 24 h) and caspase-3/7 activity was determined using a luminescence assay. Results are expressed as percentage of vehicle control. ***p < 0.001 versus vehicle; ##p < 0.01 versus IFNβ (B). Cells are treated as described in (B) and cleaved caspase-3 and cleaved PARP were assessed in cell lysates. The ratio of cleaved to total protein was quantified. Data are expressed as percentage of IFNβ. *p < 0.05, **p < 0.01, ***p < 0.001 versus IFNβ (C). All results are presented as mean ± SEM of four independent experiments.

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More importantly, we were curious to investigate if the co-treatment with OEA or PEA can still be as efficacious in enhancing IFNβ's effect in a shorter time frame. Our results report that a 12 h exposure to IFNβ at a concentration of 5ng/mL was sufficient to activate caspase 3/7, however, the potentiation elicited by co-treatment with OEA or PEA was not observed in this time frame (**Fig.15A**). It was only at 24 hours of exposure that the potentiation of IFNβ by the two lipids became significantly evident (**Fig.15B**).

Next, and based on the findings of Figure 15B, we proceeded to investigate caspase 3 and PARP, whose cleavage is a hallmark of apoptosis. Exposure to OEA or PEA alone does not promote any increase in the cleavage of these two proteins, nevertheless, cells co-treated with either of these lipids and IFNβ showed an increase in the cleaved form of caspase 3 ($p < 0.001$, OEA + IFNβ and $p < 0.01$, PEA + IFNβ versus IFNβ) as well as an increase in cleaved PARP ($p < 0.01$, OEA + IFNβ and $p < 0.05$, PEA + IFNβ versus IFNβ) (**Fig. 15C**), indicating an enhancement in the activation of the apoptotic signalling pathway.

6. OEA and PEA modulate IFNβ-regulated pro-survival and adaptive signalling pathways in SH-SY5Y cells

To further delineate the mechanisms underlying the apoptotic response, we investigated pro-survival and adaptive signalling proteins and pathways. Mcl-1, a pro-survival member of the Bcl-2 family that prevents apoptosis initiation, was significantly downregulated in SH-SY5Y cells treated with IFNβ ($p < 0.05$), while co-treatment with OEA or PEA did not produce additional changes (**Figure 16A**). We next examined survivin, a member of the IAP family known to be modulated by IFNs. IFNβ exposure increased survivin expression ($p < 0.05$; **Figure 16B**). Nonetheless, the concomitant exposure to OEA or PEA significantly attenuated this induction ($p < 0.05$, OEA + IFNβ or PEA + IFNβ versus IFNβ).

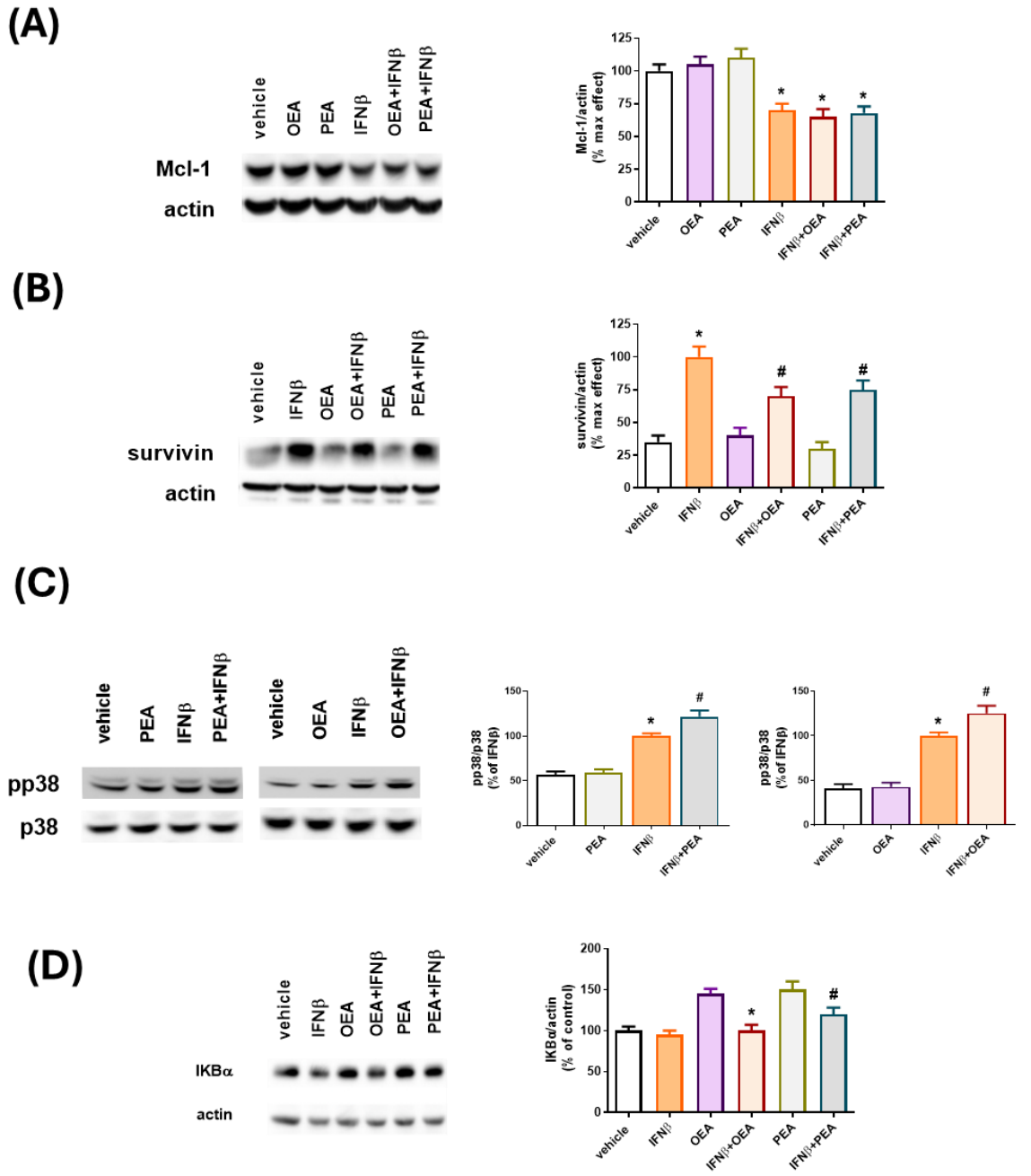

Figure 16: Modulation of pro-survival and adaptive signalling pathways elicited by treatment with IFNβ, OEA and PEA

Cells were pre-treated with OEA and PEA at 3 μM for 6 h then stimulated with IFNβ (5 ng/mL) for 24 h. Analysis of Mcl-1 levels in cell lysates, expressed relative to vehicle-treated control. *p < 0.05 versus vehicle (A). Survivin expression was quantified from cell lysates and normalized to maximal control effect. *p < 0.05 versus vehicle; #p < 0.05 versus IFNβ (B). Cell lysates were examined for phosphorylated and total p38 protein levels. The ratio between the phosphorylated and the total form of p38 protein was measured and represented in the graphs. *p < 0.05 versus vehicle; #p < 0.05 versus IFNβ (C). Analysis of IκBα levels in cell lysates, expressed relative to vehicle-treated control. *p < 0.05 compared with OEA and #p < 0.05 compared with PEA (D). All values above are represented as mean ± SEM from four independent experiments.

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Given that IFNβ has also been reported to promote counterregulatory anti-apoptotic signalling through the activation of the p38 MAPK pathway, we assessed p38 phosphorylation after treatment. As shown in **Figure 16C**, co-treatment with OEA or PEA further enhanced IFNβ-induced phosphorylation of p38 ($p < 0.05$ versus IFNβ).

Finally, we evaluated the effect of the combined treatment on IκBα, a key regulator of NF-κB–mediated inflammation, since OEA and PEA treatment has been reported to increase its levels, while PEA can also prevent its degradation (D'Agostino et al., 2009; Yang et al., 2016). Although OEA and PEA both increased IκBα, this elicited effect was blunted in the presence of IFNβ, resulting in basal IκBα levels in comparison to IFNβ-treated cells ($p < 0.05$ versus OEA or PEA; **Figure 16D**).

7. IFNβ induces PD-L1 expression in SH-SY5Y cells in a dose and time-dependent manner

We next explored other IFNβ-regulated pathways relevant to tumour immune escape. One well-characterized mechanism involves the upregulation of PD-L1, a type I transmembrane glycoprotein that binds the PD-1 receptor, thereby promoting immune evasion and enhancing cancer cell survival. Our results show that IFNβ increased PD-L1 expression in a time- and concentration-dependent manner (**Fig. 17A, B**).

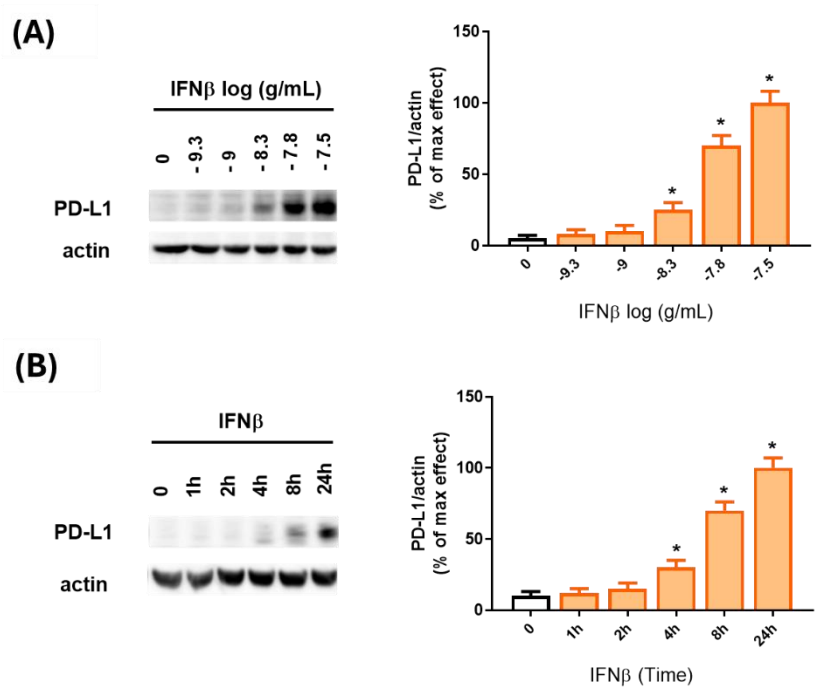

Figure 17: Time and dose-dependent upregulation of PD-L1 levels by IFNβ in SH-SY5Y cells

Cells were treated with IFNβ at different concentrations (A) or for varying time intervals (B) to assess its effect on programmed death-ligand 1 (PD-L1) expression. PD-L1 levels were quantified by Western blot and normalized to actin. Data are expressed relative to vehicle-treated control, set as 100%. Results are presented as mean ± SEM of four independent experiments (A, B). *p < 0.05 versus vehicle.

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8. OEA and PEA enhance IFNβ-induced PD-L1 levels in SH-SY5Y cells

Building on this observation, we next evaluated whether OEA or PEA could further modulate IFNβ-induced PD-L1 levels. Western blot analysis of whole-cell lysates revealed that co-treatment with IFNβ and either lipids significantly enhanced PD-L1 levels compared to what was observed with IFNβ ($p < 0.001$ for OEA + IFNβ; $p < 0.05$ for PEA + IFNβ), whereas OEA or PEA alone had no detectable effect (**Fig. 18A**).

To further characterize this response, PD-L1 surface expression was examined. Consistent with the lysate data, IFNβ alone increased PD-L1 levels at the plasma membrane, and this effect was amplified by co-treatment with OEA or PEA, resulting in 1.8- and 1.6-fold increases,

respectively, compared to IFNβ treatment ($p < 0.01$ for OEA + IFNβ; $p < 0.05$ for PEA + IFNβ; **Figure 18B**).

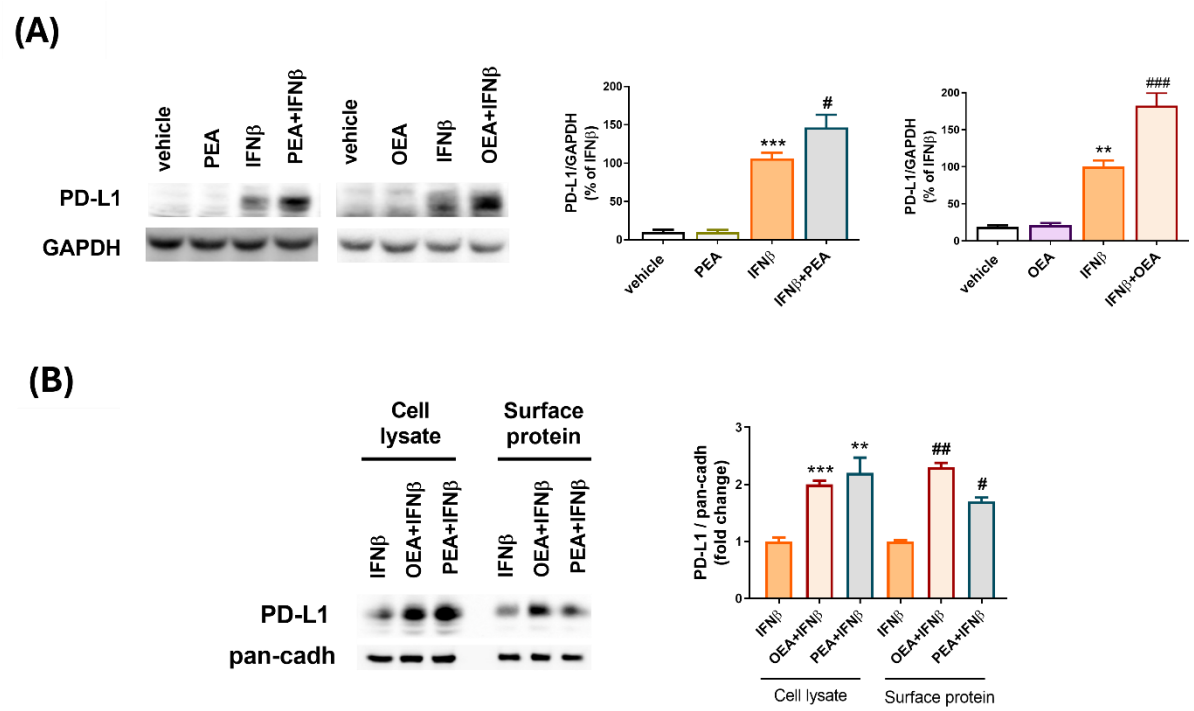

Figure 18: co-treatment with OEA, PEA and IFNβ upregulates PD-L1 levels in SH-SY5Y cells

Cells were pretreated with OEA or PEA (3 μM, 6 h) followed by stimulation with IFNβ (5 ng/mL, 24 h). Cell lysates were analysed by western blot for PD-L1. Values are expressed as percentage of IFNβ. **p < 0.01 and ***p < 0.001 versus vehicle; #p < 0.05 and ###p < 0.001 versus IFNβ. Data are presented as mean ± SEM of four independent experiments (A). Total cell extracts and biotinylated surface proteins were analysed for PD-L1. PD-L1 levels in whole cell lysates and at the plasma membrane were normalized to pan-cadherin (pan-cadh). **p < 0.01 and ***p < 0.001 in cell lysate versus corresponding IFNβ; #p < 0.05 and ##p < 0.01 in surface protein versus corresponding IFNβ. Values are shown as mean ± SEM of three independent experiments (B).

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9. OEA and PEA do not interact with the JAK-STAT signalling pathway in SH-SY5Y cells

Given the modulatory effects of OEA and PEA on apoptotic and survival-related proteins as well as their potentiation of the p38 MAPK pathway and the increase of PD-L1 levels, which are all mechanisms induced by IFNβ via the JAK-STAT pathway, we investigated whether this ability of OEA and PEA to enhance IFNβ-induced proteins and pathways could involve potentiation of the JAK-STAT cascade. To this end, SH-SY5Y cells were treated with IFNβ in

combination with either OEA or PEA, and STAT1 phosphorylation, total STAT1, as well as PKR induction were all analysed.

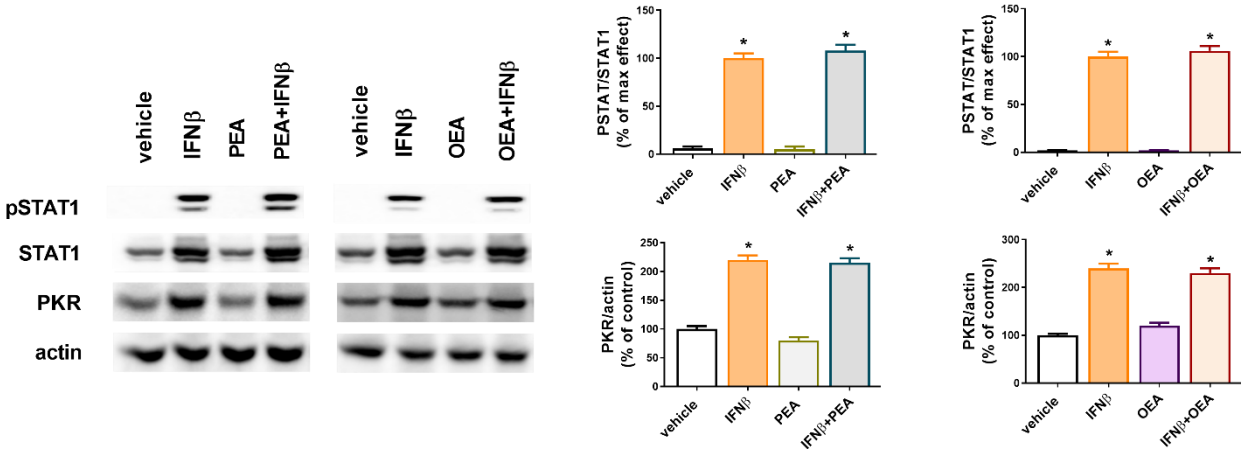

Figure 19: Co-treatment with OEA and PEA does not interfere with IFNβ-induced STAT1 phosphorylation and PKR expression in SH-SY5Y cells

Cells were pretreated with OEA or PEA (3 μM, 6 h) followed by stimulation with IFNβ (5 ng/mL, 24 h). Data are plotted as percentage of maximal effect and are represented as the mean ± SEM of four independent experiments. *p < 0.05 versus vehicle

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As shown in **Figure 19**, co-exposure with OEA or PEA did not alter the levels of STAT1 phosphorylation, total STAT1, or PKR compared with cells treated with IFNβ alone, indicating that the enhancing effects of these lipids are not mediated through amplification of the canonical JAK-STAT pathway.

10. IFNβ and PPARα do not directly interact in SH-SY5Y cells

Since IFNβ modulates a wide range of ISGs with diverse biological and therapeutic implications, we next examined whether prolonged exposure to IFNβ could influence PPARα expression, given the involvement of this receptor in mediating OEA and PEA activity. Nuclear extracts from SH-SY5Y cells treated with IFNβ for 24 h were analysed, but no changes in PPARα protein levels were detected (**Fig. 20A**).

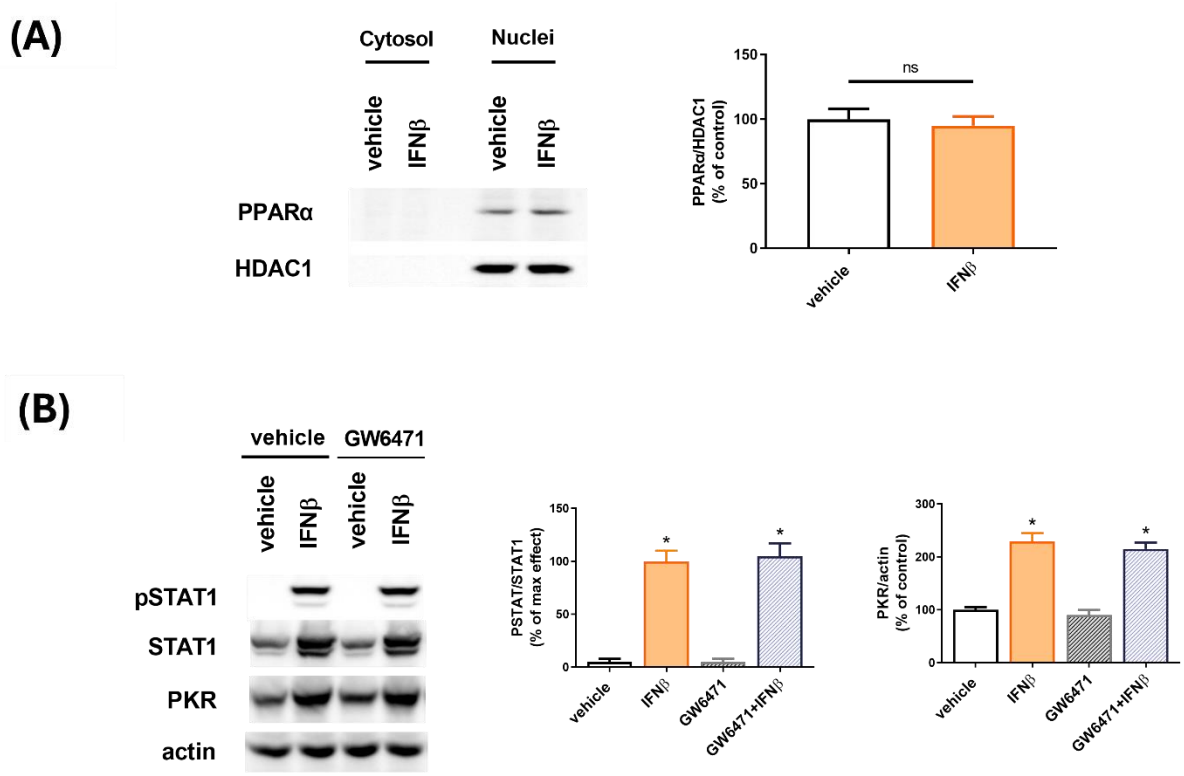

Figure 20: Lack of direct interaction between IFNβ and PPARα in SH-SY5Y cells

Nuclear extracts were analysed for PPARα protein levels following IFNβ treatment. Data are presented as the mean ± SEM of four independent experiments (ns=not significant) (A). STAT1 phosphorylation, total STAT1, and PKR were assessed following treatment with IFNβ at 5 ng/mL for 24 h, in the presence or absence of the PPARα antagonist GW6471 (6 μM); * $p < 0.05$ versus vehicle (B)

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To further explore the role of PPARα in IFNβ signalling, we employed GW6471, a selective PPARα antagonist. STAT1 phosphorylation, total STAT1, and PKR induction were assessed, yet no differences were observed in the presence of GW6471, indicating that PPARα does not interfere with IFNβ signalling in this context (**Fig. 20B**).

11. Inhibition of PPARα attenuates OEA/PEA synergism with IFN-β in mediating apoptosis and PD-L1 upregulation in SH-SY5Y Cells

Having established that PPARα does not directly interfere with IFNβ signalling, we next assessed whether its inhibition could alter the combined effects of OEA, PEA, and IFNβ on apoptotic and immune-related markers.

Treatment with the PPARα antagonist GW6471 markedly reduced PARP cleavage in cells co-exposed to OEA or PEA with IFNβ ($p < 0.001$, GW6471 + OEA + IFNβ vs. OEA + IFNβ; GW6471 + PEA + IFNβ vs. PEA + IFNβ; **Figure 21A**). In parallel, GW6471 also suppressed PD-L1 induction under the same conditions ($p < 0.01$ for GW6471 + OEA + IFNβ; $p < 0.001$ for GW6471 + PEA + IFNβ versus respective controls; **Figure 21B**).

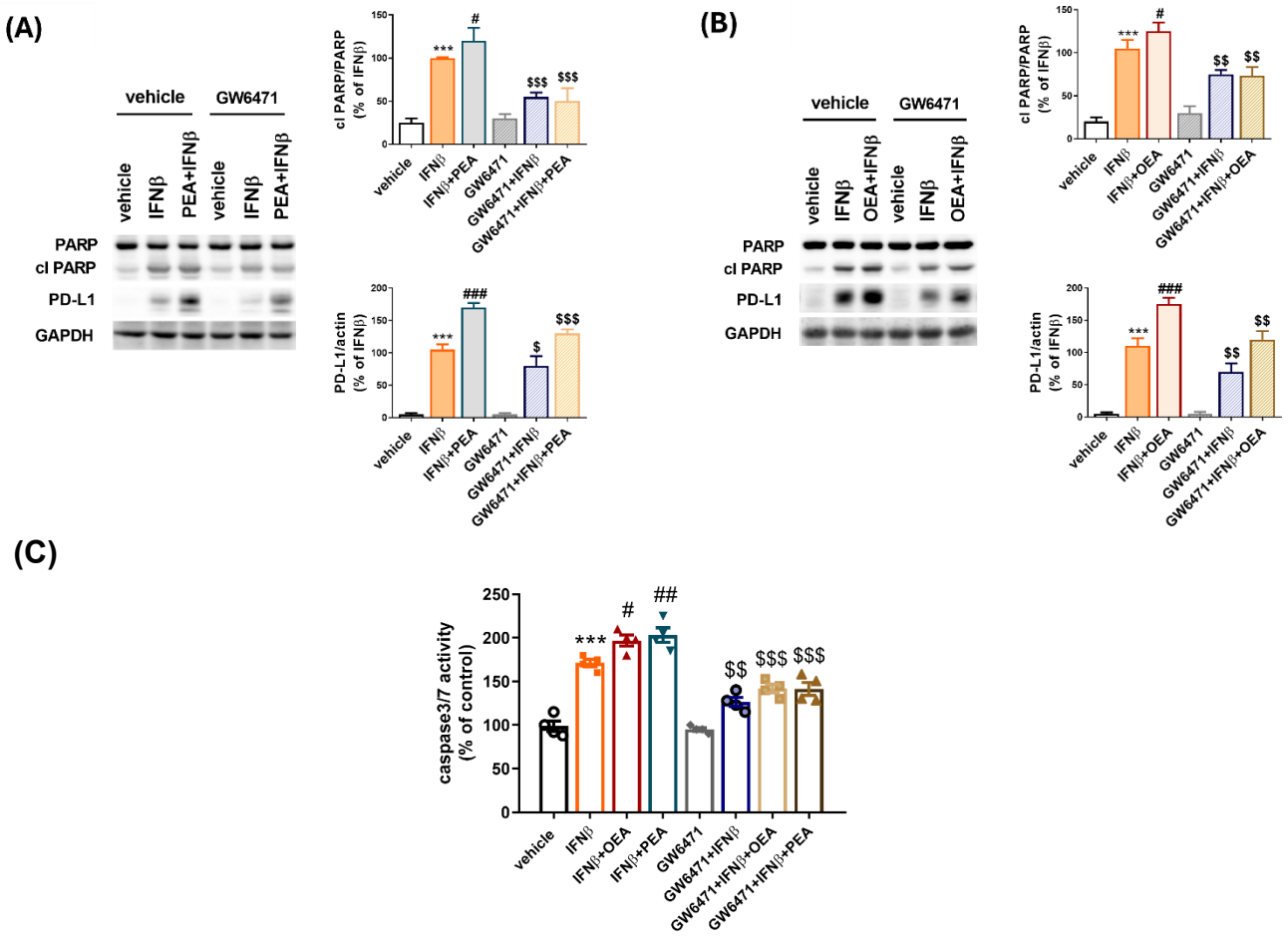

Figure 21: PPARα inhibition attenuates OEA/PEA+IFNβ-induced PARP cleavage, caspase-3/7 activation and PD-L1 expression

Cells were pre-treated with GW6471 (6 μM, 1 h) and subsequently exposed to OEA or PEA for 6 h, followed by IFNβ stimulation for 24 h. Whole-cell lysates were analysed for PD-L1 and cleaved PARP expression. ***$p < 0.001$ versus vehicle; #$p < 0.05$ and ###$p < 0.001$ versus IFNβ; $ $p < 0.05$, $$ $p < 0.01$, and $$$ $p < 0.001$ versus the corresponding control-treated group (A, B). Under the same treatment conditions, caspase-3/7 activity was assessed by luminescence assay. ***$p < 0.001$ versus vehicle; #$p < 0.05$ and ##$p < 0.01$ versus IFNβ; $$ $p < 0.01$ and $$$ $p < 0.001$ versus the corresponding control-treated group. Values are represented in this figure as the mean ± SEM of four independent experiments

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Consistent with these findings, caspase-3/7 activity measurements further confirmed that PPARα inhibition blunted the enhanced pro-apoptotic effects observed with OEA + IFNβ and PEA + IFNβ (**Fig. 21c**).

12. PPARα knockdown attenuates OEA and PEA enhancement of IFNβ-driven apoptotic and immune responses in SH-SY5Y cells

To further substantiate the involvement of PPARα in regulating PD-L1 expression and apoptotic responses, we employed a genetic silencing approach. SH-SY5Y cells were transfected with a specific siRNA targeting PPARα (**Figure 22A**). Knockdown of PPARα markedly attenuated the increase in PD-L1 levels observed with OEA + IFNβ and PEA + IFNβ, compared with respective control siRNA-transfected cells ($p < 0.01$ and $p < 0.05$, respectively). Similarly, PARP cleavage was significantly reduced in siRNA + OEA + IFNβ and siRNA + PEA + IFNβ groups relative to the corresponding controls ($p < 0.01$ and $p < 0.05$, respectively; **Figure 22B**). These findings confirm that OEA and PEA enhance IFNβ-mediated apoptosis and PD-L1 induction by involving PPARα signalling.

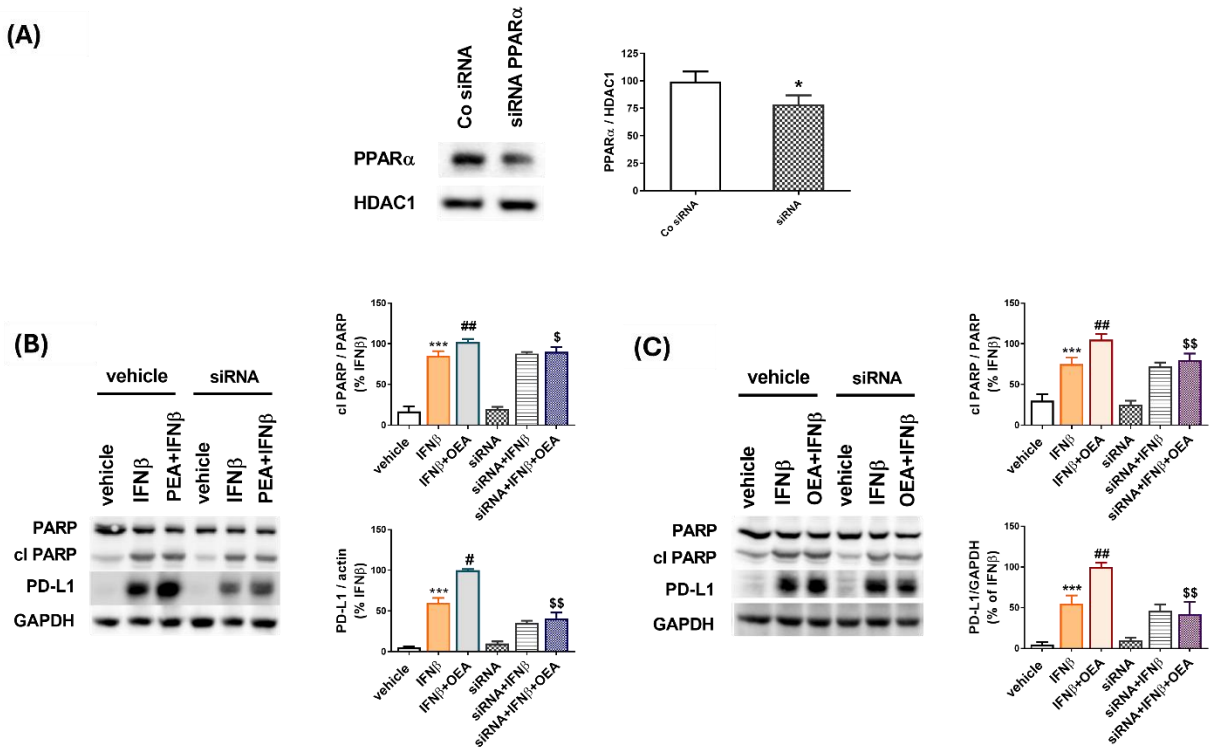

Figure 22: PPARα knockdown suppresses OEA and PEA potentiation of IFNβ-mediated PARP cleavage and PD-L1 level increase

SH-SY5Y cells were transfected with either control siRNA or PPARα siRNA. Transfection efficiency was confirmed by reduced PPARα protein levels. *p < 0.05 versus vehicle-treated cells (A). After transfection, cells were incubated for 24 h with vehicle, IFNβ, or co-treated with IFNβ and OEA or PEA as described previously. Cell lysates were analysed for cleaved PARP and PD-L1 expression (B, C). ***p < 0.001 versus vehicle; #p < 0.05 and ##p < 0.01 versus IFNβ; $p < 0.05, $$p < 0.01 versus the corresponding control siRNA condition. All data from this figure are presented as mean ± SEM of four independent experiments.

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Discussion

The anti-tumoral effects of IFNβ in NB have been well established by Dedoni and colleagues, who elucidated the pro-apoptotic potential of this cytokine in SH-SY5Y cells (Dedoni et al., 2010). The activation of apoptosis in this cell line was prompted by the JAK/STAT pathway and the induction of PKR that ultimately led to the initiation of this process (Dedoni et al., 2010). Although type I IFNs are well known for their potent antiviral and immunomodulatory effects, their therapeutic use in oncology has been limited by side effects such as flu-like symptoms, haematological toxicity, fatigue, and mood alterations, which arise from the use of high concentrations of these cytokines (Walther and Hohlfeld 1999; Sleijfer et al., 2005).

For IFNβ to activate the JAK-STAT pathway in SH-SY5Y cells, concentrations in the picomolar range were sufficient to induce STAT1 phosphorylation, while concentrations as low as 3 ng/mL were able to induce prominent PARP cleavage (Dedoni et al., 2010). Despite this, subsequent studies investigating the efficacy of IFNβ have often employed substantially higher concentrations, therefore leading to the interest in dose optimization while maintaining therapeutic feasibility, specifically in NB. The use of combination therapy to ensure efficacy at lower doses has therefore become our strategy of interest. In this regard, the bioactive lipids OEA and PEA have stood out as promising candidates thanks to their anti-tumoral properties in a variety of cancer models as proven by several in vitro cell models, including MCF-7 breast cancer cells, HCT116 colon cells and B16 melanoma cells (Di Marzo et al., 2001; Hamtiaux et al., 2012; Pagano et al., 2021). Furthermore, their favourable safety profile makes them an optimal option for combined therapy (Clayton et al., 2021; Deshmukh et al., 2024).

When present alone, PEA and OEA have been reported to significantly reduce cell viability in the N1E-115 murine NB cell line in a dose-dependent manner (Hamtiaux et al., 2011). More specifically, concentrations as low as 1 μM for OEA and 5 μM for PEA led to significant cell death. In colon cancer cells, exposure to PEA at a range of 3 μM to 30 μM was able to decrease

cell proliferation (Pagano et al., 2021). In contrast to the aforementioned cancer cell lines, our results show that SH-SY5Y cells did not exhibit any reduction in viability after treatment with either OEA or PEA, suggesting a relative resistance to these compounds. This observation highlights that the cellular response to these bioactive lipids is not solely governed by their concentration and duration of exposure but is also influenced by the intrinsic characteristics of the cancer type and cell line. These differences in sensitivity may stem from variations in receptor expression profiles, the metabolic processing of lipid amides, or the activation of distinct intracellular signalling cascades, all of which could ultimately modulate susceptibility to these bioactive lipids.

Based on their lack of cytotoxicity in SH-SY5Y cells and due to their recorded poor pharmacokinetics (Benchama et al., 2022), as well as their recorded efficacy in combination therapy in different cancers (Di Marzo et al., 2001; Keppel Hesselink 2013; Yamagata et al., 2021), the use of these compounds as adjunct molecules to an established therapeutic option such as IFNβ stands out as a promising strategy for NB treatment.

The concomitant use of OEA or PEA and IFNβ efficiently decreased cell viability as reported by our results. In addition, cell proliferation was also decreased with the co-treatment in comparison to IFNβ. Keeping in mind that these two lipids have no effect when present alone, these findings point toward a synergistic interaction between these compounds. A similar observation was made in the clonogenicity assay. Unlike proliferation assays, clonogenic assays assess the long-term ability of cells to survive, divide, and form colonies (Franken et al., 2006). Furthermore, high clonogenicity has been closely associated with tumour recurrence, underscoring its value as a marker of long-term tumorigenic potential (Noronha et al., 1985). Interestingly, in our study, the prolonged exposure to OEA and PEA alone significantly reduced colony formation in SH-SY5Y cells, despite the absence of short-term cytotoxicity. This suggests that these lipids might exert delayed effects that impair long-term

survival and proliferation, therefore affecting tumorigenicity. The underlying mechanisms behind these results remain unknown and warrant further investigations.

At the molecular level, co-treatment with OEA or PEA potentiated IFNβ-induced activation of the intrinsic apoptotic pathway, as evidenced by enhanced cleavage of caspase-3 and PARP following 24-hour exposure. These effects appear to be time-dependent, suggesting a cumulative impact resulting from the concurrent engagement of IFNβ-triggered and lipid-mediated signalling. Importantly, the co-treatment also counteracted the IFNβ-induced upregulation of survivin, a member of the IAP family known to promote cell survival and therapy resistance (Garg et al., 2016; Chen et al., 2016). Previous studies have shown that IFNs, including IFNβ, regulate survivin expression through the activation of the JAK/STAT pathway (Sharief et al., 2002; Zimmerman et al., 2010; Mormile et al., 2016). Moreover, the observed suppression of survivin in the presence of OEA or PEA aligns with findings in colorectal cancer cells, where PPARγ activation promoted apoptosis by downregulating survivin and enhancing caspase-3 activity (Wang et al., 2012). This reduction of survivin expression in our model likely contributes to the amplification of IFNβ-induced apoptosis, as the loss of survivin removes an essential block to caspase activation, thereby increasing the susceptibility of SH-SY5Y cells to the cytotoxic effects of IFNβ.

On the other hand, in our NB cell model, IFNβ alone downregulates the anti-apoptotic Bcl-2 family member Mcl-1. Reports regarding the regulation of Mcl-1 by different types of IFNs remain inconsistent in literature (Clemens et al., 2003; Allagnat et al., 2011; Bauvois et al., 2021), suggesting that this effect is highly dependent on the cellular context and experimental conditions. Notably, co-treatment with OEA or PEA did not further alter Mcl-1 expression levels. These findings support the notion that while the pro-apoptotic activity of IFNβ in SH-SY5Y cells may, at least in part, result from its capacity to suppress Mcl-1, the enhancement

of IFNβ-induced apoptosis by OEA and PEA appears to occur through Mcl-1-independent mechanisms.

We next examined IκBα levels and the p38 MAPK pathway to investigate if these pro-survival and adaptive pathways normally elicited respectively by OEA/PEA and IFNβ, are modulated by our co-treatment. IκBα is the cytoplasmic inhibitor of NF-κB, a master transcription factor that regulates genes involved in inflammation, survival, proliferation, and resistance to apoptosis (Solt and May 2008; Guo et al., 2024). Consistent with their reported anti-inflammatory properties through NF-κB inhibition (D'Agostino et al., 2009; Yang et al., 2016), exposure to OEA or PEA alone increased IκBα in SH-SY5Y cells, suggesting inhibition of NF-κB signalling. However, this effect was prevented in the presence of IFNβ, likely due to its strong pro-inflammatory activity that outweighed the anti-inflammatory action of these lipids.

On the other hand, it was previously reported that IFNβ activates the p38 MAPK-mediated stress response in SH-SY5Y cells as a feedback system to counteract its own pro-apoptotic action (Dedoni et al., 2014). In our current work, we show that the co-treatment with OEA or PEA further prompts the p38 MAPK pathway activation, which points towards the promotion of cell survival. Nevertheless, this compensatory stress response was insufficient to counteract the pro-apoptotic effects of the combined therapy.

We report for the first time that IFNβ induces a time- and concentration-dependent increase in PD-L1 levels in SH-SY5Y cells. This is consistent with the well-established role of IFNs in modulating immune checkpoint molecules. Indeed, IFNs are known to upregulate PD-L1 across various cell types through the activation of the JAK/STAT signalling pathway, contributing to adaptive immune resistance mechanisms (Shreiner et al., 2004; Wang et al., 2014). IFN-γ has been described as a potent inducer of PD-L1 in several cancer models, including NB cell lines (Srinivasan et al., 2018).

Interestingly, concomitant treatment with either OEA or PEA markedly enhanced the IFNβ-induced upregulation of PD-L1, both at the cytoplasmic and plasma membrane levels. This suggests a potential interplay between IFN signalling and lipid-mediated pathways in the regulation of immune checkpoint expression. In line with our findings, Gutting and colleagues demonstrated that PPARγ agonists can promote PD-L1 expression in gastrointestinal tumours, further supporting a possible role for PPAR-dependent signalling in PD-L1 modulation (Gutting et al., 2021). Conversely, another study reported that OEA and oleic acid reduced IFN-γ-induced PD-L1 expression in lung carcinoma cells, highlighting that the regulatory outcome may vary depending on the tumour type and the class of IFN involved (Yamagata et al., 2021).

While the classical concept of immune evasion cannot be properly studied in the current in vitro system and may hold a negative implication in in vivo translational contexts, as it is typically viewed as a mechanism facilitating tumour escape from immune surveillance, this observed upregulation of PD-L1 can be exploited differently. Therapeutic protocols for NB typically comprise a multimodal approach, integrating diverse treatment strategies to maximize therapeutic efficacy and improve patient outcomes. Thereby, the use of PD-1/PD-L1 checkpoint inhibitors as an additional therapeutic option to further enhance the therapeutic potency of OEA/PEA and IFNβ treatment could be beneficial. In addition, antibody-drug conjugates targeting PD-L1 have also been successfully used in treatment of different cancers including laryngeal squamous cell carcinoma, lung adenocarcinoma and ovarian cancer (Gandini et al., 2016; Dong et al., 2024; Xiao et al., 2021; Jin et al., 2022), and could also be beneficial as an additional treatment option to secure faster and more efficient outcomes in NB.

The activation of the main IFNβ signalling cascade, the JAK/STAT1 pathway, is responsible for mediating its effects of cell survival, proliferation, intrinsic apoptosis activation and PD-L1 induction (Dedoni et al., 2010; Morimoto et al., 2018). In our study, concomitant treatment with OEA or PEA did not appear to involve the JAK/STAT pathway in mediating the observed

responses, suggesting that these bioactive lipids may act through alternative signalling mechanisms to potentiate the anti-tumour activity of IFNβ. Given that OEA and PEA interact with multiple molecular targets, including PPARα, PPARγ, TRPV1, and GPR55, with PPARα representing their primary high-affinity receptor (Fu et al., 2005; O'Sullivan, 2007), we next sought to determine whether PPARα mediates these effects. To this end, we first evaluated whether IFNβ alters PPARα expression levels, thereby ruling out the possibility that the observed synergistic response results from cytokine-induced modulation of receptor abundance.

Pharmacological inhibition of PPARα using GW6471 partially reduced both PD-L1 induction and PARP cleavage during co-treatment, indicating its contribution to the observed effects. Consistently, caspase-3/7 activity assays further supported the involvement of PPARα in enhancing apoptotic signalling. Complementary siRNA-mediated knockdown of PPARα yielded comparable results, attenuating PD-L1 expression and diminishing apoptotic potentiation. Collectively, these findings identify PPARα as a key mediator in the combined response; however, the persistence of PD-L1 upregulation following both genetic and pharmacological silencing of PPARα suggests the involvement of additional signalling pathways, warranting further investigation into alternative receptor targets.

Our findings highlight the potential of OEA and PEA to enhance IFNβ-induced apoptosis and PD-L1 protein expression in SH-SY5Y NB cells and have elucidated the intracellular mechanisms that are involved in this synergism; however, several considerations must be addressed. Given that SH-SY5Y cells represent an intermediate-risk, a relatively resistant NB model, it is uncertain whether similar effects would be observed in other risk groups. NB is a highly heterogeneous cancer, and reliance on a single cell line limits the generalizability of these observations. The exclusive use of the SH-SY5Y cell line is based on its validation as a NB model that previously demonstrated consistent IFNβ responsiveness, intact JAK/STAT

signalling, and functional activation of apoptotic pathways (Dedoni et al., 2010; Dedoni et al., 2014). In addition, and as a model for intermediate-risk NB (Lodrini et al., 2017; Upton et al., 2020), it is characterized by greater immune modulatory responsiveness compared with high-risk NB (Dondero et al., 2015; Wienke et al., 2021). Consequently, its use enables robust investigation of downstream signalling events underlying IFNβ-driven apoptosis and PD-L1 regulation, as well as the detection of synergistic effects with OEA and PEA.

While reliance on a single in vitro model necessarily defines the scope of the present work and may limit direct extrapolation across the full biological heterogeneity of NB, this focused approach provides meaningful insights relevant to intermediate-risk NB only. Nevertheless, it is safe to say that these findings establish a strong framework for future validation in high-risk NB, in which apoptosis resistance and immune evasion are more prominent (Nicolai et al., 2015). Future studies should incorporate additional models with distinct genetic backgrounds, such as MYCN-amplified or ALK-mutant NB cell lines, to broaden the understanding of the interaction between IFNβ and OEA and PEA.

Most importantly, translation of these findings into in vivo settings is critical. Testing the therapeutic efficacy in NB xenograft models or patient-derived xenografts (PDX) would allow evaluation of not only tumour growth inhibition and survival benefit, but also the pharmacokinetics, bioavailability, and tolerability of OEA/PEA in combination with IFNβ. Moreover, given the modulation of PD-L1 observed in our system, in vivo investigations will be essential to establish the immunological significance of these effects. Such studies may clarify whether combining IFNβ with OEA/PEA and another additional therapeutic intervention, such as immune checkpoint blockade, could further enhance awaited outcomes. Such models could provide valuable insight into TME interactions as well, including potential modulation of immune responses, angiogenesis, and stromal signalling.

Collectively, these complementary approaches will be essential to delineate the underlying mechanisms and assess the translational potential of combining bioactive lipids with IFNβ in NB treatment.

Conclusion

The current work demonstrates that the endogenous lipids OEA and PEA potentiate the anti-tumoral effects of IFNβ in human SH-SY5Y NB cells mainly through PPARα-dependent mechanisms. While OEA and PEA alone did not affect cell viability, their co-administration with IFNβ significantly reduced SH-SY5Y survival, proliferation, and clonogenic capacity. This enhanced cytotoxicity was accompanied by increased cleavage of caspase-3 and PARP, together with reduced survivin levels, confirming a synergism between the lipids and the pro-inflammatory cytokine to activate the intrinsic apoptotic pathway.

The observed effects occurred independently of further JAK/STAT or PKR prompting, indicating that OEA and PEA modulate alternative signalling routes. Both lipids amplified IFNβ-induced PD-L1 expression. These findings reveal a dual role for OEA and PEA in promoting apoptosis while influencing immune checkpoint regulation. While we uncover a novel mechanism by which OEA and PEA enhance IFNβ's antiproliferative action, linking lipid metabolism, apoptosis, and immune modulation in NB through targeting PPARα, future in vivo and translational studies are warranted to validate these results and explore their therapeutic implications in NB and other neural-derived tumours. Nonetheless, these lipids have shown effectiveness as adjuvants to cytokine-based therapies, offering a promising strategy to improve treatment efficacy while potentially avoiding IFN-related toxicity.

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