



**Università
degli Studi
di Palermo**

AREA RICERCA E TRASFERIMENTO TECNOLOGICO
SETTORE DOTTORATI E CONTRATTI PER LA RICERCA
U. O. DOTTORATI DI RICERCA

Dottorato di Ricerca in Scienze Molecolari e Biomolecolari

Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF)

SSD CHEM-05/A

Next-Generation TRIDs journey: From Rational Drug Design to *in vivo* Validation

IL DOTTORE
DAVIDE RICCI

COORDINATORE
Prof. GIOVANNA PITARRESI

TUTOR
Prof. IVANA PIBIRI

CO-TUTOR
Prof. LAURA LENTINI

CICLO XXXVIII
ANNO CONSEGUIMENTO TITOLO 2025



Finanziato
dall'Unione europea
NextGenerationEU



Ministero
dell'Università
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*“The world breaks everyone, and afterward, some are strong at the broken places.”
(Hemingway, *A Farewell to Arms*, 1929)*

*Dedicated to V.M.R.,
whose resilience and fortitude have been a constant source of inspiration throughout the development
of this thesis.*

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GLOSSARY

A

- ACE-tRNA (engineered-tRNA)
- ADME (pharmacokinetic properties)
- aa-tRNA (aminoacyl-tRNA)
- AATRS (aminoacyl-tRNA synthetase)
- A site (aminoacyl site)

C

- CF (cystic fibrosis)
- CFLD (CF-associated liver disease)
- CFRD (cystic fibrosis–related diabetes)
- CFTR (cystic fibrosis transmembrane conductance regulator)
- DMD (Duchenne muscular dystrophy)
- DEPs (differentially expressed proteins)

E

- eEFs (eukaryotic elongation factors)
- eIF (eukaryotic initiation factor)
- EJC (exon junction complex)
- E site (exit site)
- ER (endoplasmic reticulum)
- ERAD (endoplasmic reticulum-associated protein degradation)
- eRF (eukaryotic release factor)

G

- G418 (geneticin)
- GTP (guanosine triphosphate)

I

- IRES (internal ribosome entry sites)

L

- LFQ (Label-Free Quantification)

M

- main ORF (main open reading frame)
- Met-tRNA-iMet (methionyl initiator transfer RNA)
- MoA (Mechanism of Action)
- Mut (nonsense mutated mice)
- MutNV848 (nonsense mutated mice treated with NV848)
- MutPTC124 (nonsense mutated mice treated with PTC124)

N

- NLU (normalized luminescence relative to untreated controls)
- NMD (nonsense-mediated decay)
- nmCF (nonsense mutation–related cystic fibrosis)
- NPQC (Nuclear protein quality control)
- NRD (nonsense mutations related disease)
- nt (nucleotide)
- NTC (natural termination codon)

O

- ORF (open reading frame)
- PABP (poly(A)-binding protein)
- PIRD (primary immunodeficiency with regulatory T-cell defect)
- PPI (protein-protein interactions)
- P site (peptidyl site)
- PTC (premature termination codon)
- PTC124 (Ataluren, TRID reference drug)

R

- R213X (specific TP53 nonsense mutation, implied)
- RQC (ribosome quality control)

- rRNA (ribosomal RNA)

S

- SDS (Shwachman-Diamond syndrome)
- tRNA (transfer RNA)
- TR (Translational readthrough)
- TRIDs (translational readthrough–inducing drugs)

U

- uORFs (untranslated ORFs, alternative)
- UTR (5' untranslated region)

W

- WT (wild-type mice)
- WTNV848 (wild-type mice treated with NV848)
- WTPTC124 (wild-type mice treated with PTC124)

ABSTRACT

Genetic diseases result from alterations in the DNA sequence that impact either single genes or entire chromosomes. In monogenic disorders specifically, various mutation types can lead to a loss of gene function. Among these, nonsense mutations account for ~11% of hereditary disorders, for which available treatments remain largely symptomatic rather than curative. Beyond genetic diseases, about 12% of tumour suppressor gene defects are also caused by nonsense mutations.

This doctoral research taking as nonsense models Cystic fibrosis transmembrane conductance regulator (CFTR) gene and TP53, combined computational drug discovery, chemical synthesis, biological validation in cellular models, and proteomic analyses in murine models to identify novel translational readthrough inducers (TRIDs), assess their therapeutic potential and further analyze the impact of the readthrough *in vivo*.

A ligand-based pharmacophore model enabled the identification of new scaffolds within the 1,2,4-triazole and diaryl urea families. Several derivatives promoted translational readthrough, and structure–activity relationship analyses highlighted extended aromatic groups, hydrogen bond acceptors, and asymmetric core substitutions as key topological features. Four triazolic compounds (IP14, IP15, IP17, IP18) showed no cytotoxicity and successfully restored full-length p53 in TP53 nonsense mutant cells, with correct nuclear localization, transcriptional reactivation, and favourable *in silico* ADMET profiles.

Proteomic studies involving cystic fibrosis mouse model carrying nonsense mutations further revealed CFTR's central role in mitochondrial bioenergetics and proteostasis. CFTR deficiency was associated with basal inflammation and tissue-specific mechanisms, including disrupted hepatic glycogen–lipid coordination and altered neuronal signalling. Both validated TRIDs, NV848 and PTC124, remodelled proteomic profiles, with NV848 showing deeper corrections, particularly in liver, and greater convergence toward wild-type proteome profile.

Overall, this work demonstrates that an integrated strategy of computational modeling, targeted synthesis, and multi-level validation can yield promising TRIDs. The study not only highlights the therapeutic relevance of TRIDs for nonsense mutation–derived diseases, such as cystic fibrosis and p53 related cancer, but also expands our understanding of the systemic consequences of CFTR loss, paving the way for innovative treatments for a broad range of nonsense mutation disorders.

CHAPTER I

INTRODUCTION

1.1 Initiation to Termination: The Journey of Protein Biosynthesis

Proteins constitute the most abundant class of macromolecules in cells and are responsible for a wide variety of functions, including structural support, enzymatic catalysis, signalling, regulation, and transport¹.

Proteins are chains of amino acids whose structural information is encoded in the genome. This information is transcribed into a messenger RNA (mRNA) sequence, organized into triplets called codons which allow the encoded instructions to reach the site of protein synthesis².

Protein synthesis occurs through a highly conserved and tightly regulated cellular process known as translation, which involves multiple types of biomolecules such as mRNA, transfer RNA (tRNA), ribosomal RNA (rRNA) and ribosomal proteins that form the ribosome, as well as translational and regulatory factors, and serves as the fundamental link between genetic information and functional molecules³.

Research focused on understanding the mechanisms underlying translation has advanced significantly over the past 60 years (Fig 1.1.1), leading to a deeper comprehension of the molecular events that drive protein synthesis and opening new therapeutic opportunities for disease treatment⁴.

Before the 1950s, studies of protein synthesis were mainly descriptive and focused on physiology, with theories invoking enzyme-mediated assembly or template mechanisms. A major shift came with the isolation of tRNA by Mahlon Hoagland and Paul Zamecnik in 1956, alongside advances like sucrose gradient centrifugation, which revealed ribosomes and mRNA⁵. These discoveries led to Jacob and Monod's 1961 messenger hypothesis, establishing the modern framework for protein synthesis⁶.

Although many mechanistic details remain to be elucidated, it is now well established that translation takes place in the molecular complex known as ribosome and proceeds through a cyclical process organised in four distinct phases: initiation, elongation, termination and recycling⁷. A single mRNA molecule can be translated simultaneously by multiple ribosomes, a process that increases the efficiency of protein synthesis⁸. The molecular complex formed by the mRNA, together with all the ribosomes bound to, is called a polysome, or polyribosome⁹.

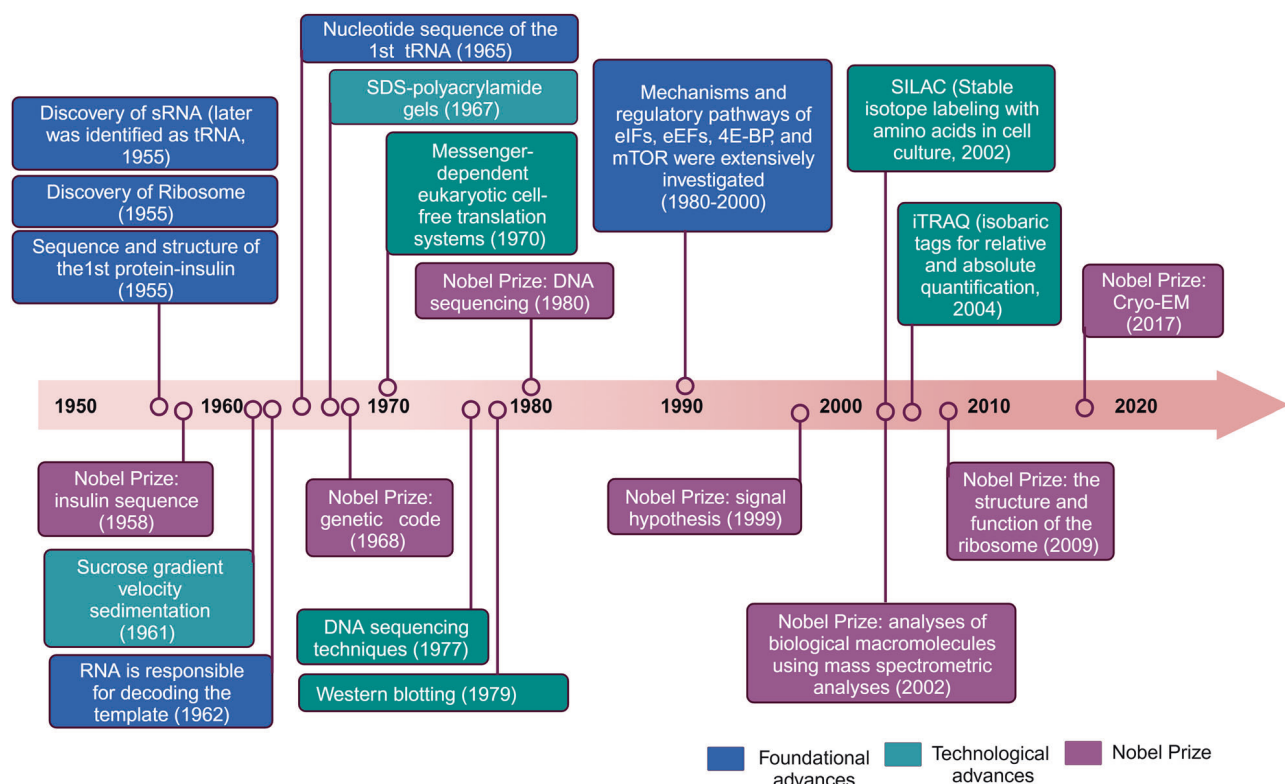


Figure 1.1.1 Timeline of discoveries and advancements in the field of protein synthesis. (Adapted from Xuechao et al. 2024)

Within a polysome, ribosomes are typically distributed along the length of the mRNA at regular intervals, each engaged in translating the same coding sequence into a polypeptide chain thus allowing the rapid production of many copies of the same protein, maximizing translational output and regulating gene expression.

Translation initiation is realized in two phase, 48S initiation complex formation and joining of 60s subunits¹⁰. It is classified as canonical or non-canonical, based on whether it requires recognition of the 5'-m⁷Gppp-cap structure present in mature mRNA, and is considered the rate determining step¹¹.

In translation initiation the small ribosome subunit 40S, constituted by 18S rRNA, folded into four major domains, and about 33 ribosomal proteins, begins with the recruitment of the eukaryotic initiation factor (eIF) eIF1, eIF1A and eIF3 and, subsequently, the ternary complex (TC) constituted by the translational guanosine triphosphatase eIF2, guanosine triphosphate (GTP) and methionyl initiator transfer RNA (Met-tRNA-iMet) forming the 43S pre-initiation complex (PIC) aided by previous mentioned eIF^{12,13}.

In canonical translation initiation (Fig 1.1.2), the 43S PIC recruits the mRNA through recognition of the 5'-m⁷Gppp-cap structure, mediated by the eIF4F protein complex together with eIF5 and either eIF4B or eIF4H¹. The eIF4F complex consists of the cap-binding protein

eIF4E, the DEAD-box RNA helicase eIF4A, and the scaffold protein eIF4G, which links eIF3, eIF4A, and the poly(A)-binding protein (PABP). eIF4B or eIF4H increase the accessibility of the cap-proximal region of the mRNA¹⁴. The cap structure is positioned between two tryptophan residues in eIF4E, with additional contacts to the cap-proximal nucleotides (nts) that stabilize the interaction. After assembly, the 43S PIC begins scanning the mRNA strand in the 3' direction, promoted by eIF1 and eIF1A, to locate the start codon, typically AUG, within an optimal nt sequence known as the Kozak sequence¹⁵.

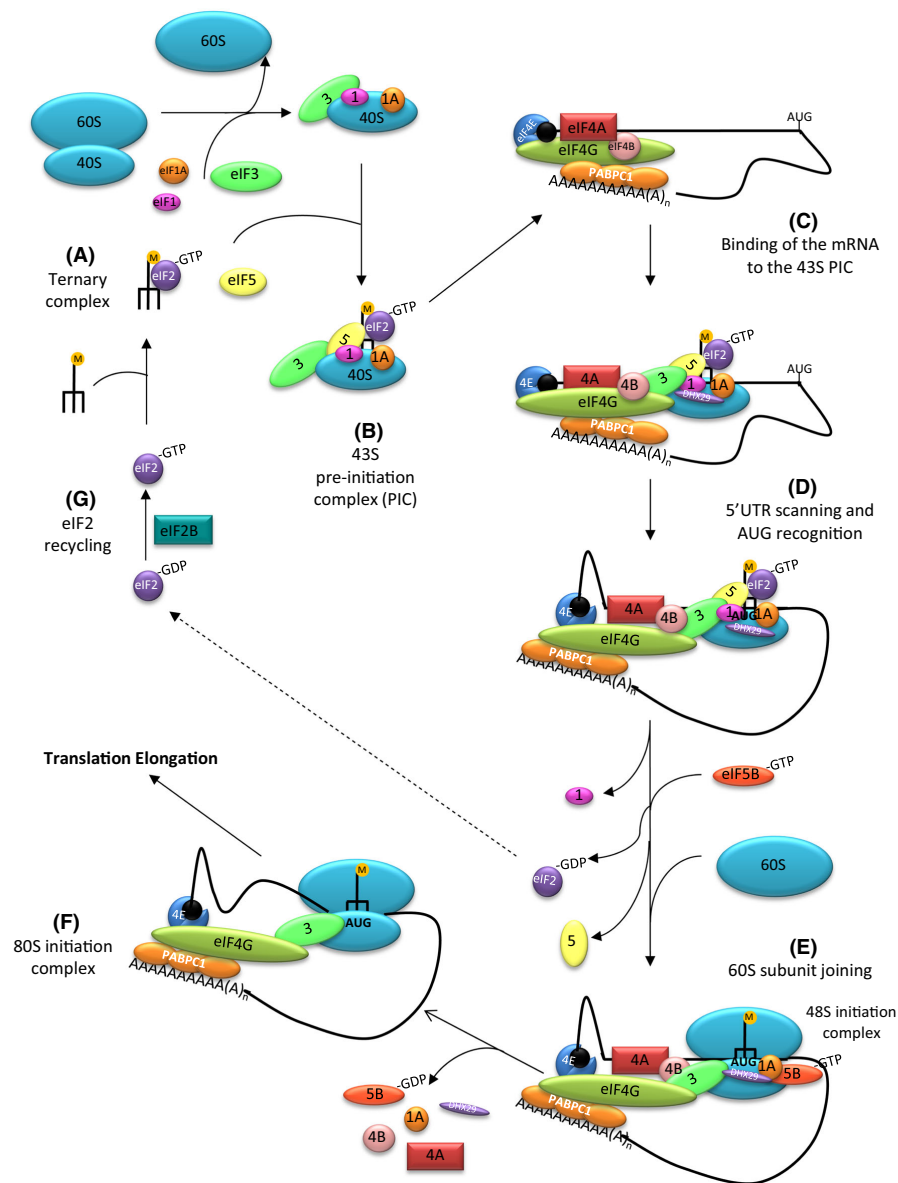


Figure 1.1.2 Canonical eukaryotic translation initiation model. (a) Formation of the ternary complex (eIF2-GTP-Met-tRNA_i); (b) assembly of the 43S pre-initiation complex with eIF1, eIF1A, eIF3, and eIF5; (c) Recruitment of the 43S complex to the mRNA 5' cap and scanning for the start codon; (d) Recognition of the first AUG in a favorable context and formation of the 48S initiation complex; (e) Joining of the 60S ribosomal subunit mediated by eIF5B-GTP hydrolysis, releasing initiation factors; (f) Formation of the 80S ribosome and entry into the elongation phase; (g) recycling of eIF2 for new rounds of initiation. (Adapted from R. Lacerda et al. 2016¹⁶)

Scanning proceeds without additional factors if the region between the 5' cap and the first optimal start codon, known as the 5' untranslated region (UTR), lacks secondary structure. If secondary structures are present, scanning requires ATP hydrolysis and the activities of eIF1, eIF1A, and DHX29, a protein that binds directly to the 40S subunit^{13,17}.

Start codon selection depends on the Kozak sequence. The most critical residues are a purine at the -3 position and a guanosine at the +4 position, with the A of AUG designated as +1¹⁸. In the absence of an optimal Kozak sequence, the 43S PIC complex will not initiate translation at that site and will instead scan downstream until a more favourable start codon is found¹⁹.

Start codon recognition leads to the formation of a stable complex known as the 48S initiation complex (IC) and gives rise to a series of complex remodelling and rearrangement. After AUG recognition, due to codon-anticodon interaction with Met-tRNA_i-Met, the 48S IC passes from an open scanning state to a closed state, leading to the misplacement of eIF1 from the P site and its release²⁰. eIF1 plays multiple roles in translation initiation, is involved in discrimination between cognate and near-cognate start codons, and suppresses eIF2-GTP hydrolysis, strongly linking it to AUG recognition²¹. After eIF1 release, Met-tRNA_i-Met located in the P site binds the eIF5B-GTP complex, which promotes eIF2-GTP hydrolysis and release²².

eIF5B and hydrolysis of its associated GTP mediate the recruitment of the 60S ribosomal subunit, which comprise the 5S, 5.8S and 25S rRNAs and 28 ribosomal protein and possess a crown-like shape, and the displacement of the remaining eIFs still linked to the 48S IC²³, leading to the formation of the 80S ribosomal complex (ribosome) and the start of the elongation phase.

The assembled ribosome possesses three important mRNA-tRNA interaction sites located at the interface between the 40S and the 60S ribosomal subunit: the aminoacyl site (A site), the peptidyl site (P site), and the exit site (E site). These sites are responsible for aminoacyl-tRNA recognition and binding, peptidyl-tRNA binding, and discharged tRNA release, respectively²⁴. Although canonical initiation is the most used mechanism in eukaryotic cells, it can be impaired or suppressed under various stress conditions, such as viral infection or cancer, leading to a drastic reduction in protein synthesis. In these situations, eukaryotic cells employ highly conserved, cap-independent modes of translation initiation, collectively referred to as non-canonical mechanisms, to express selective proteins essential for cell survival²⁵. These proteins may be encoded within the main open reading frame (ORF) or in alternative untranslated ORFs (uORFs), located upstream, downstream, or in overlapping reading frames. Non-canonical translation is also exploited by viruses to achieve preferential expression of viral proteins or to

increase the number of proteins produced from a single mRNA, thereby expanding the coding capacity of their genomes¹¹.

Non-canonical translation initiation typically relies on the recognition of high-affinity RNA sequences, unique secondary structures, or nt modifications. The best-characterized example is based on recruitment of the 40S PIC through interaction with specific elements known as internal ribosome entry sites (IRES), with or without the assistance of canonical eIFs²⁶. Two other cap-independent mechanisms are known: cap-independent translational enhancer (CITE)-mediated translation, which is used by some cellular mRNAs under apoptotic conditions²⁷, and N6-methyladenosine (m6A)-mediated initiation²⁸.

At this level, the major form of translational regulation is based on phosphorylation of the α subunit of eIF2, carried out by specific kinases such as protein kinase R-like endoplasmic reticulum kinase (PERK), general control nonderepressible-2 (GCN2), and heme-regulated inhibitor (HRI)²⁹. These kinases are activated by different signaling pathways, including PI3K, mTOR, and MAPKs³⁰. Phosphorylation of eIF2 α leads to inhibition of translation initiation by blocking eIF2B-mediated nt exchange necessary eIF2-GDP recycle³¹.

After the 80S complex is formed, the translation elongation phase begins (Fig 1.1.3). During elongation, the ribosome moves from the start codon along the mRNA molecule, adding amino acids to the nascent polypeptide chain according to the sequence of mRNA codons, thanks to the adaptor role of tRNA, which ensures the correspondence between codons and amino acids¹⁸. In particular, elongation is a cyclic process assisted, in eukaryote, by eukaryotic elongation factors (eEFs) and organized in three phases: decoding, peptide bond formation and ribosome transposition³². In the decoding step an aminoacyl-tRNA (aa-tRNA) is delivered and bound to the A site, thanks to the complementarity of mRNA codon and tRNA anticodon, as a ternary complex with eEF1A and GTP. GTP hydrolysis leading to eEF1A dissociation and ribosome conformational rearrangement bringing the aa-tRNA with a complete association with the A site. After being released, the eEF1A-GDP complex is recycled and reconverted into eEF1A-GTP by eEF1B³³.

Then the peptide bond formation step, the nucleophilic α -amino group of the aa-tRNA in the A site attacks the carbonyl carbon of the peptidyl-tRNA in the P site. This reaction yields a pre-translocation ribosome complex containing a discharged deacylated tRNA in the P site and a new, one-residue-longer peptidyl-tRNA in the A site³⁴.

Subsequently, in the ribosome translocation step, the translational machinery moves toward the next codon in the 3' direction, driven by the interaction and hydrolysis of eEF2-GTP^{35,36}. This shifts the new one-residue-longer peptidyl-tRNA into the P site and the discharged tRNA into

the E site, where it is released and recycled in a new aminoacyl-tRNA synthetase (AATRS)³⁷. AATRS are an ancient and highly conserved class of enzymes that catalyse the covalent attachment of an amino acid to its cognate tRNA via an adenylate intermediate, linking the amino acid to the 3'-OH or 2'-OH of the terminal adenosine located on the acceptor arm. Each amino acid is connected to its pool of synonymous tRNAs by a specific AARS, through a recognition mechanism based on precise interactions with the anticodon and secondary structural elements such as the D-stem. The fidelity of this process is ensured by intrinsic proofreading mechanisms. After this step, the ribosome is ready for another elongation cycle³⁸.

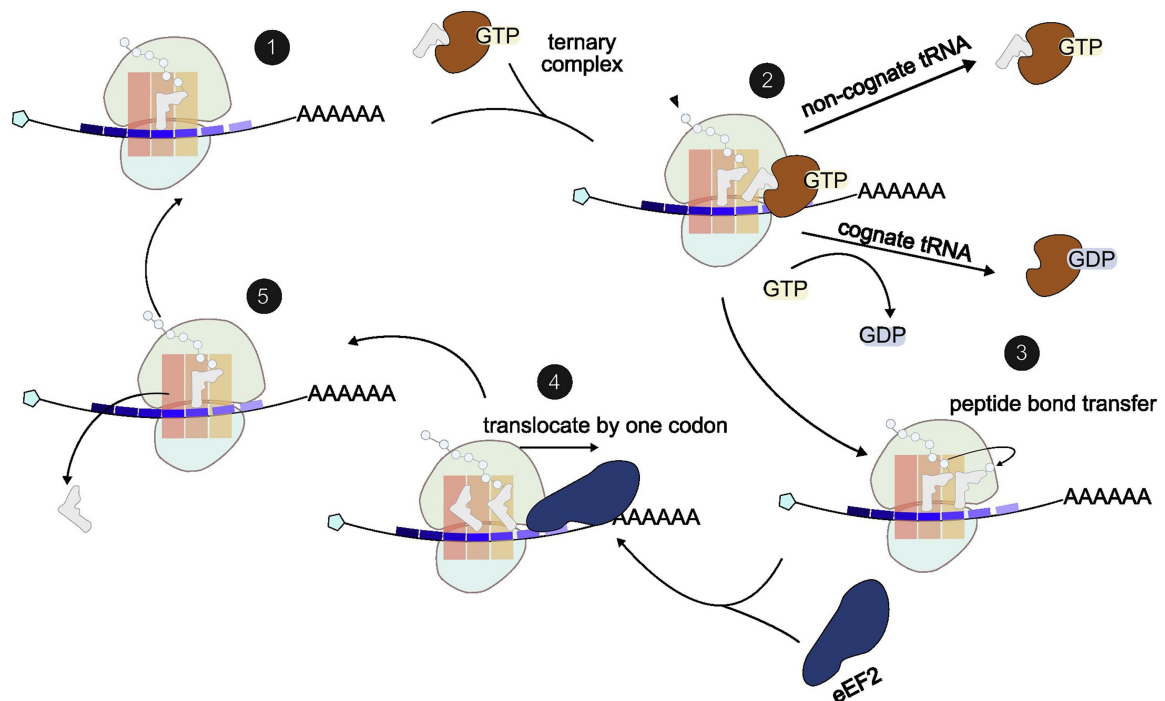


Figure 1.1.3 Eukaryotic translation elongation cycle model. 1) Ternary complex composed of eEF1A-GTP delivers an aminoacyl-tRNA to the ribosomal A-site; 2) Upon correct codon-anticodon recognition, GTP hydrolysis releases eEF1A and locks the tRNA into the A-site; 3) The peptidyl transferase center (PTC) catalyzes peptide bond formation, transferring the nascent chain from the P-site tRNA to the A-site tRNA; 4) The GTPase eEF2 promotes ribosomal translocation by one codon, shifting tRNAs ($A \rightarrow P$ and $P \rightarrow E$); 5) Ribosome restoring conformation, and freeing the A-site for the next cycle. (Adapted from L. Watkin et al. 2025³²)

The elongation phase is intrinsically regulated at different levels, based on the tRNA pool composition, post-transcriptional modifications of tRNAs, and the amino acid sequence of the nascent peptide³⁹.

tRNA pool composition has been reported to vary among tissues and between proliferating and non-proliferating cells⁴⁰. Although the genetic code is redundant, translation elongation is

influenced by the choice of synonymous tRNAs, which insert the same amino acid but differ in their decoding efficiency. Codon optimality is referred to differential decoding rates during elongation: optimal codons are decoded faster by abundant tRNAs, whereas non-optimal codons are slower due to the scarcity of their corresponding tRNAs low efficiency decoding capacity⁴¹. In this way, codon usage modulates elongation speed by regulating translation through the composition of the tRNA pool. This, in turn, affects the overall rate of gene expression and helps determine the stoichiometry of protein products during different phases of the cell cycle and across tissues⁴².

tRNAs are also extensively modified, and these modifications influence their structure and function during the elongation process. tRNA modifications can be broadly divided into two groups based on function: those that stabilize secondary structure and those that fine-tune the translational process. The latter group mainly affects position 34, the first nt of the anticodon (the wobble position), and position 37⁴³. These modifications directly impact the ability of tRNAs to accurately decode synonymous codons while preventing the misreading of near-cognate codons⁴⁴.

The amino acid sequence of the nascent polypeptide chain plays an important role in modulating the rate of translation elongation and can even cause arrest. The nascent peptide exit tunnel is composed mainly of negatively charged rRNA and contains a constriction site formed by ribosomal proteins. Certain amino acid sequences within the nascent peptide can strongly interact with the tunnel, impairing elongation and decreasing the translational rate, sometimes leading to elongation stalling⁴⁵. In certain cases, a translation pause can be beneficial, for example by ensuring proper protein localization, while in others it may result from stress conditions⁴⁶. A decrease in translation rate or translational stalling can cause collisions between adjacent ribosomes, triggering ribosome quality control (RQC). If the stalling is prolonged, these collisions can ultimately lead to ribosome removal⁴⁷.

Elongation phase is continued until a stop codon is reached, forming the pre-termination complex leading to the beginning of the termination phase.

The three stop codons, UAA, UAG, and UGA, referred to as ‘ochre,’ ‘amber,’ and ‘opal,’ respectively, do not encode any amino acid but are specifically recognized by release factors to promote translation termination. In eukaryotic cells, this process is mediated by two key proteins: eukaryotic release factor 1 (eRF1) and eukaryotic release factor 3 (eRF3)⁴⁸.

eRF1 consists of three functional domains: an amino-terminal domain responsible for recognizing the stop codon at the A site of the 80S ribosome; a middle domain containing the universally conserved GGQ motif, which drives the release of the polypeptide chain from the

peptidyl-tRNA at the P site; and a carboxyl-terminal domain implicated in interactions with eRF3 and ABCE1, a member of the ATP-binding cassette family involved in ribosome recycling (Fig 1.1.4a)^{49,50}.

eRF3 is a GTPase composed of an amino-terminal domain that stabilizes interactions with the PABP, a GTP-binding domain containing the “Switch I” and “Switch II” elements required for GTP hydrolysis and the conformational changes necessary for translation termination, and two β -barrel domains (Fig 1.1.4b)^{51,50}.

Although poorly explored, eRF1 and eRF3 can undergo post-translational modifications, such as hydroxylation of the Lys63 residue, which may modulate translation efficiency and add an additional layer of regulation to the termination process⁵². eRF1 and eRF3 interact extensively bringing the M domain and the GGQ motif of eRF1 into close proximity with the G domain and the Switch I element of eRF3 respectively⁵³.

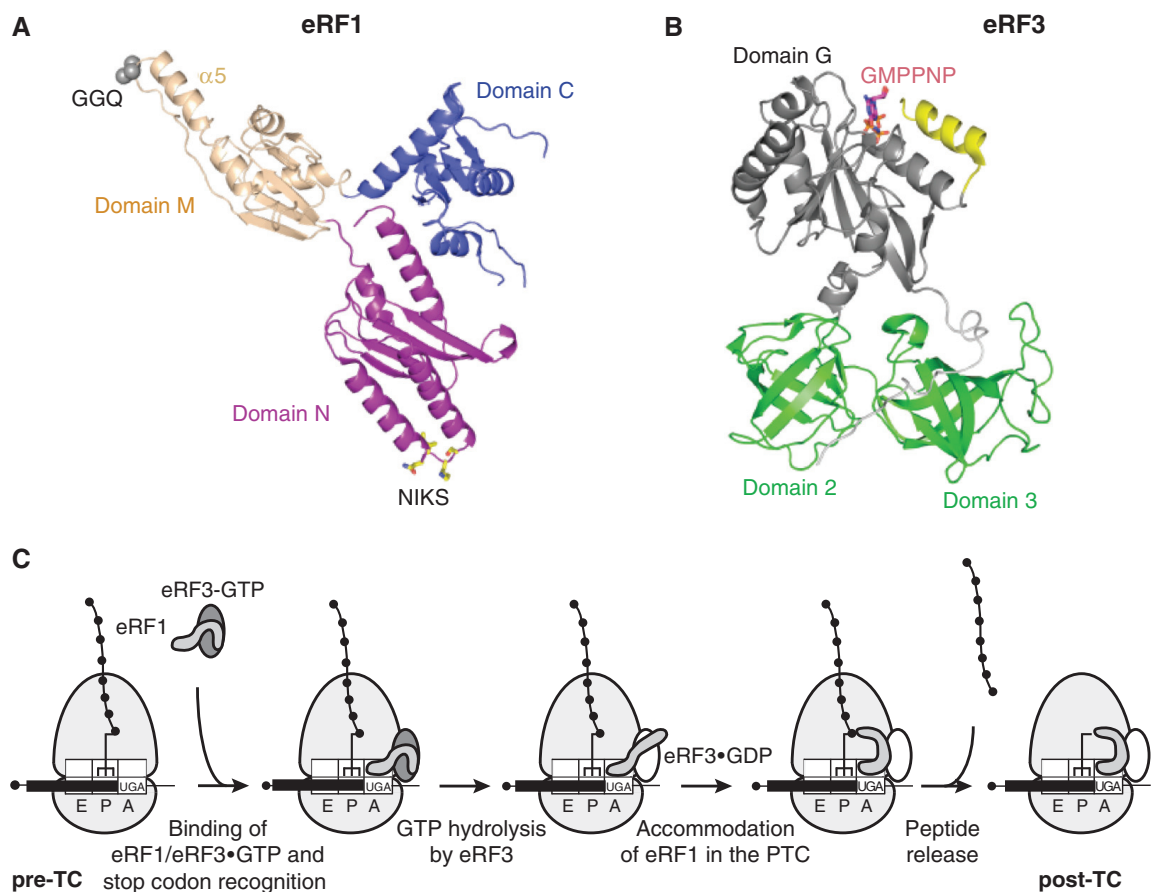


Figure 1.1.4 Eukaryotic translation termination model and proteins. a) human eRF1 structure (protein data bank; [PDB]:1DT9); b) *Schizosaccharomyces pombe* eRF3 (PDB: 1R5B); c) Translation overview: The eRF1/eRF3-GTP complex binds the A site of the pre-termination complex, where eRF1 recognizes the stop codon and adjacent +4 nucleotide. Following GTP hydrolysis by eRF3, eRF1's M domain enters the peptidyl transferase center (PTC) to catalyze peptide release, leaving eRF1 and eRF3-GDP associated with the post-termination complex. (Adapted from C.U.T Hellen 2018⁵⁰)

This arrangement not only coordinates the conformational dynamics of both factors but also enhances the affinity of eRF3 for GTP, ultimately promoting the formation of the eRF1–eRF3–GTP ternary complex required for efficient translation termination⁵⁰.

Although eRF1 can independently recognize the termination codon and catalyze peptide release, the formation of the ternary complex with eRF3 is required for efficient termination.

Indeed, eRF3 acts as a chaperone that facilitates the binding of eRF1 to the ribosome, ensuring that the process is rapid enough to outcompete tRNAs for A-site occupancy⁵⁴.

When the ternary complex reaches the ribosome, the N-domain of eRF1 recognizes the stop codon and adopts a compact conformation that also involves the nt at the +4 position. This compacted state requires a uridine at the +1 position, which is essential for stop codon recognition, as it enables stabilizing interactions with Asn61 and Lys63 of the eRF1 TASNKS motif. Such interactions cannot occur with cytidine, due to insufficient stabilization, or with purines, due to steric hindrance. In addition, the +2 and +3 positions further contribute to stop codon recognition thanks to the interaction with the YxCxxxF motif and Glu55 only when purines are present. Moreover, the presence of a purine at the +4 position enhances termination efficiency⁵⁵.

Stop codon recognition and engagement of the ribosome by the ternary complex induce conformational changes that stimulate eRF3 GTP hydrolysis. This event triggers eRF3 release and drives eRF1 into an extended conformation, positioning its catalytic GGQ motif within the peptidyl-transferase centre to catalyse peptide release. In this conformation, the GGQ motif lies in close proximity to the CCA end of the P-site peptidyl-tRNA, with Gln135 adjacent to the ester bond, thereby facilitating its exposure to nucleophilic attack by water (Fig. 1.1.4c)⁵⁶.

After peptide release, the post-termination complex is formed, consisting of eRF1, the 80S ribosomal complex, the mRNA, and the deacylated tRNA. The eukaryotic ABCE1 protein then binds to eRF1 and, upon ATP hydrolysis, induces conformational changes that promote ribosome splitting, releasing the 60S subunit and leaving eRF1 associated with the 40S subunit⁵⁷. Subsequent interactions of the 40S–eRF1 complex with initiation factors such as eIF1, eIF1A, or eIF3 lead to the release of the discharged tRNA and the mRNA, thereby resetting all translational components for a new round of protein synthesis⁵⁸.

1.2 Nonsense Mutations: Worst case scenario

Disruption of protein synthesis can compromise entire metabolic pathways, undermining cellular homeostasis and survival, resulting in severe and often life-threatening disease. This is the case of nonsense mutations, also known as stop mutations, that arise from single-nt

substitutions, frameshifts, or splicing defects that alter the ORF sequence leading to the conversion of an amino acid-coding triplet into one of the three stop codons⁵⁹.

When a gene is affected by a nonsense mutation, a stop codon will be introduced within the coding sequence of an mRNA, upstream of the natural termination codon (NTC), the resulting codon is defined as a premature termination codon (PTC). The presence of a PTC leads to the premature termination of translation, generating truncated and non-functional protein (Fig 1.2.1)⁶⁰.

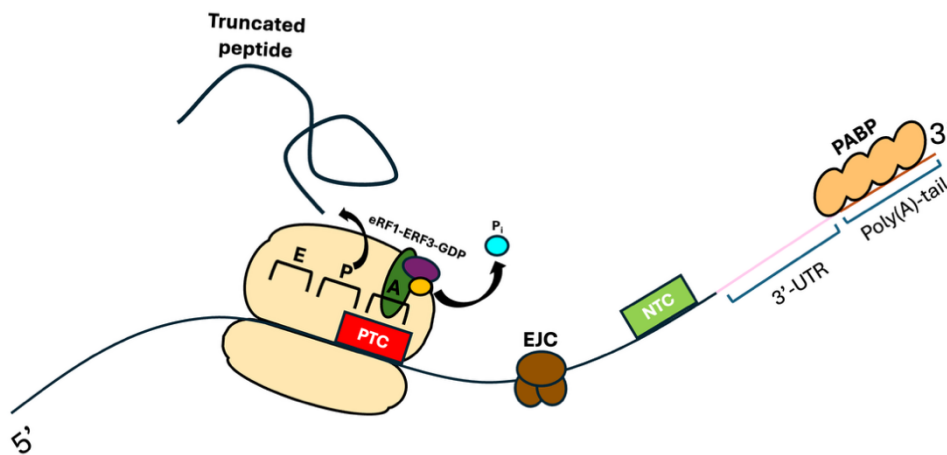


Figure 1.2.1 Premature termination of translation caused by nonsense mutations. A premature termination codon (PTC) is recognized by the eRF1–eRF3–GTP complex at the ribosomal A-site, leading to early termination and release of a truncated peptide. Downstream exon junction complexes (EJCs) mark the transcript for nonsense-mediated mRNA decay (NMD). In contrast, natural termination codons (NTCs) occur near the 3'-untranslated region (3'-UTR), where interaction with poly(A)-binding protein (PABP) facilitates proper termination and stabilizes the mRNA.

Since truncated proteins can be toxic to the cell, quality-control mechanisms act to prevent their accumulation at multiple level.

At the mRNA level, this is achieved primarily through nonsense-mediated decay (NMD) a surveillance mechanism that ensure the quality of translated transcript making sure that they are free of error and completely processed⁶¹.

NMD is responsible for PTC-containing-mRNA degradation, due to nonsense mutation as well splicing aberration like the retentions of introns or the removal exon in a transcript the can lead to the formation of a stop codon, and, more in general regulate about 10% of transcriptome⁶². NMD occurs in both the nucleus and the cytoplasm and is activated by the presence of a PTC

located upstream of the last exon and at least 50 nts upstream of the penultimate exon-exon junction.

This positional requirement reflects the role of the exon junction complex (EJC), composed of eIF4AIII, MAGOH, RBM8A (Y14), and Barentsz, which is deposited during splicing and is essential for the recognition of PTC-containing mRNAs. During the first round of translation, the ribosome removes EJCs as it scans the transcript. The presence of a PTC in a mRNA, occurring at least 50 nts upstream of the penultimate exon–exon junction, causes premature ribosome stalling and translation termination, leaving downstream EJCs intact. These retained EJCs serve as signals for transcript recognition by the NMD pathway and the recruitment of its degradation machinery⁶³.

This condition of activation is known as “50–55 nt rule” and predicts about half of PTC-containing transcripts subject to NMD⁶⁴.

This predictive rate increases to about 75% when three additional “non-canonical” rules are considered. Specifically, NMD efficiency decreases when: (i) the PTC is located within the first 150 nt of the 5' end; (ii) the PTC is situated

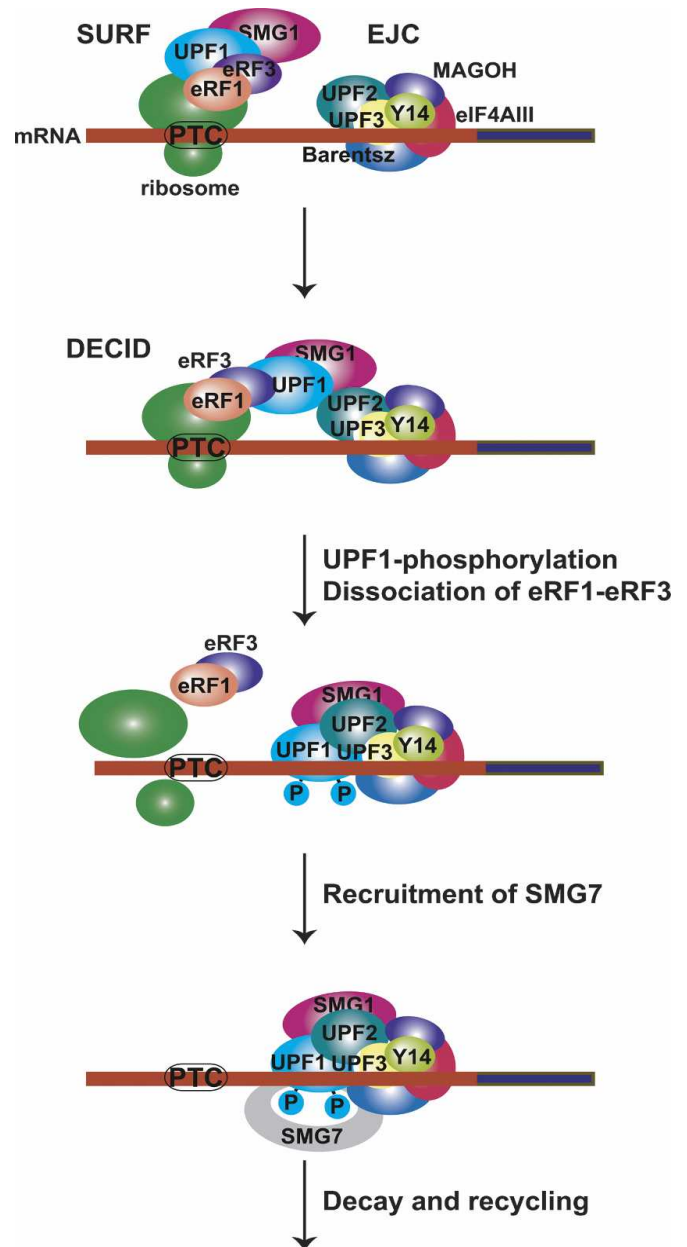


Figure 1.2.2 Mammalian NMD model. PTC initiates assembly of the SURF complex (SMG1, UPF1, eRF1, eRF3), which then interacts with UPF2, UPF3, and exon junction complex (EJC) proteins to form the DECID complex. This triggers UPF1 phosphorylation, release of eRF1/eRF3, and recruitment of SMG7 (with SMG5), which brings in decay enzymes to degrade the mRNA. SMG7 also recruits PP2A, leading to UPF1 dephosphorylation and recycling for further NMD rounds. (Adapted from I. Behm-Ansmant et al.2006)

within a long exon (≥ 400 nt) and not close to an exon–exon junction; or (iii) the mRNA has a short half-life due to competing degradation pathways⁶⁵.

To date, the precise mechanism underlying NMD activation remains unclear; however, a broadly accepted view suggests that it's induced by ribosome pause during translation termination in the presence of a PTC, due to the loss of interaction between eRF3 and PABP. A NTC, that is located at the 3' end of the mRNA, allows the contact of eRF3 with PABP that improve the translational termination efficiency. On the contrary, with a PTC, that can be located much upstream relative to NTC, the increased distance doesn't allow the interaction causing a decrease in process efficiency, giving time to the formation of the protein complex responsible for NMD activation⁶⁶.

NMD is coordinated by seven core factors: UPF1, UPF2, UPF3, SMG1, SMG5, SMG6, and SMG7. The process begins in the nucleus, where UPF3 binds to the exon junction complex (EJC) during splicing. Once in the cytoplasm, UPF2 associates with UPF3 to form a heterodimer, positioning the transcript for surveillance. When the ribosome stalls at a premature stop codon, the release factors eRF1 and eRF3 recruit UPF1, which then interacts with SMG1. This interaction allows assembly of the SURF complex (eRF1, eRF2, UPF1, UPF2, UPF3, SMG1 and EJC) and phosphorylation of UPF1 that drives the release of eRF1 and eRF3 and promotes transition to the decay-inducing (DECID) complex. The DECID complex, composed of UPF1, UPF2, UPF3, SMG1, and the EJC, subsequently recruits SMG5, SMG6, and SMG7. Among these, SMG7 acts as the principal effector, localizing to P-bodies where it directs mRNA degradation through both 5'-3' exonuclease activity and 3'-5' exosome-mediated decay. In parallel, SMG7 recruits PP2A to dephosphorylate UPF1, ensuring recycling of the surveillance machinery and maintaining the efficiency of the NMD pathway (Fig 1.2.2)^{67,61}.

At the post-translational level, aberrant proteins are selectively recognized and degraded through by compartment-specific protein quality control (PQC). Two of the most important are the endoplasmic reticulum (ER)-associated protein degradation (ERAD) and the nuclear quality control⁶⁸.

Proteins within the secretory pathway are monitored by the conserved process ERAD, which targets misfolded species for proteasome-mediated degradation but also regulates ER lipid composition and sterol homeostasis.

In particular, ER proteins are recognized and degraded through three distinct pathways, depending on the localization of their misfolded domain: in the lumen (ERAD-L), within the membrane (ERAD-M), or on the cytosolic side of the ER membrane (ERAD-C)⁶⁸. All three pathways converge on a common mechanism involving retro-translocation across the ER

membrane and ubiquitination by an E3 ubiquitin ligase, assisted by cofactors that vary according to the recognition pathway. In mammals, more than ten ER membrane-bound ubiquitin ligases have been linked to ERAD, with human HRD1 (also known as synoviolin, SYN1) being the most extensively characterized, and RNF5 (also known as RMA1) implicated in the turnover of numerous proteins, including the cystic fibrosis transmembrane conductance regulator (CFTR)^{69,70}.

Once exposed on the cytosolic side, ERAD polyubiquitinated substrates are recognized by the AAA-ATPase Cdc48/p97 (VCP in mammals)⁷¹, which is recruited to the ER membrane via its cofactors Ufd1–Npl4 and the adaptor Ubx2. Powered by ATP hydrolysis, Cdc48 extracts the misfolded protein⁷² from the membrane and releases it into the cytosol after deubiquitinase (DUB)-mediated processing of the ubiquitin chain. The fully dislocated substrate is then delivered to the 26S proteasome, where it is unfolded and degraded into peptides (Fig 1.2.3)⁷³.

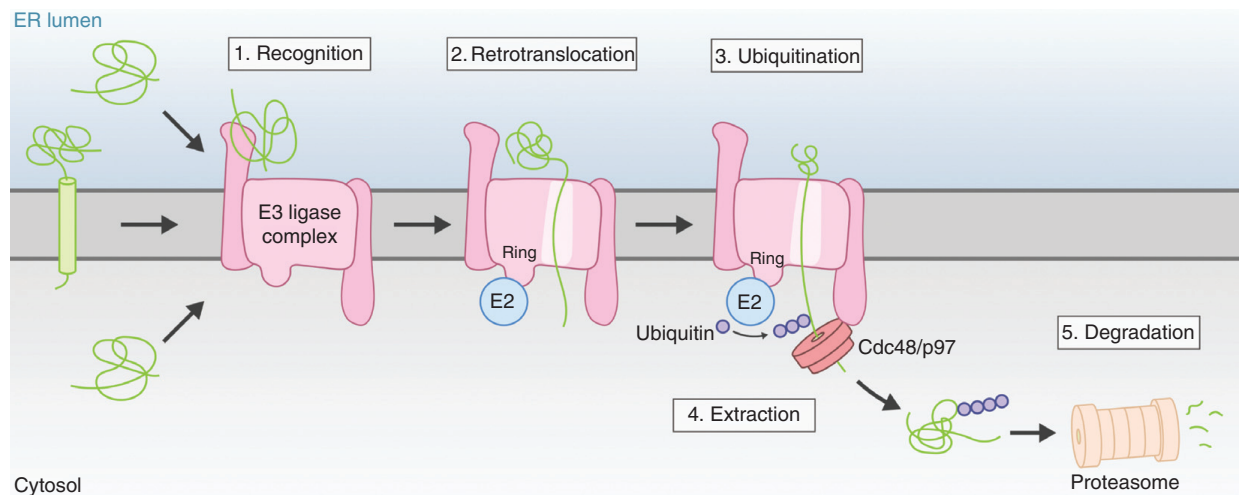


Figure 1.2.3 Generic ERAD model. 1) *Recognition:* Misfolded or unwanted substrates are identified either in the ER lumen, membrane, or cytosolic side; 2) *Retrotranslocation:* Substrates are moved toward the cytosolic face of the ER, aided by ligase complex membrane domains. 3) *Ubiquitination:* Once exposed to the cytosol, substrates are ubiquitinated; 4) *Extraction:* The Cdc48/p97 complex binds ubiquitinated substrates and pulls them out of the membrane; 5) *Degradation:* Substrates are finally delivered to the proteasome for breakdown. (Adapted from L. Krshnan et al. 2022⁶⁹)

Nuclear protein quality control (NPQC), which may be crucial for regulating proteins such as p53, is far less understood than ERAD or other PQC pathways. In mammalian cells, misfolded proteins often form RNA-containing aggregates that, under stress conditions like heat shock or proteasome inhibition, accumulate as perinuclear aggresomes⁷⁴. A recently described NPQC pathway shows that these inclusions are tagged with ubiquitin and ubiquitin-like proteins (Ubls) such as NEDD8 and SUMO. The E3 ligase HUWE1 plays a central role, especially during recovery, by attaching hybrid ubiquitin-Ubl chains that act as specific clearance signals. Unlike

bulk processes such as autophagy or nuclear export, elimination relies on nuclear HSP70 chaperones to extract or disaggregate proteins, the nuclear proteasome to degrade them, and RNA Pol I–dependent lncRNAs (e.g., IGS42), which help organize inclusions during stress and guide their disassembly and clearance afterward⁷⁵.

Given their profound impact on protein expression, completely abolishing even residual functionality through activation of NMD and PQC surveillance mechanisms, nonsense mutations are associated with a threefold higher risk of developing severe conditions, often accompanied by high mortality rates, collectively named as nonsense mutations related disease (NRD).

The pathologies triggered by this type of mutation can affect specific anatomical districts, such as the ocular system in the case of choroideremia, retinitis pigmentosa, and Leber’s amaurosis, or they can be systemic in nature, as in Duchenne Muscular dystrophy (DMD), or even lead to the development of neoplastic diseases and tumours.

Among them two clinically and biologically relevant conditions are cystic fibrosis (CF) and nonsense mutated TP53 gene associated disease.

1.3 Cystic Fibrosis: The Legacy of a Broken Channel

CF is the most common life-limiting autosomal recessive disease in Caucasian and in Europe affect 1 in 2500-3500 newborn, is due to the mutation of the Cystic fibrosis transmembrane conductance regulator (CFTR) coding gene⁷⁶.

Discovered in 1989 by Lap-Chee Tsui, Francis Collins, and Jack Riordan⁷⁷, CFTR gene is constituted of 27 exons, located in chromosome 7 q31.2 region and express for the homonymous protein. The CFTR protein belongs to the ABC transporter family and is expressed on the apical membrane of epithelial cells in several organs, including the lungs, liver, pancreas, gastrointestinal tract, brain and reproductive system. Its primary role is that of a cAMP-regulated chloride channel; however, CFTR also exerts numerous regulatory functions. These include inhibition of sodium transport through the epithelial sodium channel (ENaC), modulation of the outwardly rectifying chloride channel, regulation of ATP-sensitive channels, control of intracellular vesicle trafficking, acidification of intracellular organelles, inhibition of endogenous calcium-activated chloride channels, and regulation of bicarbonate–chloride exchange⁷⁸ (Fig 1.3.1).

The clinical manifestations of CF vary widely among affected individuals, including siblings who share the same CFTR genotype. This phenotypic variability arises from a complex interplay of molecular and environmental factors linked to the loss of CFTR function.

Impaired CFTR activity disrupts transmembrane water transport toward the epithelial surface, resulting in dehydration and increased viscosity of the mucus layer. The accumulation of this thickened mucus compromises mucociliary clearance and fosters an environment favorable to bacterial colonization and persistent infection⁷⁹.

In addition to its role in ion and water transport, CFTR dysfunction also contributes to an excessive and dysregulated inflammatory response. This imbalance is partly mediated by altered Ca^{2+} homeostasis, which triggers mitochondrial stress and upregulates pro-inflammatory mediators, as well as by the loss of CFTR's modulatory interactions with components of innate immune signalling⁸⁰.

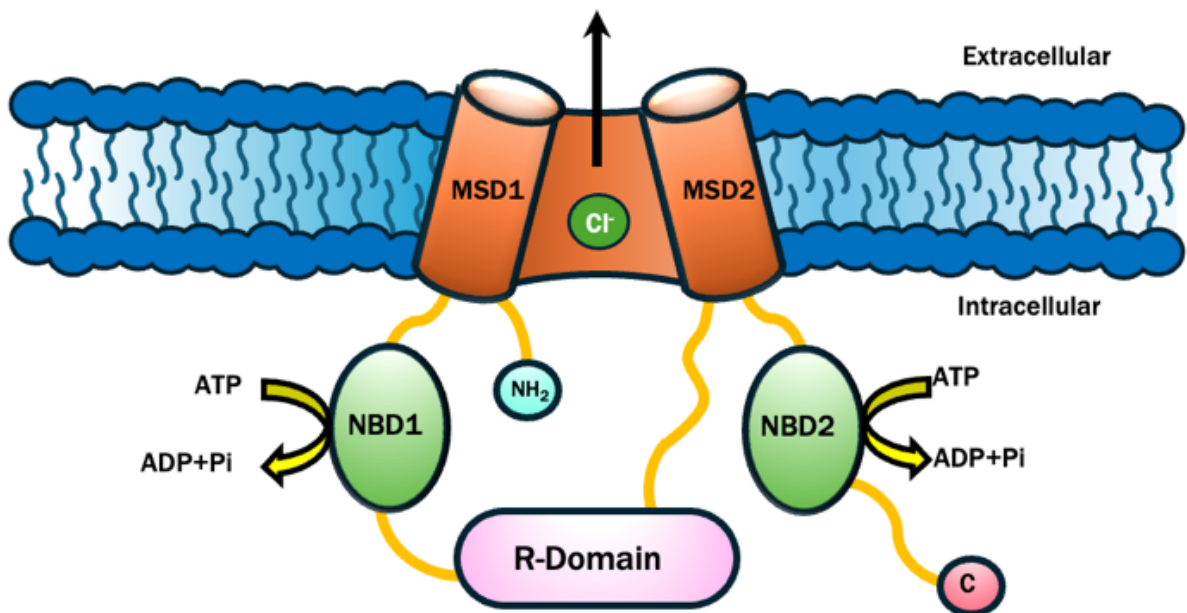


Figure 1.3.1 Schematic representation of the CFTR protein. CFTR consists of two membrane-spanning domains, MSD1 and MSD2, forming the chloride ion channel pore, two nucleotide-binding domains, NBD1 and NBD2, that hydrolyze ATP to regulate channel gating, and a regulatory R-domain that controls channel activity. ATP binding and hydrolysis drive conformational changes allowing chloride ion (Cl^-) transport across the plasma membrane.

Because CFTR is expressed in multiple tissues throughout the body, CF manifests as a multisystem disease, producing a broad range of symptoms that affect several organs and physiological processes.

Chronic lung disease represents the primary CF and is responsible for nearly 80% of CF-related mortality⁸¹. Although the lungs of newborns with CF appear structurally normal, they rapidly acquire infections and become inflamed, with polymorphonuclear cell infiltration evident

within the first weeks of life⁸². Bronchiectasis can be detected in some infants as early as ten weeks. Hallmark features of progressive pulmonary decline include air trapping, hypoxemia, and hypercarbia, while airway damage predisposes to complications such as hemoptysis and pneumothorax. Recurrent and chronic bacterial infections are a defining feature, with gram-negative pathogens such as *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) predominating. These infections accelerate disease progression, increase the frequency of pulmonary exacerbations, and significantly worsen clinical outcomes^{83,84}.

In the pancreas, thickened secretions cause autodigestion and progressive fatty replacement of the organ. Pancreatic insufficiency affects approximately 80% of CF patients and results in steatorrhea, fat-soluble vitamin deficiencies, and malnutrition^{85,86}. Before the advent of supportive therapies, malnutrition was the leading cause of death in CF, as first clinically described in 1938 by the American pathologist Dorothy Hansine Andersen⁸⁷, who was inducted into the American National Women's Hall of Fame in 2001 for this landmark discovery. Today, with the introduction of pancreatic enzyme replacement therapy, malnutrition has become a manageable complication, although careful dietary monitoring remains essential.

Importantly, even in patients with preserved pancreatic function, CF confers an increased risk of pancreatitis. While pancreatitis occurs in only about 0.5% of patients with pancreatic insufficiency, its prevalence rises to 10% among pancreatic-sufficient CF patients, particularly those carrying milder CFTR genotypes that preserve residual channel activity⁸⁸.

CF patients are at high risk of developing cystic fibrosis-related diabetes (CFRD), a complication whose prevalence increases with age, affecting around 20% of adolescents and up to 50% of adults⁸⁹. CFRD is a distinct form of diabetes that cannot be classified as either type 1 or type 2, as it combines insulin resistance with progressive loss of islet cells⁹⁰.

Gastrointestinal involvement is also frequent. Reduced gastric emptying and slower intestinal transit predispose patients to bowel obstruction. After the neonatal period, inspissated stool typically causes left-sided fecal retention and constipation, which can progress to intestinal obstruction. Viscid secretions may additionally lead to appendiceal obstruction, intussusception, and rectal prolapse⁹¹.

CF-related liver disease spans a wide clinical spectrum, ranging from steatosis to focal biliary fibrosis, and in severe cases to cholestasis and advanced cirrhosis with portal hypertension, sometimes necessitating liver transplantation⁹².

Infertility in CF is highly sex-specific: more than 95% of men are affected due to congenital bilateral absence of the vas deferens (CBAVD), which causes obstructive azoospermia despite generally preserved spermatogenesis, while women are typically fertile but may experience

reduced fertility from thick, acidic cervical mucus. Disease severity, malnutrition, and low body mass index can further compromise female fertility through anovulation⁹³.

Registered CFTR gene mutations nowadays are more than 2000 with 700 linked to disease-causing variants. CFTR mutations are classified in 5 group on the bases of consequences that these have on biogenesis, processing and functionality of the protein⁹⁴ (Table 1.3.1). Class I mutations, which include nonsense variants, are characterized by a complete absence of CFTR protein production leading to the most severe form of CF.

Table 1.3.1 CFTR mutation classification.

Class	Defect	Mutations	CF patients incidence
I	Lack of CFTR production	Gly542X, Trp1282X, 711+1G→T, 1717-1G→A	~ 10%
II	Defective CFTR trafficking, ubiquitination and degradation	Phe508del, Asn1303Lys, Gly85Glu, Leu1065Pro	~ 70%
III	Defective CFTR regulation	Gly551Asp, Ser492Phe, Val520Phe, Arg553Gly	~ 5%
IV	Reduced CFTR activity	Ala455Glu, Arg117Cys, Asp1152His, Leu227Arg	~ 2%
V	Reduced production	3849+10kb C→T, 1811+1.6kb A→G, IVS8-5T	~ 2%

The most common CFTR mutation is F508del, which accounts for approximately 70% of CF cases in Europe⁸³. The introduction of Trikafta, a combination therapy consisting of two folding correctors (elexacaftor and tezacaftor) and a potentiator (ivacaftor), has provided a decisive advance in improving patients' quality of life and mitigating symptoms, as reported by significant reduction in 2024 in the rate of pulmonary exacerbations requiring intravenous antibiotics^{95,96}. Overall improvements in treatment, together with extensive campaign of newborn screening, are increasing estimated life expectancy for individuals with cystic fibrosis from 43 years for patients born between 2012 and 2016 to 53 years for those born between 2017 and 2021⁹⁷.

Notably, nonsense mutations account for approximately 10% of CF cases, with G542X and W1282X being the most prevalent⁹⁸. Their frequency can vary considerably depending on geographical and ethnic backgrounds, reaching higher percentages in specific communities. Nonsense mutation–related CF is typically associated with a more aggressive disease course and poorer clinical outcomes⁹⁹. To date, no approved therapies are available for this subgroup of patients, underscoring the urgent need for the development of targeted therapeutic strategies.

1.4 TP53 gene associated disease

TP53 is among the most extensively investigated genes, not only for its pivotal role in tumor initiation and progression but also for its emerging relevance in diverse diseases, frequently associated with nonsense mutations.

TP53 is located in position p13.1 on the short arm of chromosome 17 and encode for the protein p53, a transcription factor discovered in 1989, also known as the “guardian of the genome”, that nowadays represent the most extensive studied cancer related protein by the scientific community. In humans p53 is constituted by 393 amino acids organized in five domains: the N-terminal Transactivation Domain (TAD), the Proline Rich Domain (PRD), the central DNA-Binding Domain (DBD), the Tetramerization Domain (TD) and the C-Terminal Regulatory Domain (CRD)¹⁰⁰.

Initially thought to be an oncogene involved in cell transformation, TP53 was later classified in 1989 as a tumor suppressor gene. Today TP53 and its associated protein network are considered the major stress responder in cells, capable of promoting adaption to a broad range of stress stimuli, including oncogene activation, genotoxic stress, ribosomal stress, hypoxia, and nutrient deprivation, impacting the expression of over 60 genes¹⁰¹.

To respond to a wide range of stress signals, p53 is capable of activating and influencing a wide variety of metabolic pathways (Fig 1.4.1).

The earliest identified functions of p53 include inducing cell cycle arrest, apoptosis, and senescence in response to stress, with DNA damage representing the most prominent activating signal. Upon stress signals, p53 transcriptionally activates the expression of p21 (CDKN1A), a cyclin-dependent kinase (CDK) inhibitor, which inhibits the activity of CDK1, CDK2, and CDK6 through the interactions of its N-terminal domain, thereby arresting cell cycle progression in the G1 phase. Cell cycle arrest provides the cell with a time window to adapt and eventually counteract cellular stress or, if the damage proves too severe, to promote cell elimination through p53-dependent cell death pathways like apoptosis, senescence, ferroptosis and necrosis¹⁰².

p53 drives apoptosis by inducing pro-apoptotic factors such as BAX, NOXA, and PUMA (intrinsic pathway) and upregulating death receptors FAS, DR4, and DR5 (extrinsic pathway). Prolonged p53 activity sustains p21 expression, leading to p16INK4A upregulation, RB activation, and irreversible cell cycle arrest, establishing senescence¹⁰³. Under nutrient stress, p53 modulates metabolism of glucose, lipids, amino acids, nucleotides, and iron, with outcomes depending on ROS levels: low ROS triggers antioxidant defense via TIGAR, while high ROS promotes cell death, including ferroptosis. In ferroptosis, p53 represses SLC7A11, limiting glutathione synthesis, and regulates GLS2, VKORC1L1, and PLTP, enhancing oxidative damage and cell death^{104,105}.

In addition to apoptosis, ferroptosis and senescence, p53 has been implicated in different form of necrosis including necroptosis, parthanatos and pyroptosis.

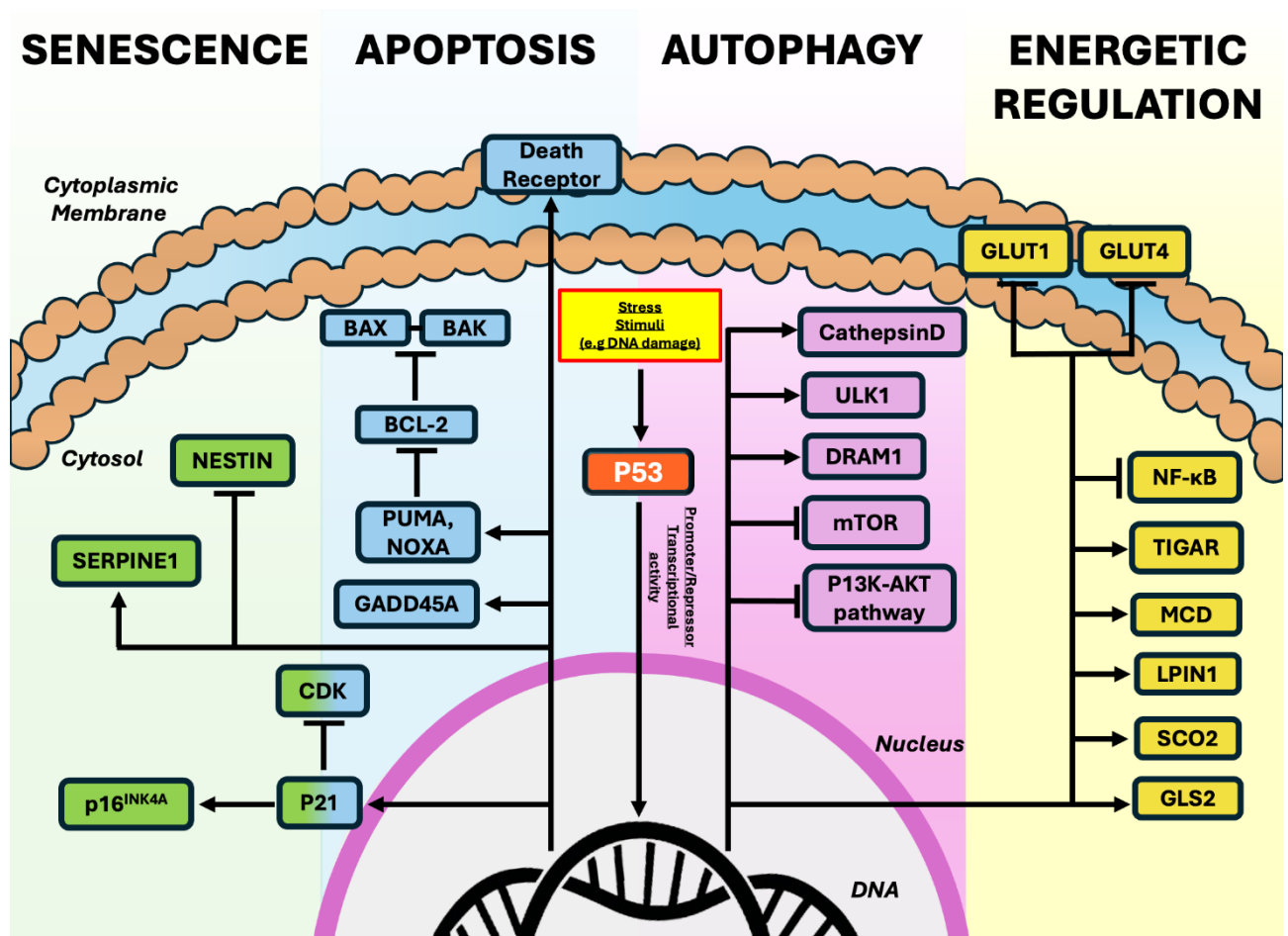


Fig. 1.4.1 Comprehensive schematic overview of P53 related metabolic pathways.

The emerging theme in p53 activation is the maintenance of organismal health and homeostasis through two complementary mechanisms: on one hand, by supporting stress adaptation, repair,

and survival; and on the other, by promoting the elimination of irreversibly damaged or uncontrolled cells.

Because of its broad influence on many metabolic pathways, the loss or impairment of p53 function caused by mutations, such as nonsense mutations in the TP53 gene, are been associated with the onset of different diseases, including type II diabetes, neurodegenerative or immune disorders, and, most prominently, cancer^{100,106,107,108,109}.

Loss of p53 function, including loss of heterozygosity, biallelic activation or deletions, promotes genomic instability and drives the evolution of tumorigenesis¹¹⁰.

It is estimated that more than half of all human cancers involve mutations in TP53, with approximately 10% represented by nonsense mutations^{111,112}. The primary hotspots for nonsense mutations include codons R196, R213, R306, and R342. Nonsense-mutated p53 variants are strongly associated with aggressive cancer phenotypes¹¹³. For example, TP53 mutations are detected in up to 80% of triple-negative breast cancers, the subtype with the poorest clinical outcomes, where nonsense mutations account for as much as 15%¹¹⁴. These observations highlight the critical need for the development of novel targeted therapeutic strategies.

1.5 From Stop to Start: Emerging Strategies for Nonsense Mutations

It is estimated that approximately 11% of inherited genetic diseases and about 12% of mutations affecting tumour suppressor genes are nonsense mutations. For such severe forms of disease, treatment is currently limited to symptom management and minimizing damage. It is therefore necessary to develop therapies specifically targeting this type of genetic defect, which would make it possible to assist a broad population of patients affected by different diseases but sharing this common genetic denominator. Moreover, the development of a treatment for nonsense mutations would also help to reduce the global economic burden associated with the high costs of patient care, which are currently aimed at symptom mitigation and do not provide a definitive solution¹¹⁵.

Several therapeutic strategies are currently under development to specifically target nonsense mutations, aiming to restore full-length protein expression and provide disease-modifying interventions for a wide range of genetic disorders. Gene specific treatment include anticodon engineered-tRNA (ACE-tRNA), gene editing and mRNA based therapy¹¹⁶.

ACE-tRNA employs a modified aminoacyl-tRNA carrying a specific anticodon designed to recognize a PTC, leading to the incorporation of an amino acid instead of triggering translation termination allowing elongation to continue and so preventing the production of truncated

proteins¹¹⁷. Recent studies have demonstrated the functionality of this strategy *in vitro*, including cystic fibrosis models where ACE-tRNAs successfully restored CFTR expression¹¹⁸. However, discrepancies have emerged between *in vitro* and *in vivo* findings, reflecting differences in molecular interactions, processing pathways, and regulatory mechanisms that are present in living systems but absent or altered under *in vitro* conditions¹¹⁹.

Gene editing, particularly CRISPR/Cas9, has rapidly advanced as a promising strategy for treatment of nonsense related genetic diseases. CRISPR/Cas9 enables correction both indel formation, via non-homologous end joining (NHEJ), and point mutation, via homology-directed repair (HDR). Recent adaptations, such as adenine base editors (ABEs), can convert A-T to G-C base pairs and have been successfully applied to rescue CFTR nonsense mutations in CF intestinal organoids, though they remain limited to base transitions^{120,121}. Prime editors (PEs) overcome this restriction by allowing a wider range of edits. Importantly, CRISPR-based therapies are already reaching the clinic, with CTX001 (NCT05477563)¹²² FDA-approved for severe sickle cell disease and beta-thalassemia^{123,124}. While gene editing holds revolutionary potential for precision medicine and rare genetic disorders such as cystic fibrosis, the field remains in an embryonic state and much work is still required before clinical application becomes a reality.

mRNA-based therapies received a decisive boost in their progression and development following the SARS-CoV-2 pandemic.

Before COVID-19, this approach had not been widely explored; however, the rapid development and success of mRNA vaccines have significantly accelerated interest in using mRNA to prevent and treat not only infectious diseases but also genetic disorders, including some nonsense mutation-related conditions¹²⁵. Despite the intuitive appeal of mRNA as a therapeutic tool, several challenges remain. Key issues include optimizing mRNA production and engineering, ensuring stability and resistance to degradation, achieving efficient tissue-specific targeting, minimizing off-target effects, and developing reliable delivery systems¹²⁶. Along gene specific treatment other therapeutical strategies has been developed to face NRDs like NMD inhibition.

Pharmacological inhibition of NMD represents a potential strategy for PTC suppression-based therapies. Several compounds have been developed to target different stages and effector of the NMD pathway, including Pateamine A (PatA)¹²⁷, caffeine, warfarin,¹²⁸ and NMD-1¹²⁹, each acting on distinct effectors of the pathway. Despite its therapeutic promise, NMD inhibition requires careful evaluation. While it may be beneficial in monogenic disorders and cancers with NMD-sensitive tumor suppressor transcripts, it also risks interfering with essential cellular

surveillance mechanisms, leading to the accumulation of aberrant transcripts with dominant-negative effects¹³⁰. Moreover, because NMD normally silences numerous nonsense single nucleotide polymorphisms (SNPs), their unintended expression could produce adverse consequences¹¹⁵.

Alongside the various gene therapy approaches that are still in their embryonic stages of research and development for the treatment of nonsense mutations, a pharmacological strategy of personalized medicine is being explored, based on pharmacological induction of translational readthrough (TR).

1.6 Finding Sense in Nonsense: TRIDs-Based Therapy

One particularly promising approach for nonsense-mutations treatment involves the induction of Translational readthrough TR. This process enables suppression of PTC at a post-transcriptional level thanks to a mismatch between the PTC and a near-cognate tRNA that successfully competes with the release factor eRF1, leading to the insertion of an amino acid in the nascent polypeptide chain instead of the premature translation termination^{115,131}.

TR occurs physiologically at a basal level and at low frequencies: less than 0.1% at natural termination codons (NTCs) and up to 1% at PTCs. The difference in TR rates between NTCs and PTCs reflects distinct termination mechanisms, primarily due to the lack of stabilizing interactions in the latter case¹³². Because the NTC is located closer to the 3' region, it permits a positive interaction between the PABP complex and eRF3, thereby facilitating efficient translational termination. By contrast, a PTC located upstream and distant from the PABP complex cannot benefit from this stabilizing interaction, resulting in ribosomal pausing and a higher likelihood of eRF release and near-cognate tRNA misincorporation¹³³.

TR efficiency is highly dependent on the stop codon sequence and its surrounding nucleotide context. UGA is generally considered the most permissive stop codon for TR, as it forms weaker interactions with the N-terminal domain of eRF1 and allows wobble pairing with several near-cognate tRNAs, including tryptophanyl-tRNA, cysteinyl-tRNA, and alanyl-tRNA, thereby increasing the likelihood of mismatching. UAG follows with lower efficiency, while UAA is associated with the lowest TR rates as it is the most common termination codon in eukaryotes, reflecting its optimization for efficient termination¹³⁴.

Moreover, along with stop codon, the +4nt, +5nt and +9nt results particularly important for TR rates. Cryo-EM studies have shown that nucleotides at positions +4 and +5 downstream of the stop codon directly influence termination efficiency⁵⁵. During termination, the +4 base stacks with Gly626 and the +5 base with Cys1698 of 18S rRNA in the ribosomal A-site. These

interactions are more stable with purines, explaining why A and especially G at +4 reduces readthrough efficiency. Conversely, A at +5 is often linked to increased readthrough, though some studies report the opposite, likely due to reporter sequence differences. Additional downstream nts, such as +9, may also influence termination by interacting with rRNA or ribosomal proteins-6, -5 and -1, as suggested by ribosome profiling and structural data¹³⁵. More broadly, the codon context spanning approximately –15 to +15 nts around the stop codon influences TR efficiency, with higher readthrough rates generally associated with increased A and U content^{136,137}.

TR can be pharmacologically induced by compounds known as translational readthrough-inducing drugs (TRIDs).

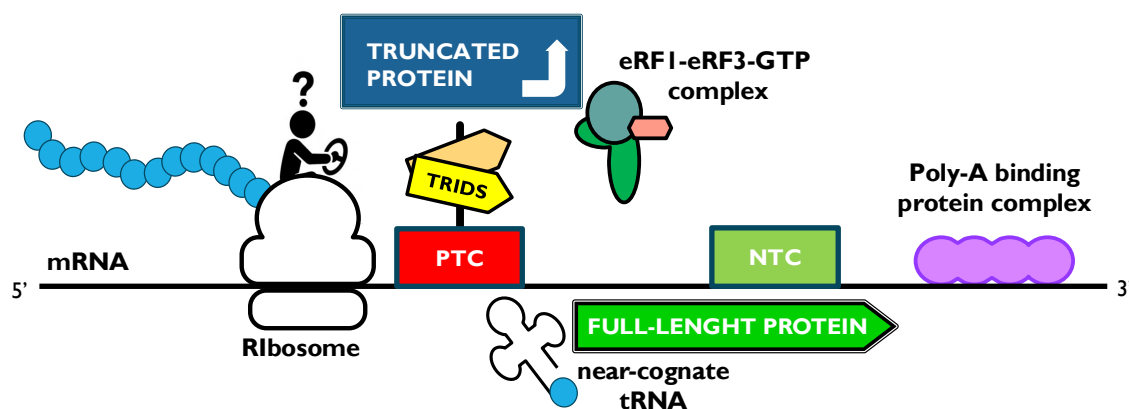


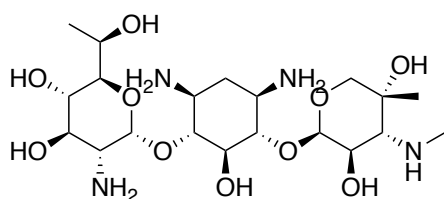
Figure 1.6.1 Schematic description of TR mechanism. A mismatch of the PTC with a near-cognate tRNA, that successfully competes with eRF1, lead to the nonsense mutation suppression and the consequent production of a complete length protein.

Aminoglycoside antibiotics were the first reported class of TRIDs. Discovered as early as 1979¹³⁸, later in 1996 Howard and colleagues demonstrated suppression of nonsense mutations in the CFTR gene using geneticin (G418), leading to the production of full-length, functional CFTR protein in a CF model system, paving the way for TR-based therapy, also called “suppression therapy”¹³⁹. Beyond G418 other aminoglycoside antibiotics such as gentamicin, paromomycin, tobramycin, amikacin, neomycin, kanamycin, and streptomycin (Fig 1.6.2) have been reported to induce translational readthrough of premature termination codons in various model systems¹⁴⁰.

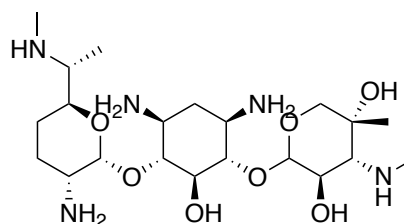
Today, aminoglycoside antibiotics such as gentamicin and G418 represent reference molecules used in assays to evaluate the effect of TR on diseases caused by nonsense mutations across various model systems¹¹⁵.

The TR activity of aminoglycoside antibiotics is due to their binding with the ribosomal decoding centre, specifically, aminoglycosides bind at the A-site of helix 44 in the 18S rRNA

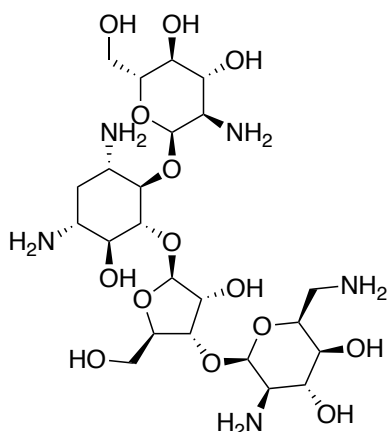
of the 40S subunit, a conserved decoding helix involved codon-anticodon pairing stabilization. Aminoglycoside antibiotics binding induces conformational rearrangements, locking A1492 and A1493 (*E. coli* numbering, corresponding to A1824 and A1825 in human 18S rRNA), two rRNA residues involved in codon-anticodon recognition, into an extrahelical conformation¹⁴¹. This reduces the fidelity of stop codon recognition by eRF1 and increases the probability of near-cognate tRNA accommodation at PTCs, thereby enabling continued elongation and synthesis of a full-length protein^{141,142}.



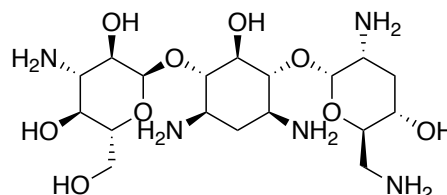
Geneticin (G418)



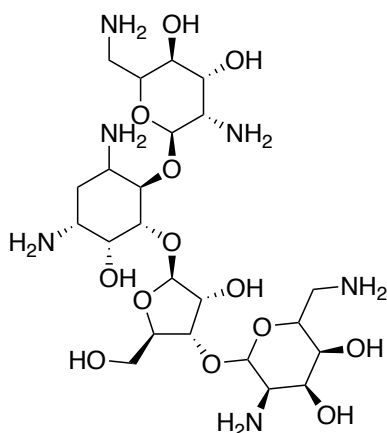
Gentamicin



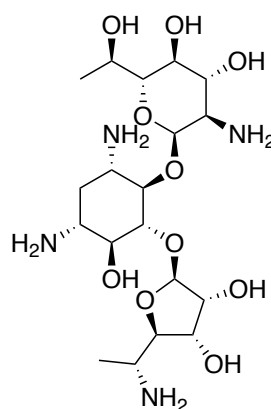
Paromomycin



Tobramycin



Neomycin



ELX-02

Figure 1.6.2 Aminoglycoside antibiotic TRIDs structure overview.

However, this mechanism is not selective for PTCs. NTCs can also be misread, leading to off-target readthrough and production of aberrant proteins. Moreover, therapeutic application of aminoglycosides is hampered by dose-limiting toxicities. The concentrations and exposure period needed for effective readthrough lead to acute ototoxicity¹⁴³, due to accumulation in cochlear hair cells, and nephrotoxicity, via proximal tubule cell damage¹⁴⁴.

These toxic effects, that are independent of their readthrough activity, are due to their polycationic nature and disruption mitochondrial ribosomes activity^{145,146}.

To overcome the side effects of aminoglycoside antibiotics, Bidou and colleagues identified NB124 (also known as ELX-02), a new-generation aminoglycoside that exhibits strong TR activity and efficacy in several nonsense mutation-related diseases¹⁴⁷. Unlike traditional aminoglycosides, ELX-02 lacks characteristic chronic toxicity, as demonstrated in a Phase I clinical trial of single- and multiple-ascending doses in healthy volunteers (NCT03309605), where treatment was well tolerated with no adverse effects¹⁴⁸. In 2023, Eloxx Pharmaceuticals announced the completion of Phase II clinical trials for cystic fibrosis (NCT04135495, NCT04126473)¹⁴⁹ and Alport syndrome (NCT05448755)¹⁵⁰, reporting significant improvements in patient conditions. In CF patients, ELX-02 enhanced lung function, specifically increasing percent predicted forced expiratory volume (ppFEV1). In Alport syndrome, kidney biopsies from treated patients showed improved podocyte foot process effacement, a key indicator of renal health. Additionally, ELX-02 has recently been evaluated in other nonsense mutation-driven diseases, including coagulation factor V deficiency, where it restored protein expression in a dose-dependent manner in both *in vitro* and *ex vivo* reporter systems.

Aminoglycoside antibiotics and their derivatives are not the only known TRIDs. Due to their ototoxicity and nephrotoxicity at the concentrations and treatment durations required to induce RT, research efforts shifted toward the discovery of new compound classes capable of acting as TRID, leading, in 2007, to the identification of Ataluren (PTC124) developed by PTC Therapeutics¹⁵¹.

PTC124, a diaryl-1,2,4-oxadiazole derivative structurally unrelated to aminoglycosides (Fig 1.6.3), was the first TRID to ever receive conditional EMA approval. Early clinical trial demonstrated its favourable safety profile in both adult and children. In particular, Phase I trial on healthy adults (NCT01247207)¹⁵² demonstrated that PTC124 administration show no evidence of systemic toxicity or treatment-related adverse effects, even at multiple dose levels. Similarly, in a Phase II trial on paediatric patients with nonsense related DMD (NCT02819557)¹⁵³, PTC124 was well tolerated, with no major safety concerns reported¹⁵⁴.

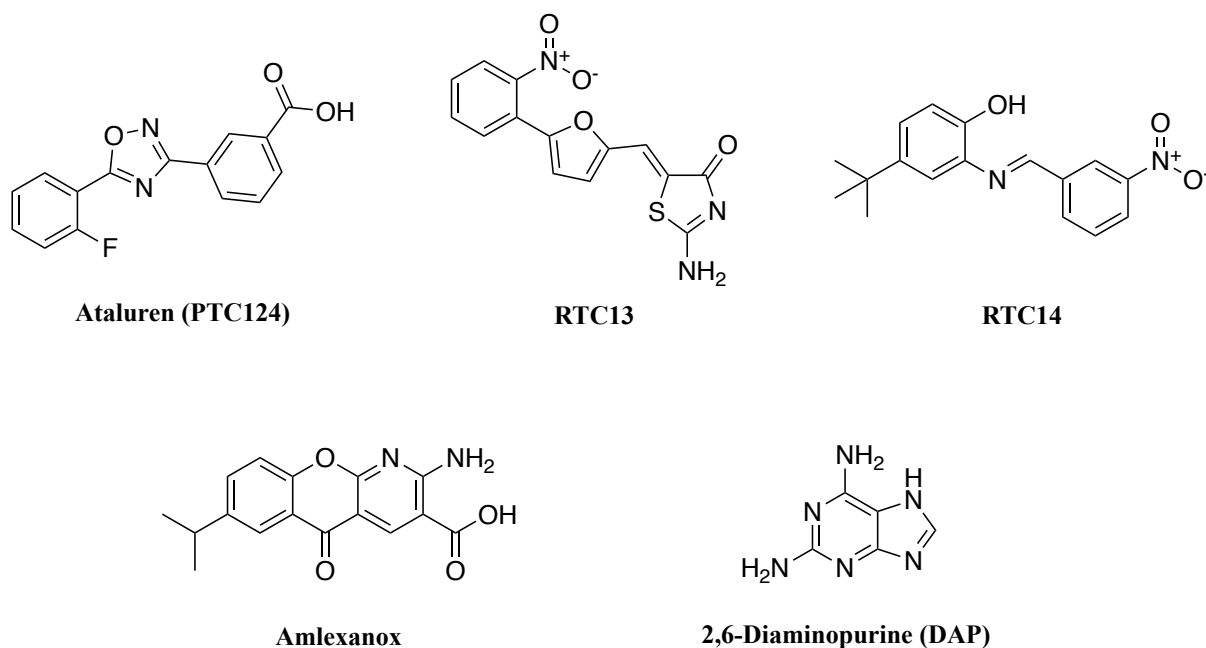


Figure 1.6.3 non-aminoglycoside antibiotic TRIDs structure overview

In the light of above PTC124 was considered a highly promising candidate for the treatment of genetic disorders caused by nonsense mutations.

Ataluren demonstrated efficacy as a RT inducer in *in vitro* tests conducted on models of DMD and CF. Its RT activity, combined with its overall low toxicity profile, enabled progression to clinical trials for both diseases.

From clinical trials evaluating its efficacy in DMD, Ataluren was shown to reduce the loss of muscle function and restore dystrophin expression, as demonstrated in the phase III trial NCT01826487, leading to its conditional approval for clinical use¹⁵⁵.

Conversely, clinical investigations assessing Ataluren in CF therapy, including the phase III trial NCT00803205 and the extension study NCT02139306, despite promising *in vitro* and early-phase results (phase I–II), failed to achieve regulatory approval due to inconsistent outcomes^{156,157}.

Ataluren has been shown not to alter mRNA levels, suggesting that it does not act at the level of transcription or mRNA stability (NMD), but instead induces TR at the translational level. Current studies hypothesize that Ataluren acts through multiple targets, by directly interacting with stop codons via π – π stacking interactions and functioning as a competitive inhibitor of release factors^{115,158}.

Nevertheless, Ataluren has long been at the center of controversy regarding its true clinical efficacy and its ability to meet trial endpoints. This led the Committee for Medicinal Products for Human Use (CHMP) of the EMA to issue a negative opinion on the renewal of its

conditional approval in September 2023, reconfirmed in January 2024, and ultimately to the definitive withdrawal of authorization on March 28, 2025¹⁵⁹.

Despite its recent withdrawal, debate surrounding Ataluren (PTC124) remains ongoing. Patients already on therapy were permitted to continue treatment to ensure continuity of care, while PTC Therapeutics submitted a request for EMA reconsideration, keeping discussion of its clinical utility alive. Indeed, following its 28 March 2025 decision not to renew Translarna's conditional marketing authorisation, the European Commission, as requested by patient and family organisations, indicated that individual countries within the European Economic Area may leverage Articles 117(3) and 5(1) of Directive 2001/83/EC to allow continued use of Translarna for patients who are already being treated.

Supporting this, PTC124 has shown therapeutic benefit in D-bifunctional protein (DBP) deficiency, a fatal autosomal recessive peroxisomal disorder typically leading to death by age two and characterized by neonatal hypotonia, seizures, craniofacial abnormalities, psychomotor delay, deafness, and blindness. In one reported case, a patient treated with PTC124 demonstrated improved swallowing, as well as progressive motor and speech development over a two-year treatment course¹⁶⁰.

PTC124 has also shown promise in Shwachman-Diamond syndrome (SDS), a rare inherited bone marrow failure disorder characterized by neutropenia, exocrine pancreatic insufficiency, skeletal defects, and an increased risk (15–20%) of MDS/AML due to SBDS mutations, with the nonsense variant c.183-184TA>CT (K62X) accounting for ~50% of cases. In patient-derived cells, Ataluren restored SBDS protein expression, corrected ribosome maturation, improved CFU-GM growth, reduced neutrophil dysplasia, enhanced chemotaxis, and lowered p53 levels, while showing no effect on erythropoiesis. Responses varied (~16% non-responders), consistent with heterogeneity also seen in DMD and CF. Notably, Ataluren increased SBDS expression in osteoblasts, suggesting additional benefit for skeletal abnormalities¹⁶¹. These findings highlight its therapeutic potential in SDS and support further clinical evaluation in Phase I/II trials¹⁶².

More recently, our research group demonstrated PTC124's efficacy in primary immunodeficiency with regulatory T-cell defect (PIRD) caused by nonsense-mutated LRBA. We reported the outcome of a 39-year-old male with a homozygous nonsense mutation in LRBA (c.5047C>T; p.Arg1683*) who presented with recurrent infections, autoimmune cytopenias, granulomatous lymphocytic interstitial lung disease (GLILD), severe chronic diarrhea, and nodular regenerative hyperplasia with portal hypertension. Despite multiple immunosuppressive regimens, diarrhea persisted as the dominant and disabling symptom.

Following initiation of PTC124 at 1000 mg daily, treatment was well tolerated and clinical improvement was rapid: diarrhea decreased within two weeks and completely normalized within two months, accompanied by correction of thrombocytopenia and reduction in soluble CD25 levels. Clinical stabilization allowed the patient to undergo successful liver transplantation in January 2022, a procedure previously denied due to his poor clinical condition. To date, under continued Ataluren therapy, the patient remains free of diarrheal relapse and respiratory complications, underscoring PTC124's potential as a targeted therapy for nonsense mutation driven LRBA deficiency¹⁶³.

Other non-aminoglycoside TRIDs have been identified. RTC13 and RTC14 (Fig 1.6.3) were able to restore dystrophin in DMD (mdx) mice and improved muscle function, even if RTC14 showed limited efficacy compared to RTC13 and PTC124¹⁶⁴. Amlexanox (Fig 1.6.3), an anti-allergic and anti-inflammatory drug, has also been investigated for its ability to inhibit NMD and promote PTC readthrough, but proved largely ineffective in restoring full-length proteins, even in combination therapies¹⁶⁵. A more promising candidate is 2,6-diaminopurine (DAP) (Fig 1.6.3), a purine derivative that enhances UGA readthrough in the TP53 gene by inhibiting FTSJ1¹⁶⁶. DAP shows low toxicity, stable plasma levels, and wide tissue distribution. Preclinical studies in mice, organoids, and CF patient-derived cells demonstrated its ability to restore expression and function of nonsense mutated CFTR, with efficacy even via maternal milk delivery¹⁶⁷.

Interest in TRIDs has risen substantially in recent years, as reflected by the increasing number of compounds demonstrating readthrough activity *in vitro* and progressing to preclinical development. The recent non-renewal of PTC124 by the EMA—previously a therapeutic option for patients with DMD, and perspective options for other nonsense derived severe genetic disorders—has left a significant therapeutic gap for individuals affected by diseases caused by nonsense mutations. Considering that more than 7,000 rare genetic disorders affect an estimated 350 million people worldwide¹⁶⁸, tens of millions of patients could potentially benefit from therapies specifically targeting nonsense mutations, highlighting the urgency for new and TRIDs discovery. In this context, my research group has identified, synthesized, and patented three novel 1,2,4-oxadiazole derivatives, NV848, NV914, and NV930, as next-generation TRIDs (Fig 1.6.4)^{169,170}.

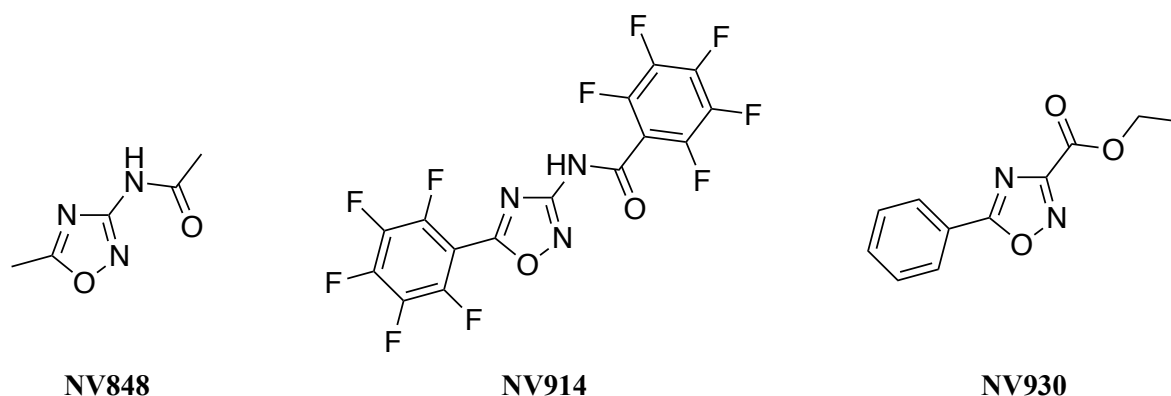


Figure 1.6.4 Pibiri's group next-generation TRIDs structure.

These molecules, first identified in 2020 through screening studies using a firefly luciferase assay, demonstrated the ability to induce TR and restore full-length, functional CFTR protein expression from mutant CFTR cDNAs carrying UGA stop mutations in CF cellular models, specifically in the G542X and W1282X variants (Fig. 1.6.5)¹⁶⁹.

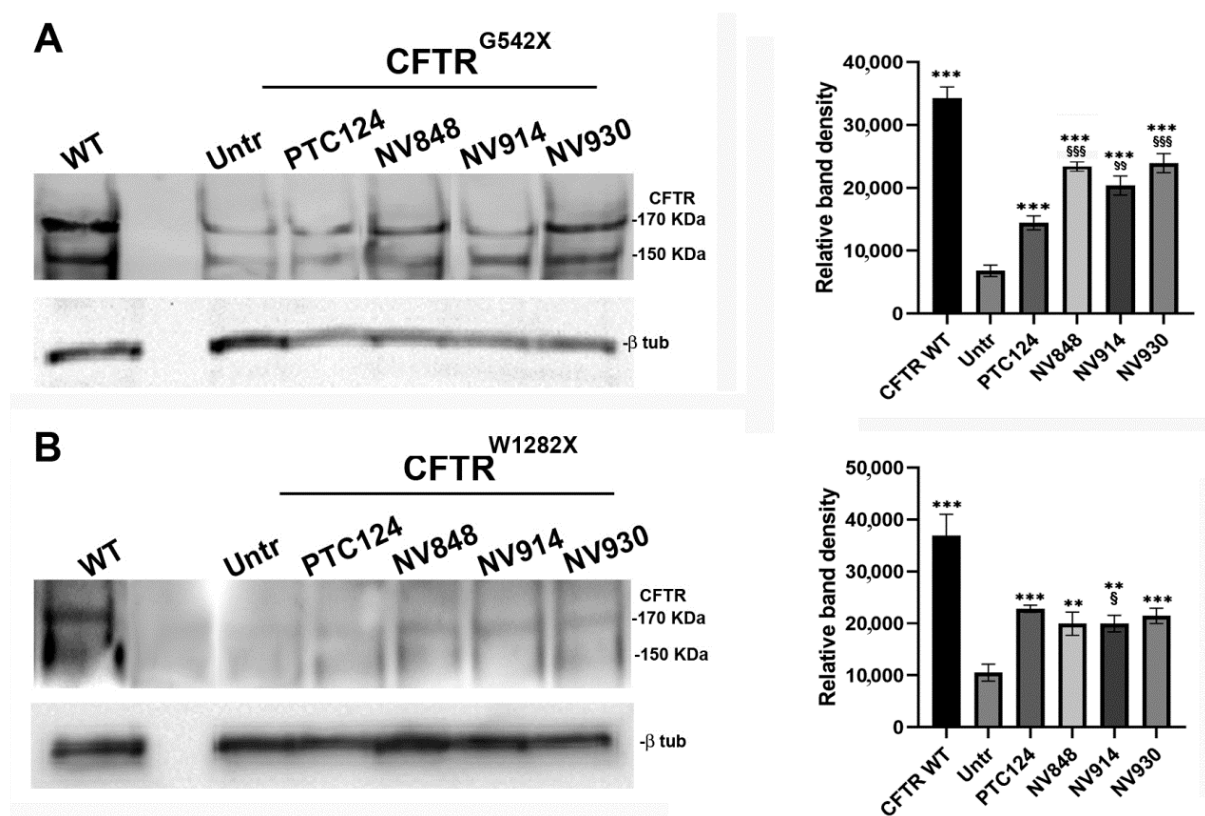


Figure 1.6.5 *in vitro* CFTR recovery upon NVs treatment. (a) Western blot analysis of WT CFTR and FRT CFTR^{G542X} cells, untreated (Untr) or treated for 24 h with 12 μ M PTC124, NV848, NV930, or NV914. (b) Western blot analysis under the same conditions in FRT CFTR^{W1282X} cells. Data represent mean $\pm$ SEM from three independent experiments. * p < 0.01, p < 0.001 vs. untreated; § p < 0.01, §§ p < 0.001 vs. PTC124 (Student's t -test and ANOVA with Dunnett's post hoc test). (Adapted from I. Pibiri et al. 2020¹⁶⁹)

Importantly, the rescued protein was correctly localized to the membrane and showed no detectable off-target effects. Moreover, western blot analysis in validated *in-vitro* model for CF demonstrated that these molecules restored higher levels of CFTR protein compared to Ataluren at the same concentration (Fig 1.6.6)¹⁶⁹. As for Ataluren, the mechanism of action of NVs molecules has not been fully elucidated, but computational and *in vitro* studies suggest that they could promote TR via a multitarget mode of action. One of the possible molecular targets is FTSJ1, a conserved RNA 2'-O-methyltransferase responsible for specific modifications of rRNA within the ribosomal decoding centre. Recent studies suggest that inhibition or modulation of FTSJ1 activity can alter ribosomal dynamics at PTCs, thereby facilitating TR^{171,172}.

However, FTSJ1 is unlikely to be the sole target, since in preliminary unpublished results we also observed TR activity in a eukaryotic cell-free protein synthesis system model (PURE-LITE), composed only of ribosomes, mRNA, a pool of aminoacyl-tRNAs, and eRFs, following treatment with NVs molecules.

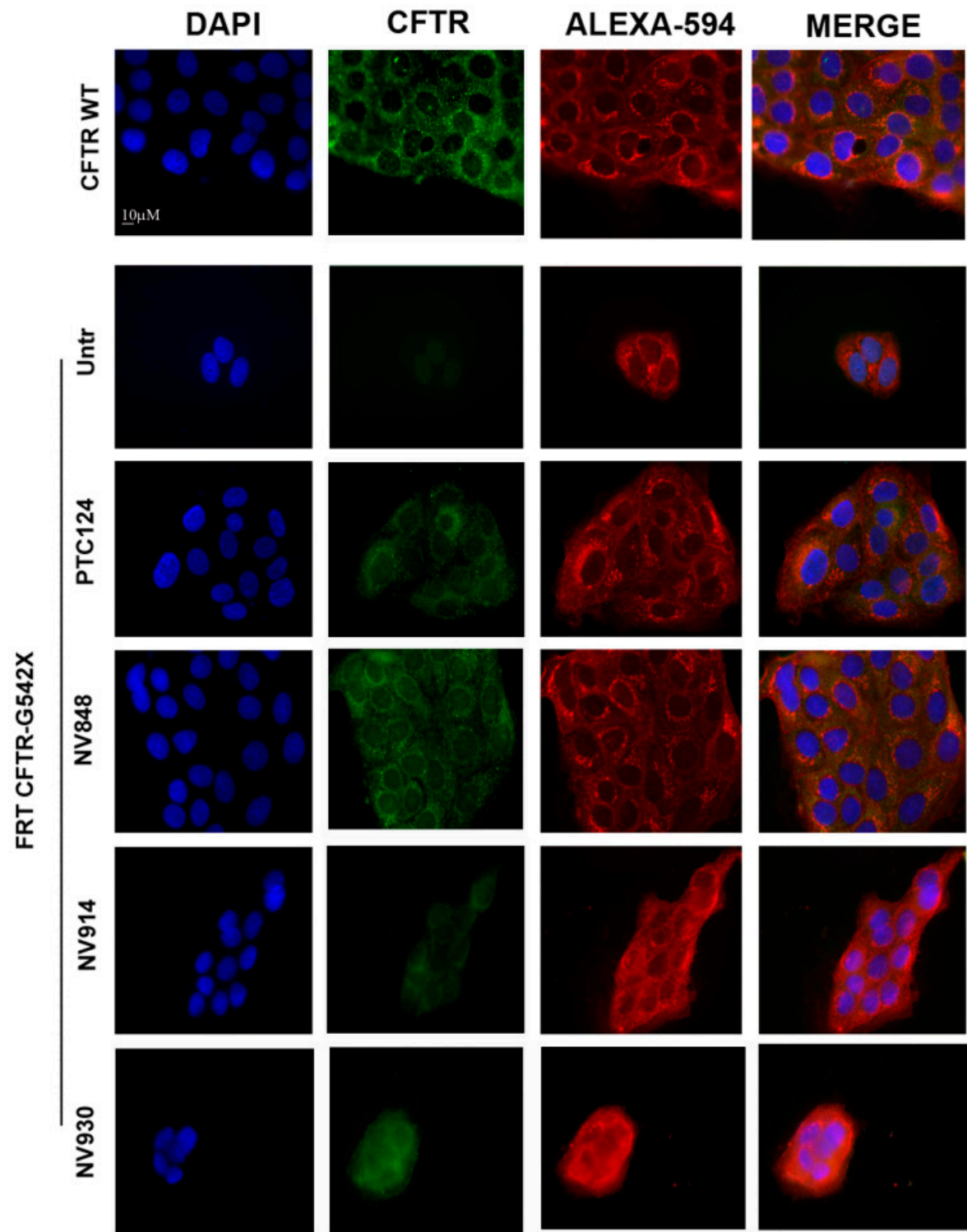


Figure 1.6.6 CFTR expression and localization recovery upon NVs treatment. Immunofluorescence analysis of full-length CFTR protein in wild-type (CFTR-WT) and CFTR^{G542X} Fisher rat thyroid (FRT) cells, untreated (Untr) or treated for 24 h with 12 μ M PTC124, NV848, NV930, or NV914. CFTR was detected with a specific antibody (ab570, green), nuclei were stained with DAPI (blue), and the plasma membrane was labeled with wheat germ agglutinin (WGA)-Alexa 594 (red). (Adapted from I. Pibiri et al. 2020¹⁶⁹)

The three next-generation TRIDs, NV848, NV914, and NV930, display high specificity for PTCs while preserving normal NTC termination as we demonstrated, overcoming aminoglycoside antibiotics TRIDs major limitations. In particular NVs molecules did not alter p53 molecular weight, nuclear localization, or transcriptional activity, nor did they induce size changes in housekeeping proteins like Cystatin-C and β -2 Microglobulin, indicating no detectable NTC readthrough, in contrast to G418, which partially disrupted p53 localization and is consistent with its known off-target effects and toxicity¹⁷³.

Furthermore, all three molecules did not show acute toxicity in C57BL/6 mouse models, indicating good tolerability with no mortality observed following acute administration at 300 mg/kg for NV848 and NV930, and 2000 mg/kg for NV914. Mice treated with 2000 mg/kg of NV914 displayed various symptoms, including hypoactivity, abdominal pain, and tremors, while mice treated with 300 mg/kg of NV848 or NV930 exhibited hyperactivity, abdominal pain, and tremors¹⁷⁴. However, all symptoms resolved within 4 hours of acute dose administration, and no major changes were observed in mouse organs before or after post-acute administration (Fig. 1.6.7). Based on these findings, NV848 and NV930 were classified as category 4 and NV914 as category 5 under the Globally Harmonized System (GHS) of Classification and Labeling of Chemicals, according to OECD criteria, indicating good tolerability and low health risk¹⁷⁴.

Due to its promising results, NV848 has been validated *in vivo*, where chronic treatment rescued CFTR expression in a CFTR^{G542X} nonsense mutant mouse model. In particular, NV848 has demonstrated to restore the expression of full-length CFTR in apical membrane of pulmonary ducts from in-vivo transgenic G542X murine models following chronic oral administration (60 mg/kg for 15 days). These findings support its efficacy in suppressing PTCs (Fig 1.6.8)¹⁷⁵.

Pharmacokinetic profiling revealed that NV848 exhibits markedly greater metabolic stability than Ataluren. In human liver microsome assays, NV848 showed minimal consumption under CYP and combined CYP/UGT conditions, and no glucuronide metabolites were detected, indicating resistance to UGT-mediated conjugation. Complementary *in vivo* biodistribution studies demonstrated rapid but sustained uptake of NV848 across multiple target organs, including pancreas, lungs, kidneys, intestine, and brain, with organ-specific retention persisting for up to two hours post-administration and, in some tissues, beyond¹⁷⁶.

These properties underscore NV848's potential to achieve therapeutically relevant exposure in organs critically affected by CF.

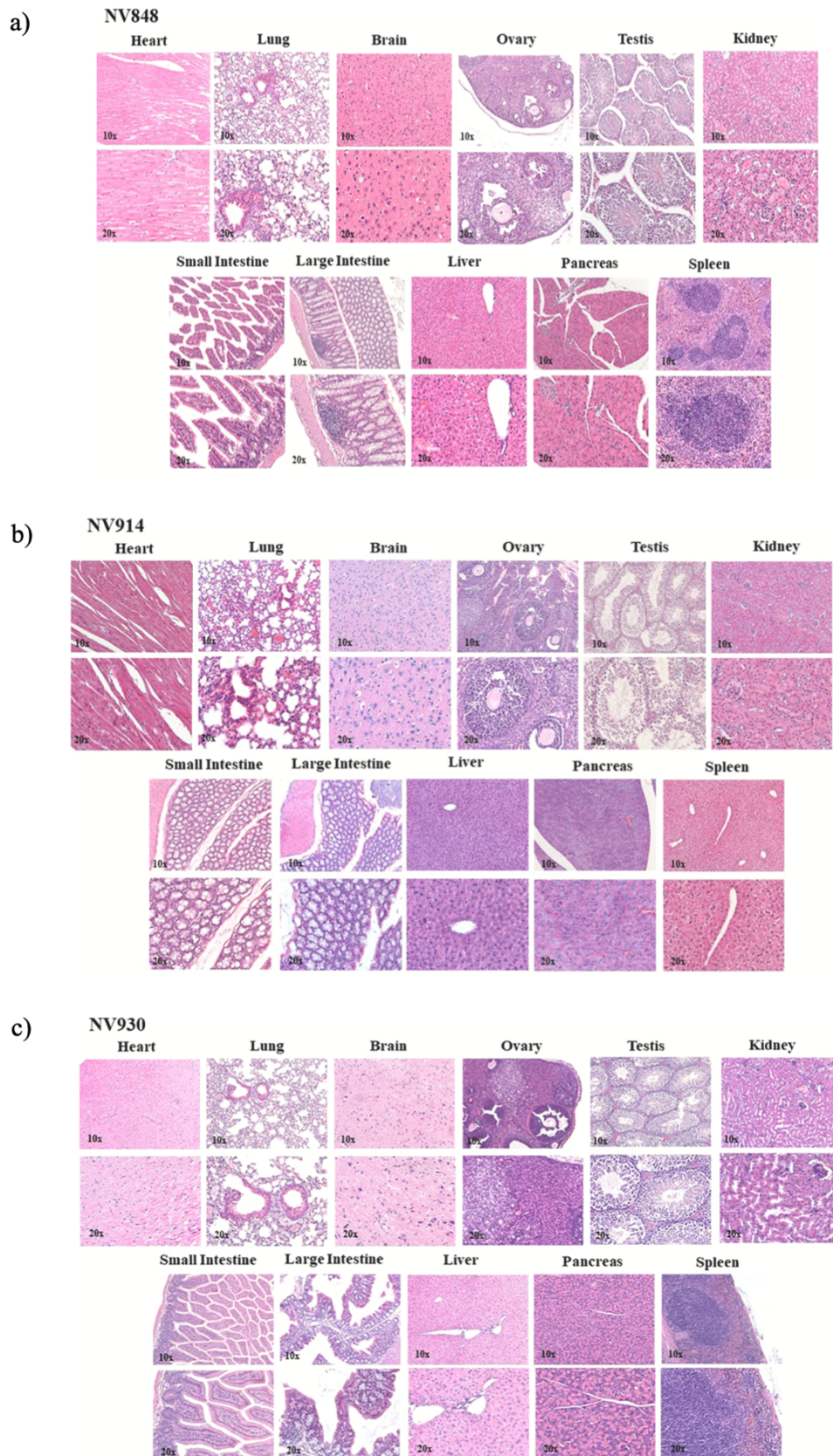


Figure 1.6.7 Histological analysis confirms safety of NV848, NV914, and NV930. Histological panel of the indicated organs in mice treated with NV848 (a), NV914 (b) and NV930 (c), 14 days post-acute dose administration (10X-20X). None of the analyzed organs showed morphological or structural alterations. (Adapted from Corrao et al. 2022⁷⁴)

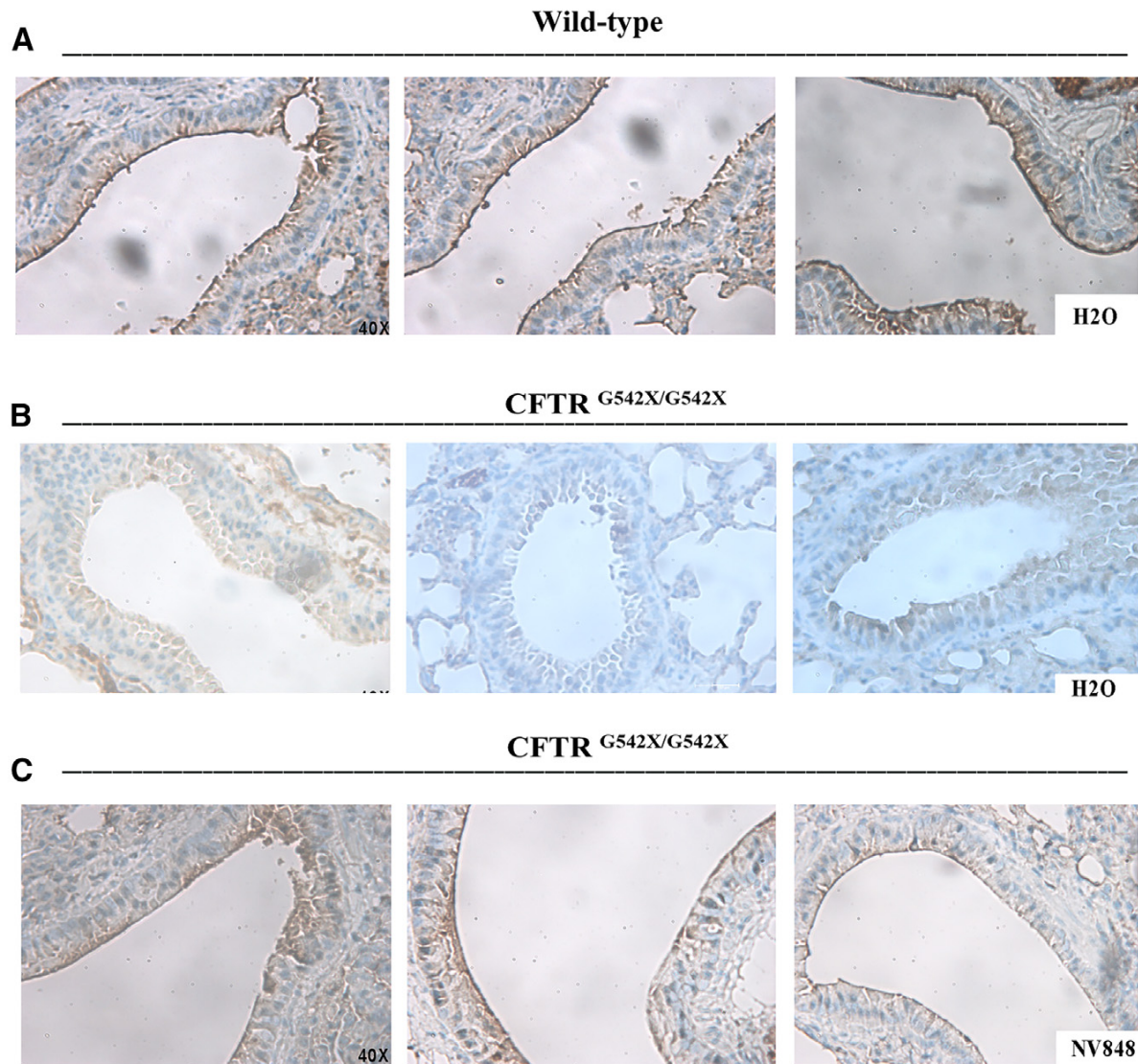


Figure 1.6.8 Expression recovery of CFTR protein in lung of mice model carrying nonsense mutated CFTR^{G542X/G542X}. a) CFTR staining (brown) in CFTR^{WT} lung sections. b) CFTR^{G542X/G542X} untreated (H₂O) control. c) NV848-treated CFTR^{G542X/G542X} lung sections showing CFTR expression at the apical surface of lung ducts (brown). Images shown at 40× magnification. (Adapted from Fiduccia et al. 2024¹⁷⁵)

NVs molecules result effective not only in the treatment of nonsense related CF but also other NRD like SDS. In SDS cellular models (LCLs and PDLSCs) NV848 and NV914 significantly restored SBDS protein expression in LCLs and primary PBMCs from SDS patients¹⁷⁷. Notably, NV848 also improved ex vivo myeloid differentiation of hematopoietic stem cells to a similar extent as Ataluren, although no effect was observed on erythroid differentiation, in line with previous observations that TRID activity is cell-type dependent. Importantly, NV848 enhanced granulocyte maturation, reducing dysplastic features typical of myelodysplastic syndrome (MDS), suggesting a potential role in delaying disease onset. In addition, NV848 promoted SBDS expression in mesenchymal PDLSCs, indicating potential benefit in non-hematopoietic tissues as well¹⁷⁷.

Overall, NV848 showed comparable efficacy to Ataluren but offered several advantages, including greater hydrophilicity, lack of toxicity up to 1 mM in zebrafish experiments, and superior pharmacokinetic properties (ADME). These findings identify NV848 as the most promising candidate for further development in SDS therapy and potentially extendable to other inherited bone marrow failure syndromes (IBMFS) caused by nonsense mutations, such as Diamond-Blackfan anemia, Fanconi anemia, and dyskeratosis congenita¹⁷⁷.

Given their promising pharmacological profile and the ability to restore protein expression in multiple cellular and animal models, NV compounds are currently being explored beyond CF and SDS. Ongoing studies are evaluating their therapeutic potential in a broader spectrum of NRDs, including DMD, PIRD, and cancers associated with nonsense mutations in TP53. By targeting distinct genetic contexts where premature termination codons disrupt protein synthesis, these molecules could provide a versatile and much-needed therapeutic strategy across rare inherited disorders and oncology, ultimately addressing a substantial unmet medical need.

CHAPTER II

STUDY OBJECTIVES

TRID research is rapidly advancing, with efforts focused on improving their efficacy both as standalone agents and in combination therapies. Such progress marks a new frontier in personalized medicine, where nonsense mutation-targeted treatments could be tailored to individual genetic contexts and disease profiles.

However, a critical therapeutic gap remains to be filled. Primarily, the non-renewal of PTC124 by the EMA represents a major problem for patients with NRDs, as no other approved TRIDs are currently available, underscoring the urgent need to develop new treatments capable of advancing into clinical trials. To date, the application of TRID-based therapies has focused almost exclusively on inherited diseases, conceived as life-long treatments, while their potential use in oncology, either alone or in combination with other therapies, remains largely unexplored. This is particularly relevant given that nonsense mutations account for approximately 12% of cancer cases, corresponding to an estimated 6600 new patients diagnosed daily worldwide¹⁷⁸. Furthermore, the precise mechanism of action of currently known TRIDs remains debated, limiting the efficient discovery and rational design of new active molecules. Building on the work developed by my research group and aiming to address these gaps, my doctoral project pursues three main objectives:

- (i) pharmacophore modelling and structure–activity relationship (SAR) analysis for the identification of novel TR inducers and their synthesis;
- (ii) evaluation of biological activity *in vitro* using validated reporter systems to recover TP53 expression carrying a nonsense mutation;
- (iii) *in vivo* proteomic evaluation of one of the validated next-generation TRIDs for CFTR recovery in mice model non-canonical target organs: pancreas, brain, and liver.

The three objectives are structured as a complementary continuum: from rational drug design (pharmacophore and SAR modeling) to synthesis and functional validation *in vitro* (TP53 recovery), and finally to *in vivo* biological assessment (CFTR proteomics of NV848). Although applied to different molecules, this integrated approach provides a strategic framework that supports both the discovery of new TRIDs and the systematic evaluation of their mechanisms of action. Together, these objectives strengthen translational readiness and broaden the therapeutic potential of TRIDs across distinct NRDs.

To achieve this objective, I have employed a multidisciplinary strategy integrating computational analysis, organic synthesis, biological assays, and proteomic studies, with the goal of identifying novel active TRIDs, defining key molecular moieties linked to TR activity,

assessing their applicability in oncology, and contributing to the preclinical development of NV848. To make the process more sustainable, I have implemented an optimized drug development workflow that extensively employs Computer-Aided Drug Design (CADD) methodologies. These are applied not only to the rational design of synthesized molecules but also to toxicity study, in which *in silico* toxicity predictions integrates early-stage *in-vitro* toxicity assessment to support compound evaluation at the earliest phases and providing new active molecules prone for further development.

Moreover, since nonsense mutations represent a common pathogenic mechanism across many NRDs, the newly identified TRIDs capable of restoring p53 could be used to rescue other critical proteins, extending their applicability to rare genetic disorders such as CF, DMD, and other inherited conditions going beyond oncology.

To investigate the biological impact of NV848 *in vivo* in CF models, I employed mass spectrometry-based proteomics. This technology has become a cornerstone in biomedical research, enabling the identification of differentially expressed proteins associated with disease initiation, progression, and therapeutic response¹⁷⁹. Such proteins may serve both as biomarkers for diagnosis and as molecular targets for drug development. In the context of NRDs, proteomic approaches are particularly valuable for elucidating the cellular effects of TRIDs, such as NV848. By providing a global and unbiased view of protein expression and pathway modulation, proteomics offers critical insights into the mechanisms of action of TRIDs and helps assess their potential therapeutic applicability, including in oncology. To maximize protein identification and analytical robustness, I implemented innovative methodologies for protein extraction, digestion, and peptide identification, such as eFASP-assisted digestion¹⁸⁰ and *de novo* peptide sequencing. The outcomes of these analyses are threefold: confirming the therapeutic efficacy of NV848 in CF by providing indirect evidence of *in vivo* CFTR functional recovery in addition to protein expression, shedding light on previously underexplored roles of CFTR in extra-pulmonary tissues, thereby uncovering novel CFTR-related pathways and identify metabolic pathways associated to the TRIDs MoA.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Identification of new Translational Readthrough Promoters

In order to identify new TR-promoters we implemented an in silico drug discovery workflow enabling rapid identification and characterization of candidate molecules while minimizing time and material consumption.

Since the Mechanism of Action (MoA) and molecular targets of TRIDs remain poorly understood, largely because the full complexity of ribosomal function and translational fidelity is still not completely elucidated, we developed pharmacophore models through a ligand-based approach to guide the rational design of novel compounds¹⁸¹. Ligand-based design uses structural features of known active molecules to guide the design of new candidate scaffolds, even without defined targets. The library used for model construction and validation was assembled from molecules with well-documented TR activity, sourced from both in-house collections and public databases, as described in Chapter V. We focused on PTC124 analogues^{169,182,183,184,185} and other non-aminoglycosidic small molecules^{186,187,188}, as these compounds have demonstrated superior TR activity at lower doses with reduced toxicity compared to aminoglycoside antibiotics, which were excluded from the library. Moreover, non-aminoglycosidic small molecules appear to act specifically at PTCs, as demonstrated by the Cooperman group, whereas aminoglycoside antibiotics primarily affect general proofreading activity, also involving NTCs¹⁵⁸.

In particular, during the molecule's conformers generation and alignment of well-known TRIDs emerge that the so-called "ataluren-like" and "chromenopyridine" derivatives, derived from PTC124 and EX 1.37 respectively, exhibit similar features disposition that align to the same pharmacophore model.

Starting from this observation, the pharmacophore model (Model 1) was built considering only these two classes of compounds and consists on nine features: three hydrophobic interactions, two aromatic interactions, three hydrogen bond acceptors, and one hydrogen bond donor as shown in Fig. 3.1.1a.

The pharmacophore model was validated using a dataset consisting of 61 known active molecules and 2,651 decoys generated with the enhanced Database of Useful Decoys (DUDe). Model performance was assessed using enrichment factor (EF) and receiver operating characteristic (ROC) curve analysis (Fig. 3.1.1b). At the top 1% of ranked compounds, the model achieved an EF of 28 and an AUC of 1.0, correctly identifying 38 true actives among 242 predicted hits. At full coverage (100%), the model maintained an AUC of 0.79 and an EF of 7.0, demonstrating reliable predictive power in discriminating active from inactive

compounds. Moreover, Model 1 was able to recognize the parent compounds of both the chromenopyridine (Fig. 3.1.1c) and the ataluren-like (Fig. 3.1.1d) classes of TRIDs.

Overall, the pharmacophore model exhibited strong early enrichment, with a clear preference for active molecules in the top-ranked subset, supporting its utility for prospective hit identification.

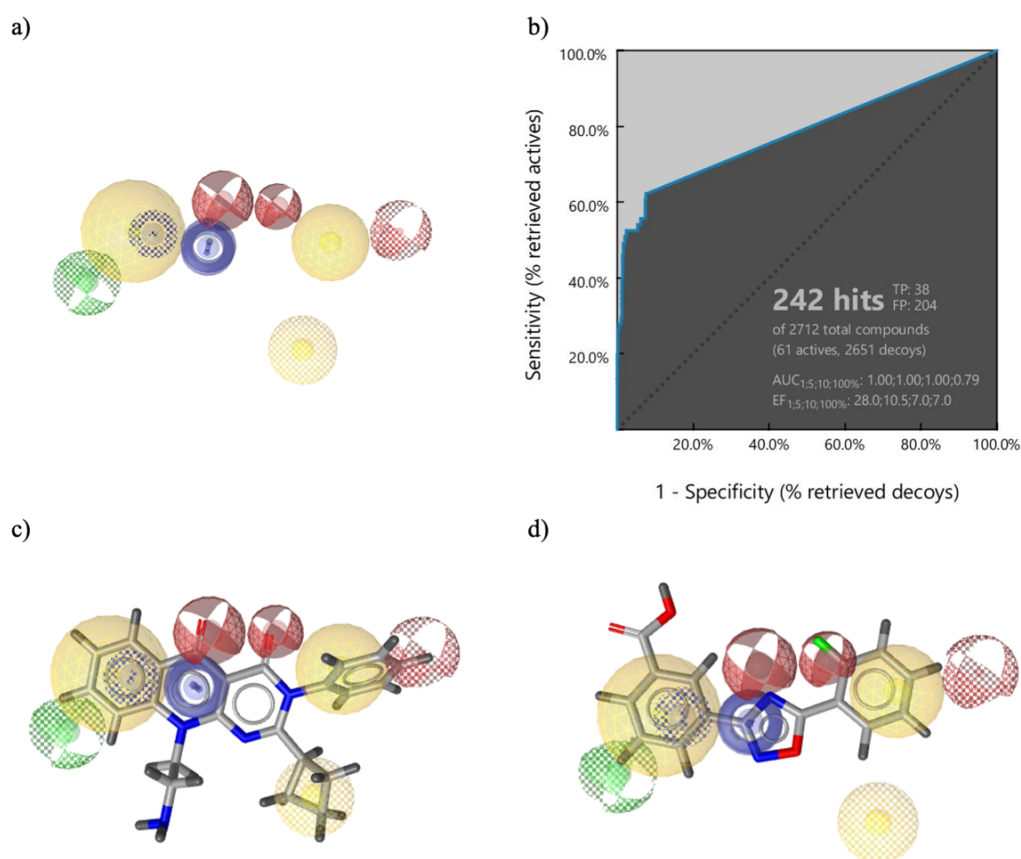


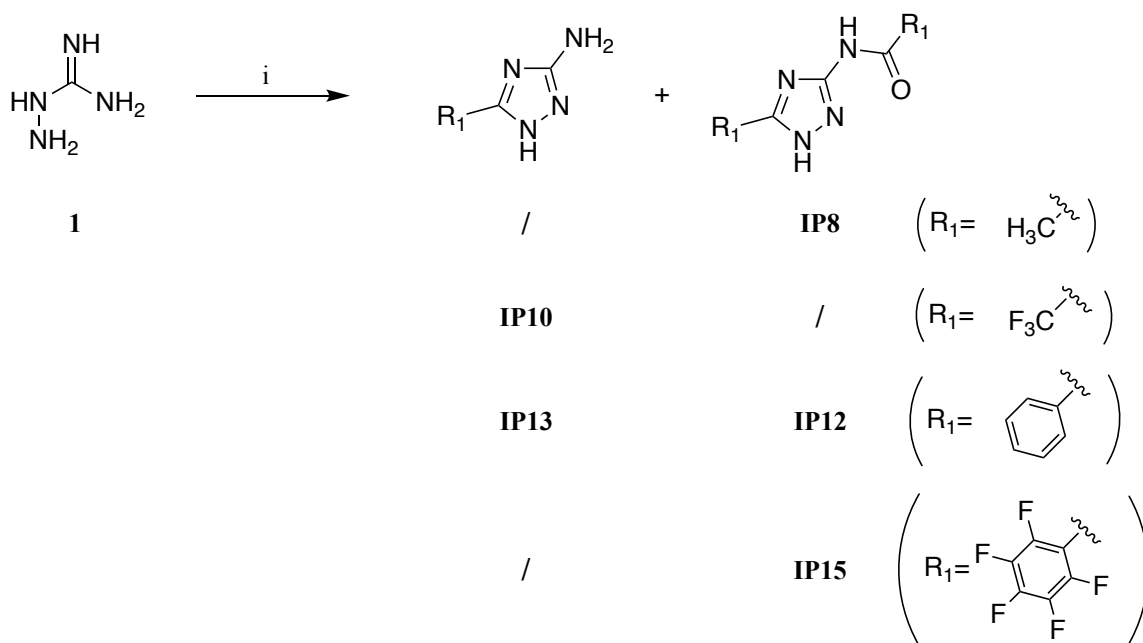
Figure 3.1.2. Pharmacophore model design and validation. a) Ligand-based pharmacophore model (Model 1), comprising three hydrophobic features (yellow spheres), two aromatic ring interactions (blue rings), three hydrogen bond acceptors (red spheres), and one hydrogen bond donor (green sphere). b) ROC curve for Model 1, generated to evaluate its ability to distinguish active compounds from decoys. c) Pharmacophore features of model 1 aligned with compound EX 1.37, a representative of the “Chromenopyridine” class of TRIDs. d) Pharmacophore features of model 1 aligned with PTC124, representing the “Ataluren-like” class of TRIDs.

The validated pharmacophore model was used as a query to screen both our “in-house” compound library and the ZINC database¹⁸⁹, with the aim of identifying novel candidate molecules with translational readthrough–inducing activity.

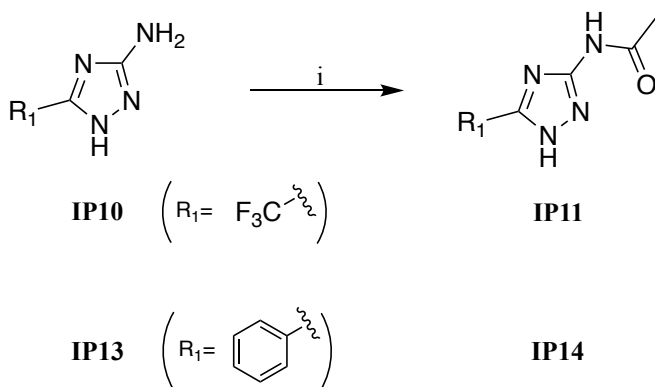
From the in-house library screening, we identified and synthesized eleven 1H-1,2,4-triazole derivatives and six diaryl urea derivatives, to which two additional compounds (IP28 and IP29)

were added as byproducts of the reactions, as detailed in the synthesis and characterization section of Chapter 4.

Seven 1H-1,2,4-triazole derivatives were prepared as aminoguanidine derivatives, either by direct cyclization (Scheme 3.1.1) or by acetylation of the corresponding amine precursor (Scheme 3.1.2), with the aim of evaluating whether triazolic analogues of next-generation TRIDs (NV848, NV914, and NV930) and their precursors are tolerated in terms of TR activity.

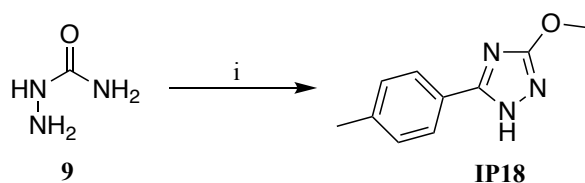


Scheme 3.1.1. Reagents and conditions: (i) RCOX (X = Cl or OH), toluene, reflux.

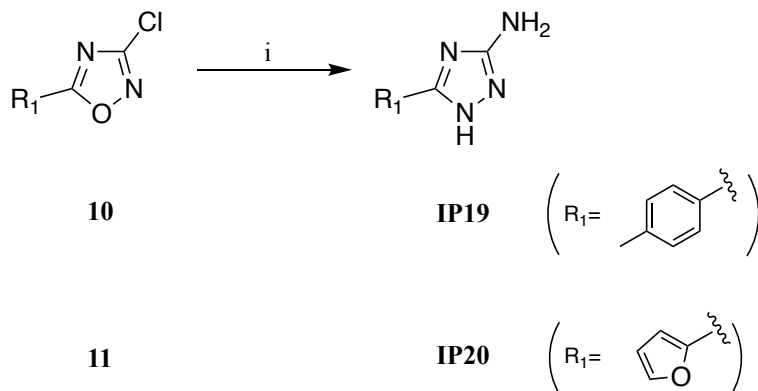


Scheme 3.1.2. Reagents and conditions: (i) Ac₂O, AcOH, 40° C, 12h.

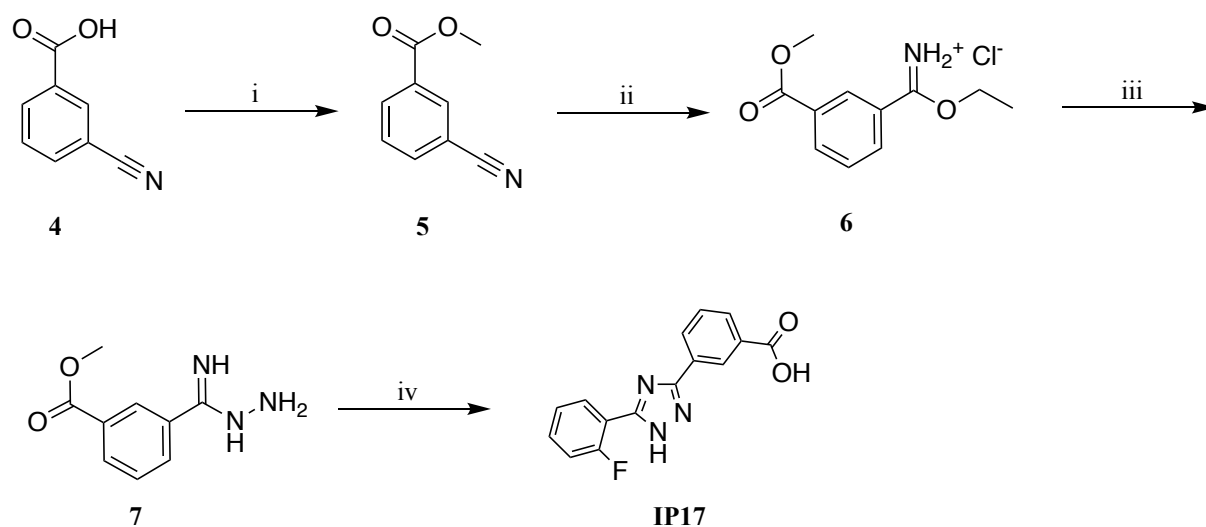
To this set, IP18, IP19 and IP20, which were identified by the pharmacophore model and represent functionalizations not previously explored in TRIDs were synthesised as described in Scheme 3.1.3 and 3.1.4. The pharmacophore model also identified the triazolic analogues of PTC124, which were synthesized as reported in Scheme 3.1.5.



Scheme 3.1.3. Reagents and conditions: (i) 4-methylbenzaldehyde, DMAP, MeOH, reflux, 24 h.



Scheme 3.1.4. Reagents and conditions: (i) N_2H_4 , DMF, r.t, 2 h.

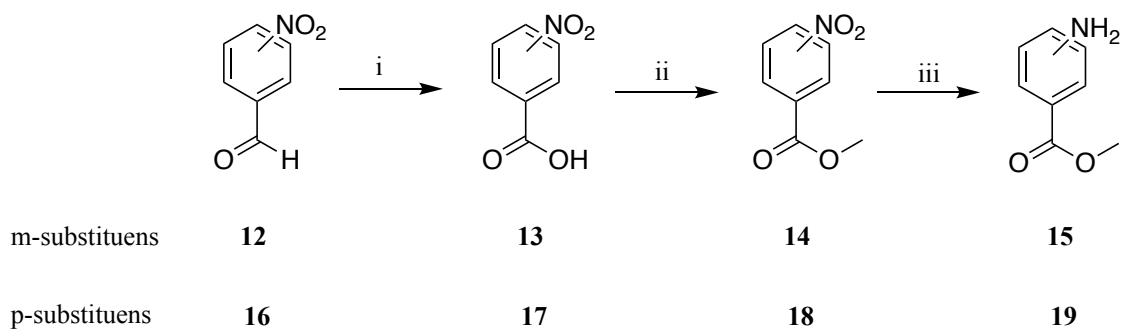


Scheme 3.1.5. Reagents and conditions: (i) MeI, K_2CO_3 , DMF, r.t, 2 h; (ii) EtOH/HCl, r.t, 12 h; (iii) N_2H_4 , Py, r.t, 2h; (iv) 2-fluorobenzoyl chloride, toluene, reflux, 12h.

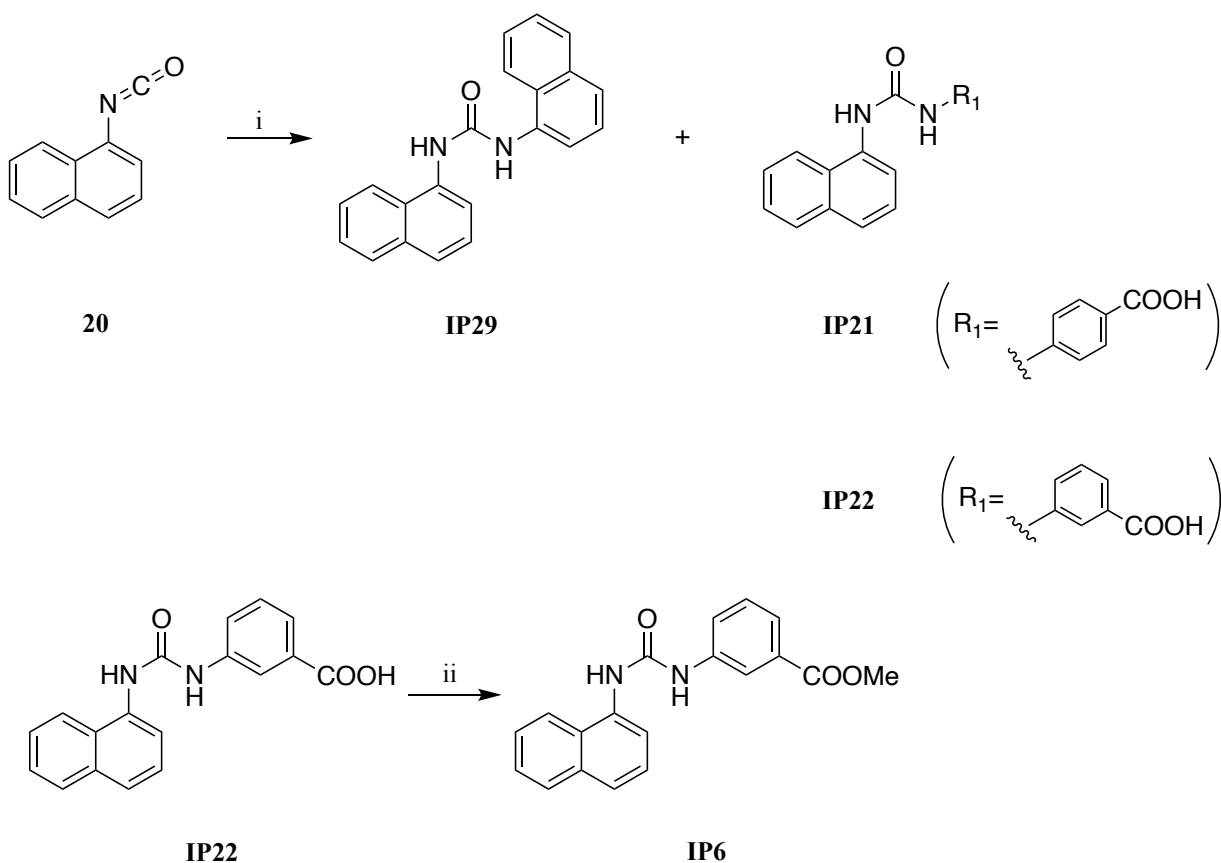
Six diaryl urea derivatives suggested by Model 1 were synthesized to investigate the effect of the relative substituent position of the naphthyl and phenyl groups on TR activity. Methyl-4-aminobenzoate and methyl 3-aminobenzoate were synthesised starting from the respective nitrobenzaldehyde (Scheme 3.1.6) in order to prepare the corresponding urea derivatives. Two synthetic strategies were employed: the 1-naphthyl-substituted derivatives were obtained by direct use of 1-naphthyl isocyanate (Scheme 3.1.7), while the 2-naphthyl-substituted derivatives were synthesized via a Curtius rearrangement of 2-naphthoic acid (Scheme 3.1.8).

In addition, two byproducts arising from the dimerization of naphthyl isocyanate into urea dimers were also isolated and included in the biological evaluation.

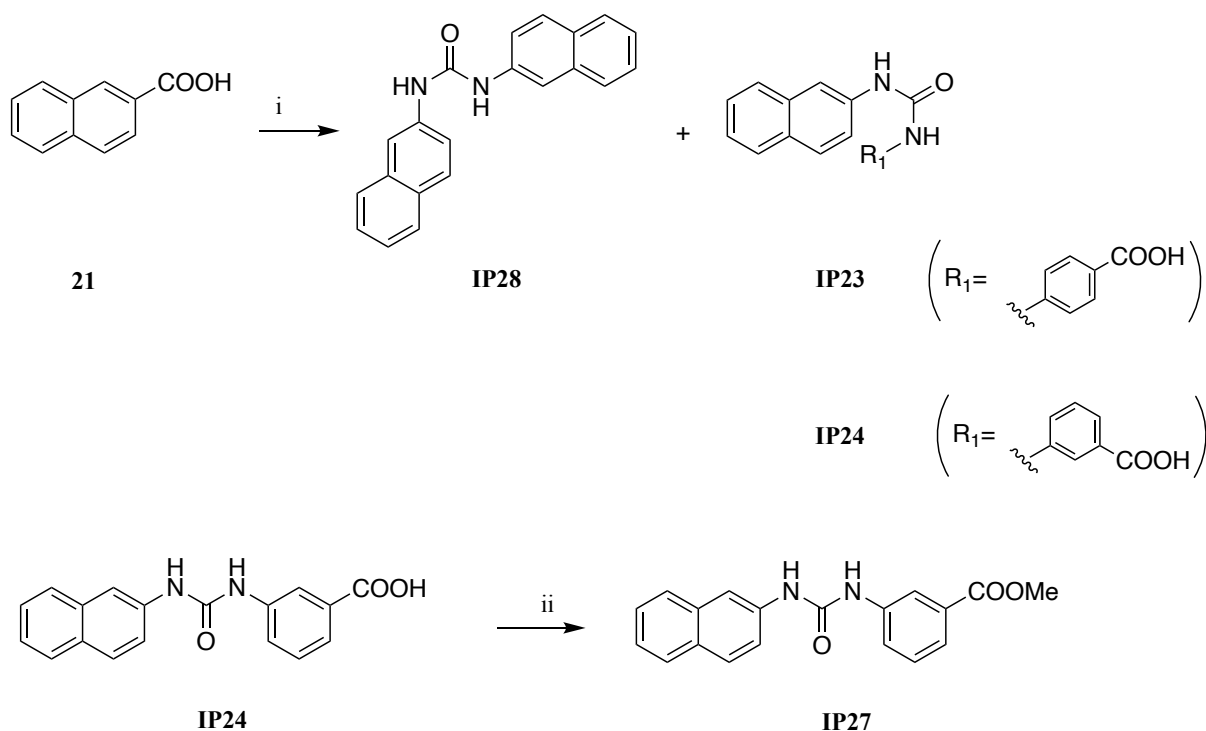
In the synthesis of diaryl urea derivatives, additional steps of hydrolysis and esterification were required to facilitate purification of the final products.



Scheme 3.1.6. Reagents and conditions: (i) AgNO_3 , KOH_{aq} (7%), H_2O , 60°C , 2h; (ii) MeOH , H_2SO_4 (1%), reflux, 16h; (iii) NaBH_4 , Ni_2B (mol 5%), H_2O , r.t., 2h.



Scheme 3.1.7. Reagents and conditions: (i) 1. methyl 4-aminobenzoate or methyl 3-aminobenzoate, dry THF, $0^\circ\text{C} \rightarrow \text{r.t.}$, 24 h. 2. LiOH_{aq} (1M)/ MeOH (1:1), 60°C , 16h; (ii) MeOH , H_2SO_4 (1%), reflux, 72h.

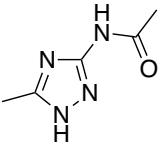
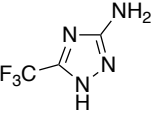
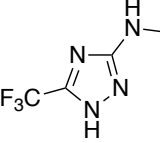
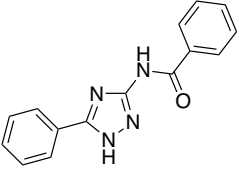
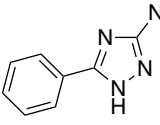
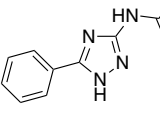
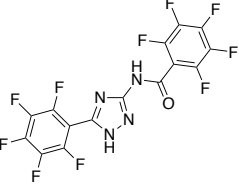
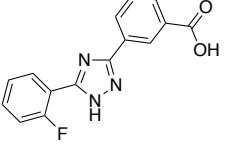
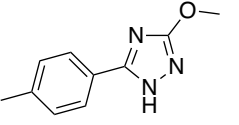
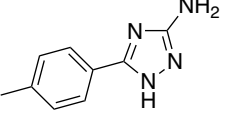


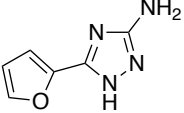
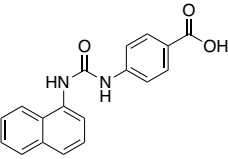
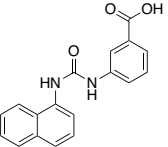
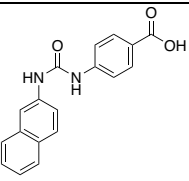
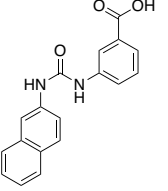
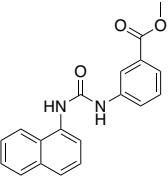
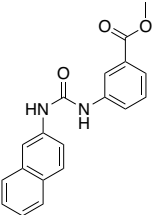
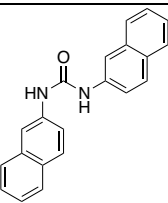
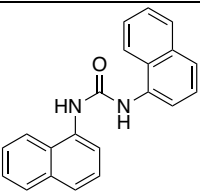
Scheme 3.1.8. Reagents and conditions: (i) 1. methyl 4-aminobenzoate or methyl 3-aminobenzoate, DPPA, toluene (dry), 100° C, m.w, 10min. 2. LiOH_{aq} (1M)/MeOH (1:1), 60° C, 16h; (ii) MeOH, H₂SO₄ (1%), reflux, 72h.

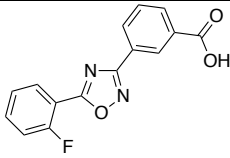
Together, the triazole and diaryl urea families provide complementary scaffolds that expand the chemical space of TRIDs, offering new opportunities for structure–activity relationship exploration and potential lead optimization.

To assess the efficacy of the newly synthesized small molecules in promoting translational readthrough of premature termination codons, we employed a qualitative firefly luciferase (FLuc) reporter assay. HeLa cells were transiently transfected with either the pFLuc-WT plasmid, used as a positive control, or the pFLuc-opal plasmid carrying a UGA stop codon mutation. Twenty-four hours after transfection, cells were treated with the synthesized compounds at a concentration of 12μM, while PTC124 was included as a reference TRID control. FLuc expression was quantified 24h post-transfection as normalized luminescence relative to untreated controls (NLU). Fluc assay were conducted in duplicate. As expected, cells transfected with pFLuc-WT exhibited robust luciferase activity (x70 NLU), whereas untreated pFLuc-opal cells displayed only low background activity, indicating a scarce basal level of TR. Treatment of pFLuc-opal cells with PTC124 or the lead compounds resulted in a significant increase in luciferase activity, particularly in five 1H-1,2,4-triazole derivatives and three diaryl urea derivatives, compared with untreated controls as shown in Table 3.1.1. Although qualitative, this assay provides a convenient and reliable screening method for the preliminary identification of readthrough-promoting molecules.

Table 3.1.1. Cell-based readthrough-inducing activity in HeLa cells transfected with the pFLuc-opal reporter. Compounds were tested at the indicated concentrations, and activity is expressed as the ratio relative to untreated controls.

ID.	IUPAC Name	Structure	NLU (12μM)	t-test significance (p<0,05)
IP8	<i>N</i> -(5-methyl-1 <i>H</i> -1,2,4-triazol-3-yl)acetamide		x1,33	No
IP10	5-(trifluoromethyl)-1 <i>H</i> -1,2,4-triazol-3-amine		x1,54	No
IP11	<i>N</i> -(5-(trifluoromethyl)-1 <i>H</i> -1,2,4-triazol-3-yl)acetamide		x1,30	No
IP12	<i>N</i> -(5-phenyl-1 <i>H</i> -1,2,4-triazol-3-yl)benzamide		x1,30	No
IP13	5-phenyl-1 <i>H</i> -1,2,4-triazol-3-amine		x1,81	Yes
IP14	<i>N</i> -(5-phenyl-1 <i>H</i> -1,2,4-triazol-3-yl)acetamide		x1,58	Yes
IP15	2,3,4,5,6-pentafluoro- <i>N</i> -(5-(perfluorophenyl)-1 <i>H</i> -1,2,4-triazol-3-yl)benzamide		x1,66	Yes
IP17	3-(5-(2-fluorophenyl)-1 <i>H</i> -1,2,4-triazol-3-yl)benzoic acid		x1,57	Yes
IP18	3-methoxy-5-(<i>p</i> -tolyl)-1 <i>H</i> -1,2,4-triazole		x1,74	Yes
IP19	5-(<i>p</i> -tolyl)-1 <i>H</i> -1,2,4-triazol-3-amine		x1,34	No

IP20	5-(furan-2-yl)-1H-1,2,4-triazol-3-amine		x1,31	No
IP21	4-(3-(naphthalen-1-yl)ureido)benzoic acid		x1,43	Yes
IP22	3-(3-(naphthalen-1-yl)ureido)benzoic acid		x1,28	No
IP23	4-(3-(naphthalen-2-yl)ureido)benzoic acid		x1,29	No
IP24	3-(3-(naphthalen-2-yl)ureido)benzoic acid		x2,07	Yes
IP6	methyl 3-(3-(naphthalen-1-yl)ureido)benzoate		x1,33	No
IP27	methyl 3-(3-(naphthalen-2-yl)ureido)benzoate		x1,66	Yes
IP28	1,3-di(naphthalen-2-yl)urea		x1,22	No
IP29	1,3-di(naphthalen-1-yl)urea		x1,31	No

PTC1 24	3-(5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl)benzoic acid		x6,6	Yes
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Analysis of the tested 1H-1,2,4-triazole derivatives indicates that substitution at the C-5 position plays a critical role in modulating readthrough activity. Compounds bearing phenyl or extended aromatic substituents at this position (e.g., IP13, IP14, IP15, IP17, IP18) exhibited higher activity than unsubstituted or aliphatic analogues, underscoring the importance of π - π stacking and hydrophobic interactions. Substituents capable of functioning as hydrogen bond acceptors at C-5, such as perfluorophenyl or other heteroaryl groups, were beneficial in some cases, suggesting that directional hydrogen bonding may contribute alongside aromatic stacking to activity. In addition, the presence of a hydrogen bond acceptor at the C-3 substituent consistently correlated with activity, further supporting the role of acceptor interactions in enhancing ribosomal binding.

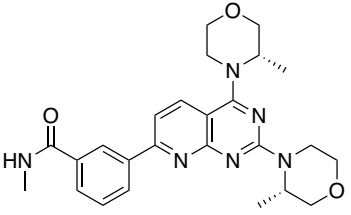
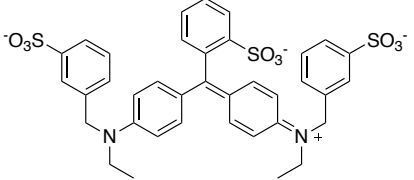
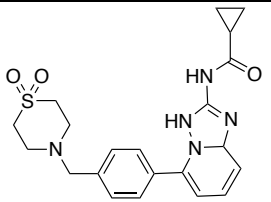
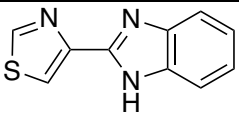
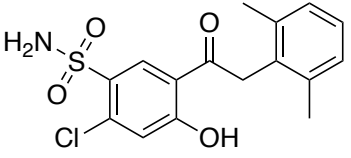
Taken together, these observations suggest that bulky aromatic groups at C-5, combined with hydrogen bond acceptor functionality either at C-5 or C-3, enhance TR-inducing activity. Moreover, pairing C-5 aromatic substituents with a hydrogen bond donor may provide complementary interactions to further improve efficacy.

For diaryl urea derivatives, the substitution pattern and the nature of the aromatic rings strongly influenced TR activity. Analogues carrying 2-naphthyl substituents (IP24, IP27) were more active than those bearing 1-naphthyl substituents, suggesting that the extended π -surface and planarity of the 2-naphthyl group provide more favourable interactions for TR activity. Furthermore, compounds with substituents in the meta-position of the phenyl ring were more active than their para-substituted counterparts (e.g., IP21), highlighting the role of substitution geometry in optimizing molecular orientation for binding. Symmetric diaryl ureas (IP28, IP29) exhibited little or no activity, underscoring the importance of scaffold asymmetry and the contribution of hydrogen bond donor or acceptor groups on the phenyl rings to productive binding. Taken together, these results indicate that extended aromatic moieties, asymmetric substitution, and carbonyl meta-substitution on the phenyl rings, in particular with 2-naphthyl groups, confer advantages for TR-inducing activity in diaryl ureas.

Across different scaffolds, a recurring leitmotif emerges: the presence of a heteroatom-rich core, such as 1H-1,2,4-triazole, urea, or 1,2,4-oxadiazole, capable of forming strong directional interactions. This structural leitmotif appears fundamental for enabling small molecules to act as translational readthrough inducers.

Moreover, the pharmacophore model was applied to screen the ZINC database, which contains millions of drug-like, purchasable compounds, including FDA-approved molecules. This screening yielded 54 hits ; the FDA-approved compounds with potential for drug repurposing are reported here (table 3.1.2), while the complete list is provided in Table A4 of Appendix A

Table 3.1.2 FDA-approved compounds with the highest fit scores to the pharmacophore model. Their current approved clinical indications are reported, highlighting their potential suitability for drug repurposing as candidate translational readthrough-inducing drugs (TRIDs).

Name	Structure	Actual approved use
Vistusertib		dual mTORC1/2 inhibitor developed by AstraZeneca as an anticancer agent.
Bludigo		Agonist of the alpha-adrenergic receptor, guanylyl cyclase inhibitor, used as a diagnostic agent. ¹⁹⁰
Filgotinib		Janus kinase 1 inhibitor used in the treatment of rheumatoid arthritis. ¹⁹¹
Thiabendazole		Inhibitor of fumarate reductase flavoprotein subunit, used as an antiparasitic agent. ¹⁹²
Xipamide		Decreases sodium reabsorption in the kidney, used as a diuretic for the treatment of edema. ¹⁹³

3.2 TR based treatment for the recovery of TP53 in cancer treatment

We assessed the potential of newly synthesized 1H-1,2,4 triazole derivatives described in section 2.1 in TR for the recovery on nonsense mutated p53.

Four 1H-1,2,4-triazole derivatives were selected for their synthetic accessibility and to allow a systematic comparison of the triazole core with the 1,2,4-oxadiazole scaffold of PTC124, the reference compound and the only EMA-approved TRID, in order to evaluate the impact of core substitution on TR activity.

To assess the potential of newly synthesized 1H-1,2,4 triazole derivatives as TRIDs, we first performed *in vitro* toxicity testing four selected compounds, IP14, IP15, IP17 and IP18 through two independent experiments: cell counts at 24, 48, and 72 hours of treatment, and MTT assay. In this context, early-stage *in vitro* toxicological screening, a crucial step in candidate drug evaluation, revealed no detectable toxicity in two independent assays for any of the newly synthesized compounds.

Cells treated with the compounds showed no loss of viability or proliferation at any time point, with results comparable to controls (Fig. 3.2.1a). These findings were confirmed by MTT assay, which showed no significant change in metabolic activity (Fig. 3.2.1b). The absence of cytotoxicity at the tested concentrations supports the further evaluation of these triazole derivatives for TR activity.

To assess the ability of the novel triazole derivative to restore expression of a nonsense-mutated p53 protein, H1299 lung adenocarcinoma cells stably expressing the p53^{R213X} variant were treated with 12 μ M of the compound for 24 hours. DMSO was used as a negative control, while G418 (300 μ g/ml) and Ataluren (12 μ M) served as positive controls for translational readthrough. HCT116 cells were included as a positive control for endogenous p53 expression, and H1299 p53-null cells (p53^{-/-}) were used as baseline controls for the reporter system.

Immunofluorescence analysis, after 24 hours treatment, revealed that HCT116 cells exhibited expression of p53, while both H1299 p53^{-/-} and H1299 p53^{R213X} cells showed no detectable signal, confirming the specificity and reliability of the reporter system (Fig. 3.2.1c).

In the same conditions, treatment with the 1H-1,2,4-triazole derivatives led to a significant increase in p53 fluorescence in H1299 p53^{R213X} cells, with both cytoplasmic and nuclear localization, consistent with the induction of full-length p53 protein (Fig. 3.2.1d). Overall, fluorescence analysis confirmed that all tested compounds effectively induced TR.

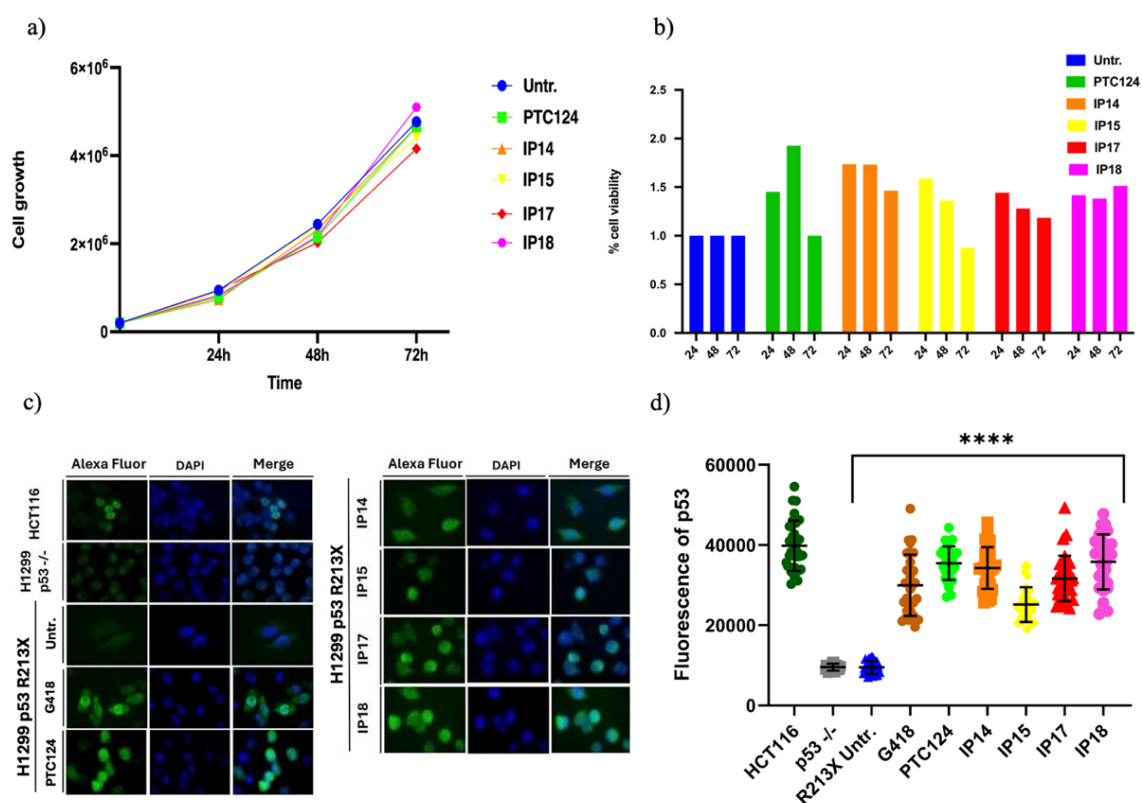


Figure 3.2.1. in vitro toxicity and preliminary evaluation of translational readthrough (TR) activity. **a)** Cell growth quantification using the Trypan Blue exclusion assay in HCT116 cells treated with newly synthesized 1,2,4-triazole (12 μ M) derivatives and PTC124 (12 μ M) as control. **b)** Cell viability assessment using the MTT assay under the same treatment conditions. **c)** Immunofluorescence staining for p53 protein (green) and nuclei (DAPI, blue) in control cell lines (HCT116, positive control; H1299 p53^{-/-} and untreated H1299 p53^{R213X}, negative controls and in H1299 p53^{R213X} cells treated with triazole derivatives (12 μ M), PTC124 (12 μ M) and G418 (300 μ g/ml) (readthrough control). **d)** Quantification of p53 fluorescence intensity (mean $\pm$ SD) in H1299 p53^{R213X} cells treated with triazoles, compared to negative controls (H1299 p53^{-/-}, blue; H1299 p53^{R213X} untreated, light blue), positive control (HCT116, dark green), and readthrough control (G418, brown). Statistical significance was determined using one-way ANOVA on 30 cells per condition (experiments performed in duplicate) compared to H1299 p53^{R213X} untreated cells. Fluorescence quantification was carried out using ImageJ software.

Notably, IP14, IP15, IP17, and IP18 demonstrated p53 levels comparable to G418 and Ataluren, making them strong candidates for further development.

We next investigated whether the triazole-based compounds (IP14, IP15, IP17, and IP18) could restore not only the expression of p53 protein but also its functional responsiveness to DNA damage induction. To this end, H1299 p53^{R213X} cells were treated with each compound, under the same condition as above, in combination with the DNA-damaging agent doxorubicin (0.2

µg/ml). HCT116 cells served as positive control, H1299 p53^{-/-} and untreated H1299 p53^{R213X} cells served as negative controls, respectively.

Following 24h co-treatment, all four triazoles induced a marked increase in full-length p53 levels in H1299 p53^{R213X} cells, comparable to levels observed with G418 and HCT116 cells (Fig. 3.2.2a, 3.2.2b). No p53 expression was detected in H1299 p53^{-/-} and untreated H1299 p53^{R213X} cells confirming the specificity of the response and the reliability of the model. Immunofluorescence analysis revealed a significant increase in nuclear fluorescence intensity following DNA damage induction ($p < 0.001$), indicative of p53 accumulation in the nucleus and suggesting reactivation of its transcriptional function and stress responsiveness activity (fig. 3.2.2c).

Triazole-treated cells exhibited a more pronounced and statistically significant increase in nuclear p53 fluorescence intensity after doxorubicin treatment, compared to G418 and Ataluren-treated cells, which maintained consistently high nuclear p53 levels regardless of treatment (fig. 3.2.2d).

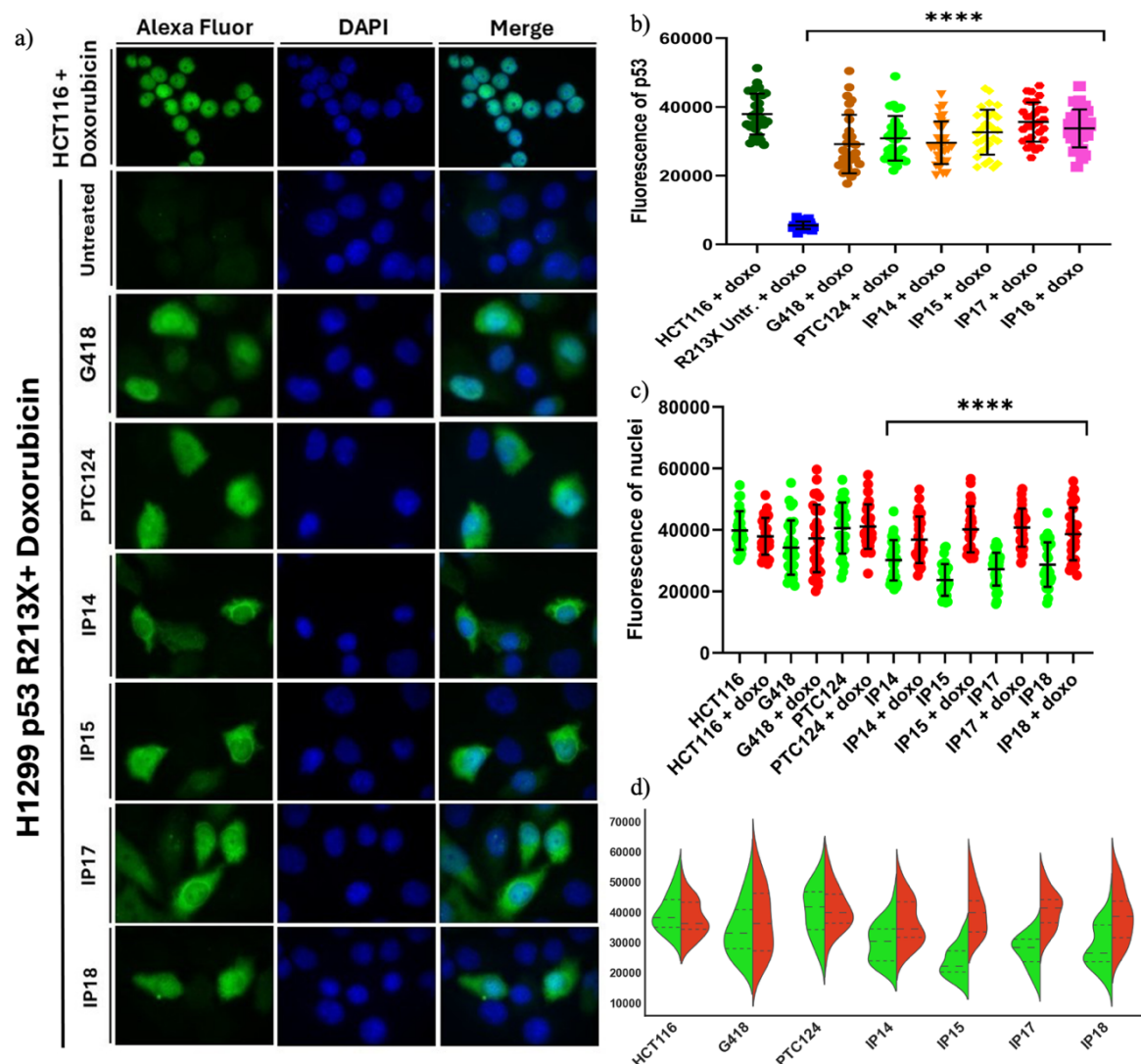


Figure 3.2.2. p53 immunofluorescence for the evaluation of stress responsiveness. **a)** Immunofluorescence staining of p53 (green) and nuclei (DAPI, blue) in control cell lines (HCT116, positive control; H1299 p53^{-/-} and untreated H1299 p53^{R213X}, negative controls) and in H1299 p53^{R213X} cells treated with triazole(12μM) derivatives, PTC124 (12μM) and G418 (300μg/ml) (readthrough control) in combination with doxorubicin to induce DNA damage. **b)** Quantification of total p53 fluorescence intensity (mean ± SD) in H1299 p53^{R213X} cells treated with triazoles, positive control (HCT116, dark green), and readthrough control (G418, brown), all co-treated with doxorubicin, compared to negative controls (H1299 p53^{R213X} untreated, blue). **c)** Comparison of nuclear p53 fluorescence intensity in H1299 p53^{R213X} cells treated with triazoles, G418, or HCT116 before (green) and after (red) induction of DNA damage with doxorubicin. **d)** Violin plot showing the distribution of nuclear p53 fluorescence intensity in H1299 p53^{R213X} cells and HCT116 cells, treated with triazoles, G418, or vehicle control, before (green) and after (red) induction of DNA damage with doxorubicin.

These findings were independently validated by Western blotting. In untreated H1299 p53^{R213X} cells, only a weak band corresponding to full-length p53 was observed, likely due to a physiological basal level of readthrough, along with a prominent ~23 kDa band representing

the truncated form of the protein (Fig 3.2.3a). Treatment with IP14, IP15, IP17, or IP18 enhanced full-length p53 expression significantly, especially upon DNA damage, confirming recovery of both protein expression and stress responsiveness compared to that observed after treatment with G418 (Fig 3.2.3b).

In contrast to the immunofluorescence results, in WB assay PTC124 treatment seems not to lead to a significant restoration of p53 expression (Fig 3.2.3b). This discrepancy may be explained by the use of a primary antibody in immunofluorescence that recognizes an N-terminal epitope of p53, while the R213X mutation is located in the C-terminal region. As a result, the antibody can detect both the truncated and the full-length protein. Additionally, differences in antibody sensitivity between immunodetection techniques may contribute to the observed variation.

Overall, induction of DNA damage with doxorubicin, treatment with these compounds enhanced the expression of full-length p53, confirming their capacity to restore stress-induced p53 responsiveness. Notably, IP17 and IP18 induced a more significant increase in p53 levels. Moreover, following genotoxic stress, a significant increase in nuclear p53 localization, visualized through immunofluorescence analysis was observed only in IP treated cells, indicating both successful rescue of the full-length protein and recovery of its stress responsiveness activity.

Finally, to assess p53 functionality, we performed a Real Time RT-PCR analysis of the canonical p53 targets CDKN1A¹⁹⁴ (p21) and GADD45A¹⁹⁵. In this context, surprisingly, p21 transcript levels were elevated not only in G418-treated cells but also in untreated and non-transfected controls (Fig 3.2.3c), suggesting p53-independent activation of CDKN1A and limiting its value as a readthrough-specific marker. In contrast, GADD45A showed a clear and significant increase after treatment with G418 and IP compounds, compared to untreated cells (Fig 3.2.3d). Moreover, DNA damage further enhanced GADD45A expression in IP- plus doxorubicin-treated p53^{R213X} cells, reaching levels comparable to G418 co-treatment.

G418 is known to induce oxidative stress¹⁹⁶ and promote p53 stabilization and target gene activation, such as CDKN1A, independently of both DNA damage stimuli and potentially even p53 itself, as evidenced by p21 activation in untreated and non-transfected cells. This was further confirmed by immunofluorescence, where elevated p53 signals were detected even before DNA damage-induced stress, complicating the interpretation of p21 induction as a marker of TR-mediated p53 recovery versus a general stress response.

Moreover, p21 expression is indirectly supported by the enhanced expression of GADD45A, as p21 acts as a cofactor together with p53 in its transcriptional activation¹⁹⁷.

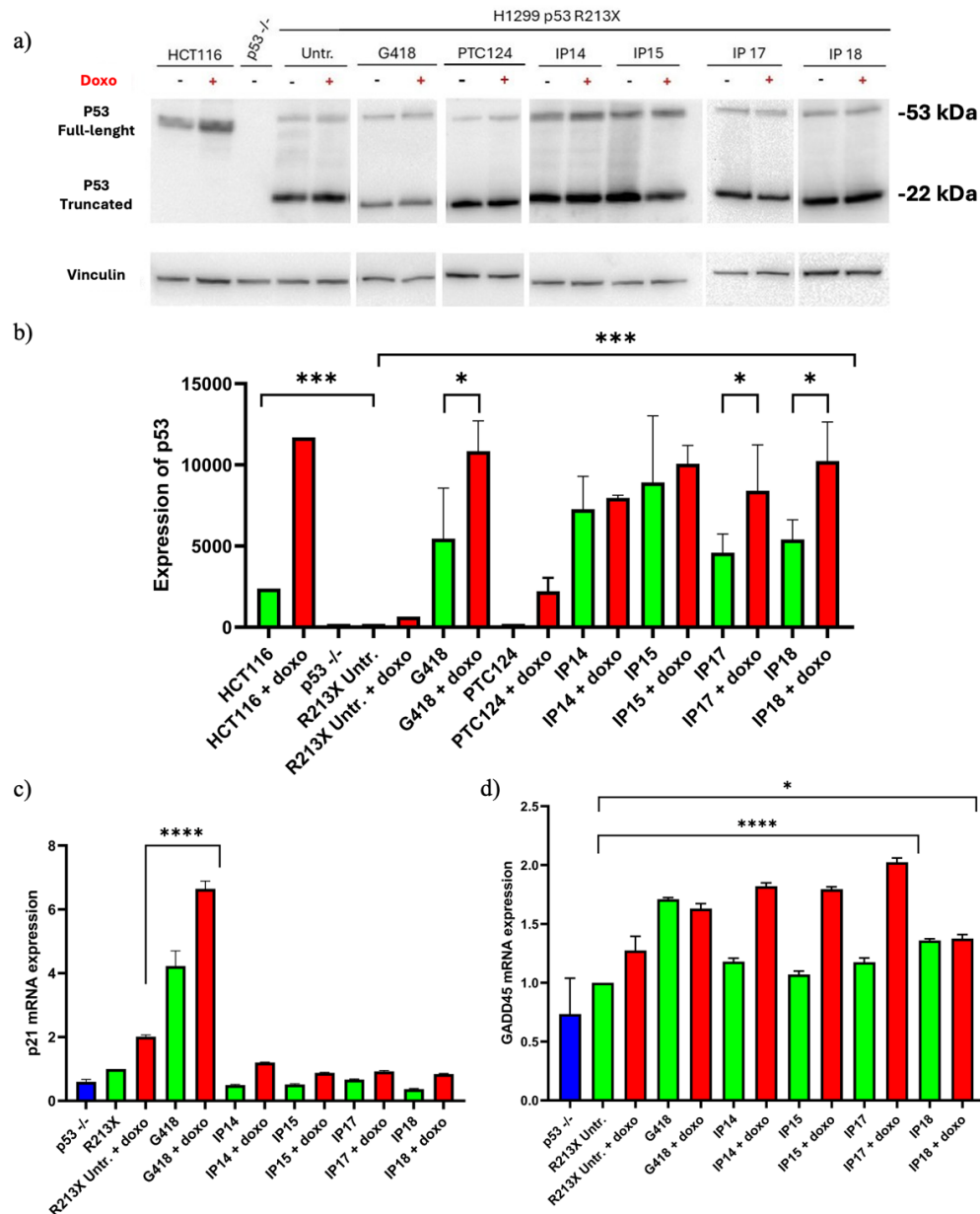


Figure. 3.2.3 p53 Western blotting quantification and qRT-PCR of genes target for functionality recovery evaluation. a) Western blot analysis of p53 expression in control cell lines (HCT116, positive control; H1299 $p53^{-/-}$ and untreated H1299 $p53^{R213X}$, negative controls) and in H1299 $p53^{R213X}$ cells treated with triazole derivatives (12 μ M), PTC124 (12 μ M) and G418 (300 μ g/ml) (readthrough control), alone or in combination with doxorubicin to induce DNA damage. **b)** Quantification of p53 protein levels (mean $\pm$ SD) by densitometry from Western blot analysis in controls and in H1299 $p53^{R213X}$ cells treated with triazoles and doxorubicin. Protein levels were normalized to the housekeeping protein vinculin. **c)** qRT-PCR analysis of CDKN1A (p21) transcript expression (mean $\pm$ SD) in control cell lines HCT116, positive control; H1299 $p53^{-/-}$ (blue), untreated H1299 $p53^{R213X}$, negative controls; H1299 $p53^{R213X}$ G418 and Ataluren-treated, readthrough control, and in H1299 $p53^{R213X}$ cells treated with triazole derivatives before (green) and after (red) induction of DNA damage with doxorubicin. **d)** qRT-PCR analysis of GADD45A transcript expression (mean $\pm$ SD) under the same conditions as in panel c. Statistical significance was calculated using one-way ANOVA (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$).

IP-treated cells, however, maintained low basal p21 expression while retaining the ability to induce it upon DNA damage. Importantly, despite being tested at lower concentrations, IP compounds significantly upregulated GADD45A both before and after DNA damage, supporting their role in restoring p53-dependent transcription.

This conclusion is further substantiated by the absence of known tumor-associated missense mutations at TP53 codon 213, suggesting that random amino acid incorporation via readthrough at this site is unlikely to impair p53 activity.

Having demonstrated the biological efficacy of these triazoles, we proceeded to evaluate *in silico* their pharmacokinetic and *in vivo* toxicological profiles. ADMET properties were assessed using SwissADME¹⁹⁸, and ADMETlab 3.0¹⁹⁹ analyzing general and specific parameters such as lipophilicity, water solubility, gastrointestinal absorption, blood–brain barrier permeability, and Lipinski's rule compliance in order to have a comprehensive overview on predicted properties for future development. All compounds displayed comparable pharmacokinetic profiles, with the exception of IP15, which exhibited suboptimal absorption predictions (Table 3.2.1).

Table 3.2.1. In-silico pharmacokinetic properties for IP14, IP15, IP17 ed IP18.

PROPERTIES	PARAMETERS	IP14	IP15	IP17	IP18
	MW (g/mol)	202.21	444.19	281.28	189.21
	Heavy atoms	15	30	21	14
	Arom. Heavy atoms	11	17	17	11
	Rotable bonds	3	4	3	2
	H-bonds acceptors	3	13	4	3
	Molar Refractivity	56.13	75.61	77.41	53.28
Lipophilicity	Log Po/w	0.89	1.10	1.93	1.68
Water soluble	Log S (ESOL)	-2.17	-5.16	-3.93	-2.92

Pharmacokinetics	GI absorption	High	Low	High	High
	BBB permeation	Yes	No	Yes	Yes
	P-glycoprotein substrate	Likely	Likely	Likely	Likely
	Major CYP substrates	CYP2C9	-	-	CYP1A2, CYP2C9
	Major CYP inhibitors	CYP1A2, CYP2C8	CYP2C9, CYP2C19, CYP2C8	CYP2C9, CYP2C8	CYP1A2, CYP2C19, CYP2C8
	Plasma clearance (ml/min/kg)	4.395	4.666	0.568	7.034
	Half-life (t _{1/2} , h)	1.297	1.163	1.22	0.996
Drug likeness	Lipinski violation	0 violations	1 MLOGP>4,15	0 violation	0 violations

Toxicity assessments using the ProTox 3.0²⁰⁰ platform predicted that IP14, IP15, IP17, and IP18 belong to toxicity class 4, with relatively high LD50 values and no major alerts for organ or systemic toxicity endpoints (Table 3.2.2). These findings support the potential of these compounds as viable, low-toxicity therapeutic candidates.

Table 3.2.2. *In-silico toxicity properties for IP14, IP15, IP16, IP17.*

ENDPOINT		TARGET	IP 14	IP 15	IP 17	IP 18
Predicted	LD50		1000	2000	1000	580
		(mg/kg)				
Predicted	Toxicity		4	4	4	4
	Class					

Predicted accuracy (%)		67,38	54,26	54,26	67,38
Organ Toxicity	Hepatotoxicity	Active	Active	Active	Active
	Nephrotoxicity	Light Active	Inactive	Active	Inactive
	Respiratory toxicity	Light Active	Active	Inactive	Inactive
	Cardiotoxicity	Inactive	Inactive	Inactive	Inactive
Toxicity Endpoint	Carcinogenicity	Active	Light Active	Inactive	Inactive
	Mutagenicity	Inactive	Inactive	Inactive	Inactive
	Cytotoxicity	Inactive	Inactive	Inactive	Inactive
	Ecotoxicity	Inactive	Light Active	Inactive	Inactive
	Clinical toxicity	Active	Light Active	Light Active	Inactive

3.3 *in vivo* proteomic evaluation of translational readthrough therapy with next generation TRID NV848 for CFTR recovery in mice model non-canonical target organs: pancreas, brain, and liver

In previous studies conducted by our research group, NV848 has emerged as a promising candidate for further development in the treatment of cystic fibrosis caused by nonsense mutations. (nmCF)^{169,174,176}.

In this context, proteomic analysis of NV848-based therapy in *in vivo* models represents a powerful and innovative strategy to evaluate its therapeutic efficacy. By capturing global protein-level changes, proteomic profiling offers a comprehensive view of how NV848-based treatment reshapes key biological processes, hallmark of CF, including protein biosynthesis, trafficking, redox homeostasis, and inflammatory signalling, in an *in vivo* model of nonsense-mutated CFTR^{201,202}.

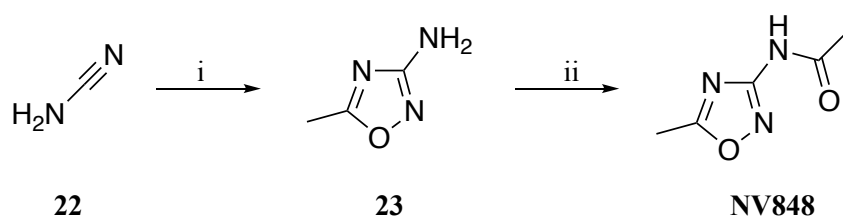
To our knowledge, this constitutes the first attempt to integrate translational readthrough therapy with a proteome-wide analysis in a mammalian model of nmCF. The resulting data not only validate NV848's therapeutic potential but also generate novel insights into the broader functional consequences of CFTR rescue at the systemic level, thereby offering a foundation for future translational studies and the rational design of combinatorial therapies. Moreover, these analyses can offer novel insights into Ataluren's efficacy, used as a benchmark, and uncover new CFTR-dependent metabolic pathways.

To this aim, at the laboratories of NOVA University of Lisbon under the supervision of Prof. L. Capelo-Martinez and Prof. H.M. Santos, we analysed the proteomics of pancreas, liver, and brain of wild-type (WT) mice and CF mouse models carrying G542X nonsense mutations (Mut), untreated, treated with NV848, and treated with PTC124, this last used as a comparative benchmark for TR.

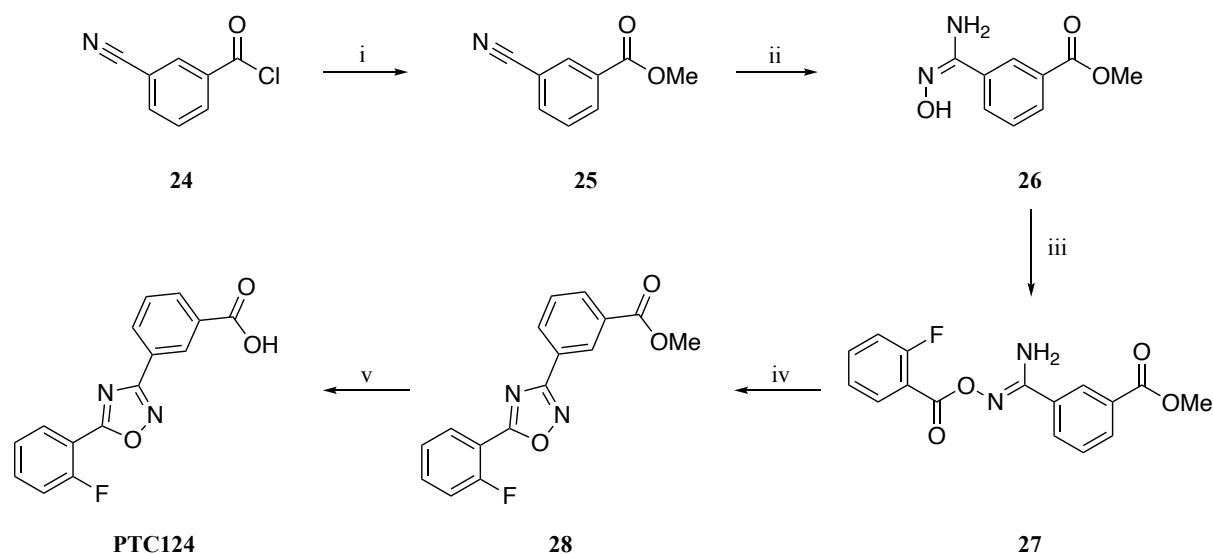
Although the involvement of the pancreas and liver in CF is well recognized, the precise role of CFTR in these organs remains poorly understood. The brain, in contrast, has not been explored to date, as current research efforts have largely focused on the lungs and intestine, the organs most directly responsible for CF morbidity and mortality in humans. However, as life expectancy in CF has risen markedly in recent decades, from a median age at death of ~24 to ~37 years²⁰³, previously overlooked manifestations are now emerging in noncanonical organs. In this context, proteomic investigation of the pancreas, liver, and brain provides an opportunity to uncover novel aspects of CF pathology and expand our understanding of CFTR function beyond the classical tissues.

The organs were kindly provided by Prof. Zizzo's Animal Facility Lab at the Aten Center – University of Palermo, using both WT and MutG542X mice, in which CFTR expression recovery in the lungs following treatment with NV848 and PTC124 had previously been demonstrated, as reported in an earlier published study¹⁷⁵.

Briefly, following genotyping, mice were treated starting at day 21 after birth with a 15-day of chronic oral administration of NV848 or PTC124 at 60 mg/kg, a dose well below reported safety limits. Both NV848 (Scheme 3.3.1) and PTC124 (Scheme 3.3.2) used for mice treatment were freshly synthesized according to previously reported methodologies¹⁶⁹.



Scheme 3.3.1 Reagents and conditions: (i) 1. Ac_2O , AcOK , acetone, r.t., 24 h; 2. $\text{NH}_2\text{OH}\cdot\text{HCl}$, pyridine, EtOH (abs), r.t., 24 h; (ii) Ac_2O , AcOK , 40 °C, 24 h.



Scheme 3.3.1 Reagents and conditions: (i) MeOH , TEA , r.t., 24h; (ii) $\text{NH}_2\text{OH}\cdot\text{HCl}$, Na_2CO_3 , EtOH_{abs} , r.t., 16h; (iii) 2-fluorobenzoyl chloride, TEA , THF_{dry} , r.t., 1h; (iv) toluene, reflux, 1h; (v) NaOH_{aq} (1.5 M), THF , r.t., 16h.

The mice analysed were of mixed sex, all fed the same diet, and maintained under identical housing conditions. A minimum of three mice per experimental condition were included, with a detailed list provided Appendix B.

Organs from treated and untreated mice, both wild-type and G542X mutants, were collected and immediately frozen at -80 °C. Freshly frozen organs were mechanically homogenized, and

protein extraction was performed using ultrasonication-assisted, surfactant-aided lysis (fig. 3.3.1a).

The extracted proteins were digested using the eFASP methodology with an enzymatic cocktail of Lys-C and trypsin. Peptide mixtures were then analyzed by high-resolution tandem nanoLC-MS. Spectral data were processed using machine learning-based de novo sequencing in PEAKS software, and protein abundances were quantified by Label-Free Quantification (LFQ) analysis to assess relative changes in expression across experimental conditions (fig. 3.3.1b).

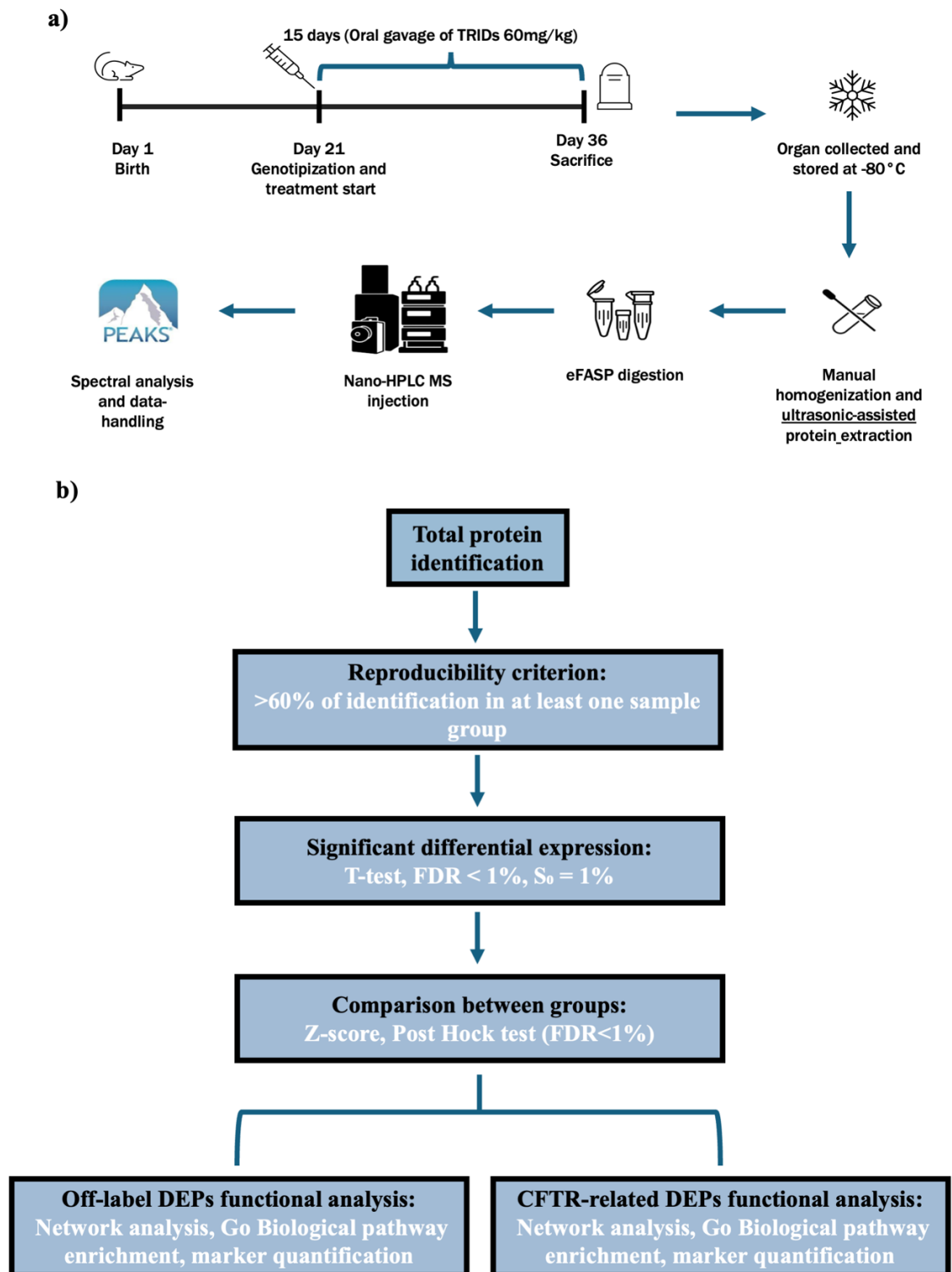


Figure 3.3.1 Sample preparation and statistical analysis. a) Schematic overview of mice growth, treatment, tissue preparation, and protein extraction and digestion followed by nanoLC-MS/MS analysis; b) Statistical proteomic workflow used for the identification of differentially expressed proteins (DEPs) from off-label treatment and CFTR-related analysis.

Pancreas

Pancreatic involvement and functional impairment in CF have been recognized since 1938, when Andersen first documented them⁸⁷. CFTR is expressed in islet duct β -cells and in the apical luminal membrane of acinar cells, where it is fundamental for $\text{Cl}^-/\text{HCO}_3^-$ homeostasis across the membrane. This process depends on a two-step mechanism involving SLC26A6 and is involved into Ca^{2+} regulation through adenylyl cyclase 6 (AC6)²⁰⁴.

Loss of CFTR function impairs both HCO_3^- and Ca^{2+} transport, leading to defective ductal secretions and obstruction, epithelial injury caused by premature pancreatic enzyme activation, and progressive tissue destruction accompanied by inflammation, chronic cytokine exposure, fibrosis, and fatty infiltration or replacement and islet shrinkage^{205,206}. Overall, this effect results in chronic pancreatic inflammation, ultimately impairing insulin secretion and predisposing to diabetes and pancreatitis.

Diabetes is a common comorbidity in CF, referred to as CFRD. It occurs in 40-50% of adult patients and in up to 85% of those with class I CF mutations. CFRD is distinct from both type 1 and type 2 diabetes, as it combines β -cell loss with varying degrees of insulin resistance²⁰⁷. Pancreatitis is a less frequent condition, with a prevalence of about 1.9%, but CFTR mutations increase its incidence up to threefold compared to healthy individuals²⁰⁸.

In this context, we evaluated the recovery of CFTR in the pancreas through proteomic analysis to validate the TR activity of NV848 and to identify novel protein interactions associated with CFTR loss of function. For this purpose, we examined pancreatic tissue from both WT and G542X mice, untreated and treated with NV848, using PTC124 as a comparative control for TR for a total of six conditions.

While most murine models develop only mild exocrine pancreatic disease compared to humans, CFTR knockout mice display a markedly more severe phenotype in models of acute pancreatitis. This is characterized by a heightened inflammatory response, reduced levels of apoptosis, and an increased basal expression of pro-inflammatory cytokine genes when compared to wild-type controls, reflecting a dysregulated immune environment and impaired resolution of pancreatic injury.^{209,210}

Nano-LC-ESI-HR-MS/MS was used to analyse extracted digested protein from mice pancreas sample from three to five different mice for each condition. Two technical replicates for each sample were produced.

Pearson correlation coefficient ranged between 0,80 and 0,97 across each replicate ensuring the reproducibility and the homogeneity of the sample analysed as it's possible to appreciate from normal distribution of $\log_2$ of intensities (Fig. 3.3.2a).

Protein identification and LFQ were performed using PEAKS (v.12.00, Bioinformatics Solutions Inc.) with integrated de novo sequencing.

A total of 3295 proteins were identified across different conditions, of which 3205 proteins were present in the 60% of the samples of at least one condition, that were taken in consideration for statistical analysis to ensure robustness of the results. PCA and hierarchical clustering (fig. 3.3.2b) did not reveal clear separation of the conditions, with technical replicates largely overlapping and no condition-specific clusters emerging. In contrast, statistical testing (t-test, $FDR < 1\%$, $S_0 = 1\%$) identified significant differences between conditions (fig. 3.3.2c), indicating that changes are present but do not dominate the overall variance structure. This discrepancy likely reflects widespread, non-specific variability across replicates rather than strong condition-driven shifts. A plausible explanation is the intrinsic heterogeneity of the pancreas, where its small size may lead to inclusion of different cell populations during isolation. Together, these factors suggest that while condition-dependent differences exist at the protein level, they are subtle compared to the technical and biological variability inherent to the samples.

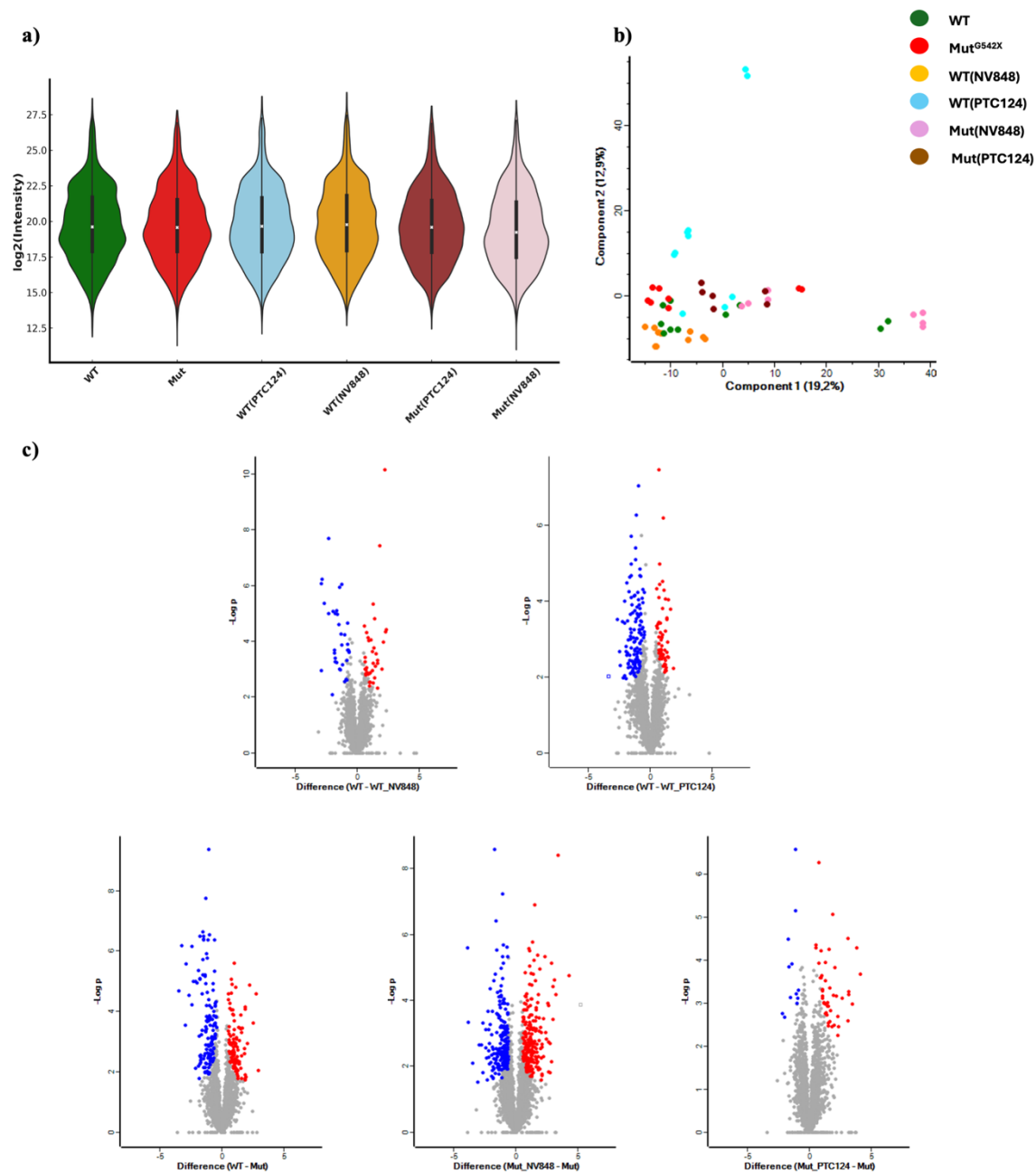


Figure 3.3.2 Statistical analysis of pancreatic tissue. a) Violin plot of $\log_2$ intensities illustrating distribution across conditions; b) Principal component analysis (PCA) showing clustering of WT, Mut, WTNV848, WTPTC124, MutNV848, and MutPTC124 samples; c) Volcano plot highlighting significant differences (t -test, $FDR < 1\%$, $S_0 = 1\%$) in protein expression against WT for off-label DEPs and Mut for CFTR-related DEPs.

We first evaluated the effects of NV848 (WTNV848) and PTC124 (WTPTC124) treatment on wild-type pancreatic tissue to highlight potential off-target effects. Compared with untreated WT pancreas, 112 differentially expressed proteins (DEPs) were found in the WTNV848 group and 280 in the WTPTC124 group. The observed differences were consistent across biological replicates for each condition, as illustrated by the heatmap in fig. 3.3.3.

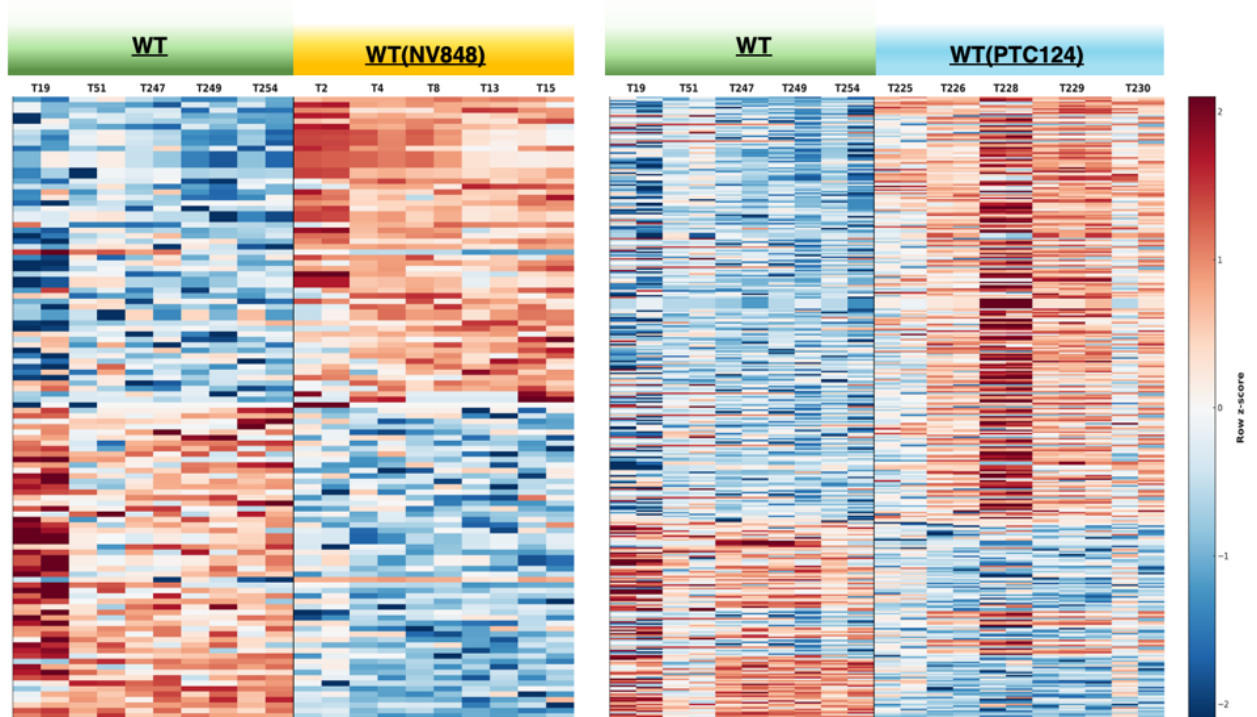


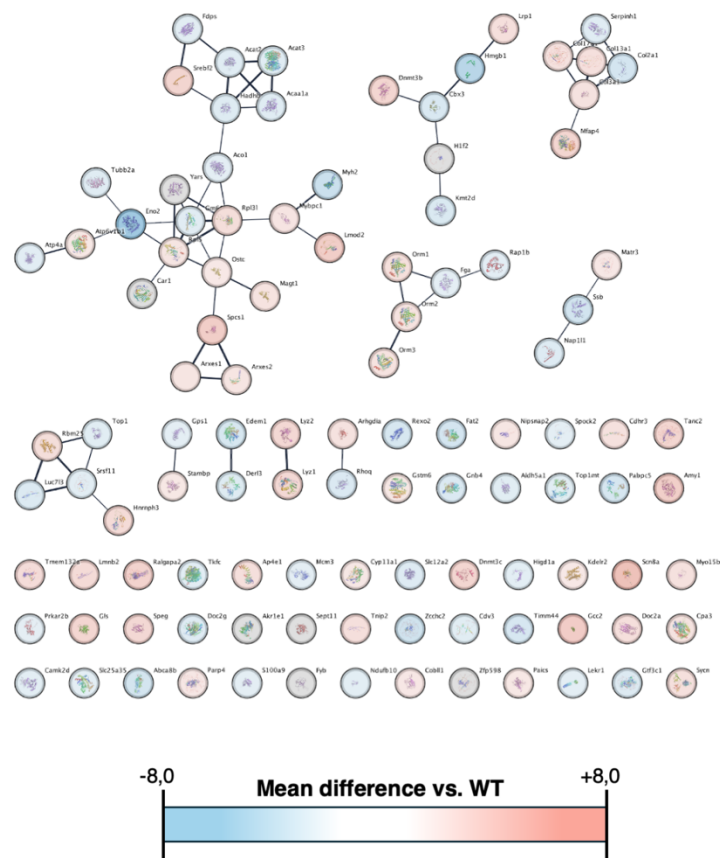
Figure 3.3.3 Comparison of Pancreatic DEPs for off-label treatments effect. Normalized (Z-score) abundance heatmap of DEPs (*t*-test, $FDR < 1\%$, $S_0 = 1\%$) in WT mice treated with NV848 (WTNV848) or PTC124 (WTPTC124), compared with untreated WT.

Notably, only two proteins were shared between the two treatment conditions, underscoring the distinct proteomic signatures induced by NV848 and PTC124 and highlighting potential differences in RT mechanisms.

Network-based functional analysis was performed using STRING on DEPs, with protein-protein association data integrated into Cytoscape to identify functional clusters and enriched biological pathways using Go Biological process.

WTNV848-related networks (fig. 3.3.4a) did not form a homogeneous protein-protein interactions (PPI) structure, and no biological processes were significantly enriched. Instead, proteomic analysis highlighted the marked upregulation of AMY1 (amylase), LMOD2 (leiomodin 2), and SCN8A (sodium voltage-gated channel alpha subunit 8) following NV848 treatment (Fig 3.3.4b).

a)



b)

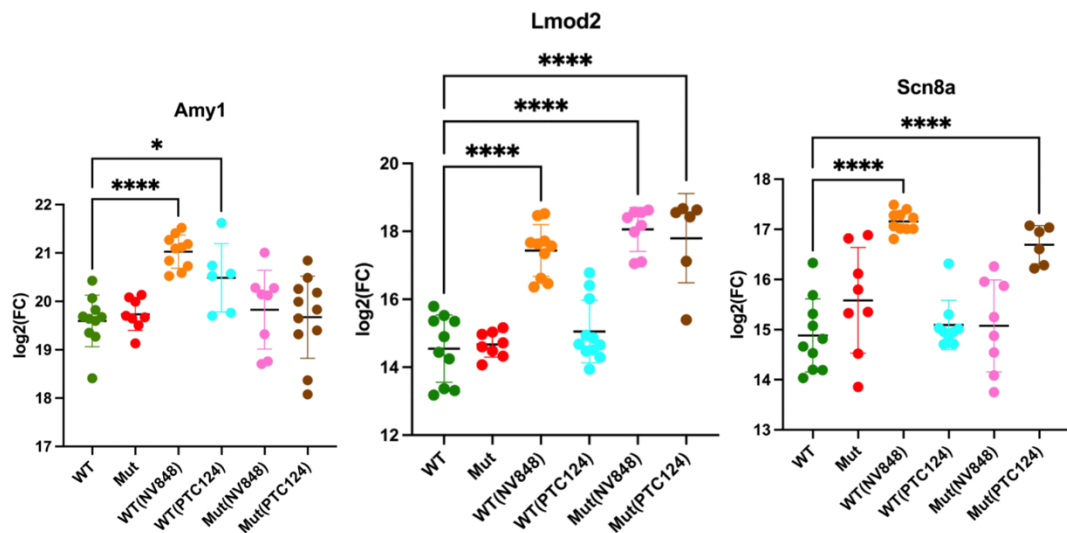


Figure 3.3.4 Pancreatic WTNV848 off-label-related PPI network and significant DEPs. a) PPI network of DEPs in WTNV848 compared to WT, generated using the STRING database. Significantly upregulated and downregulated proteins (FDR < 0.01) are shown in red and blue, respectively. b) LFQ comparison of WTNV848 DEPs. Data were analyzed with GraphPad Prism 6 and are expressed as mean $\pm$ SEM. Statistical significance relative to WT is indicated as follows: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001 (ANOVA with Dunnett's post hoc test).

In contrast, WTPTC124-related networks (Fig. 3.3.5) formed a coherent PPI network with two distinct topological clusters. Cluster 1 was enriched for pathways linked to gene expression and response to stress, whereas Cluster 2 was associated with response to chemical stimuli and negative regulation of biological processes, indicating that PTC124 induces moderate, controlled stress responses and mild proteomic remodelling (Appendix B). Together, these findings demonstrate that, even in normal tissue, TRID administration elicits regulatory and adaptive responses beyond basal proteomic and transcriptional processes, likely reflecting cellular recognition of the compounds and potential off-target interactions. Importantly, neither NV848 nor PTC124 induced clusters related to massive apoptosis, extensive tissue damage, or overwhelming stress, supporting the notion that both compounds are well tolerated and consistent with the expected safety profile of TRIDs.

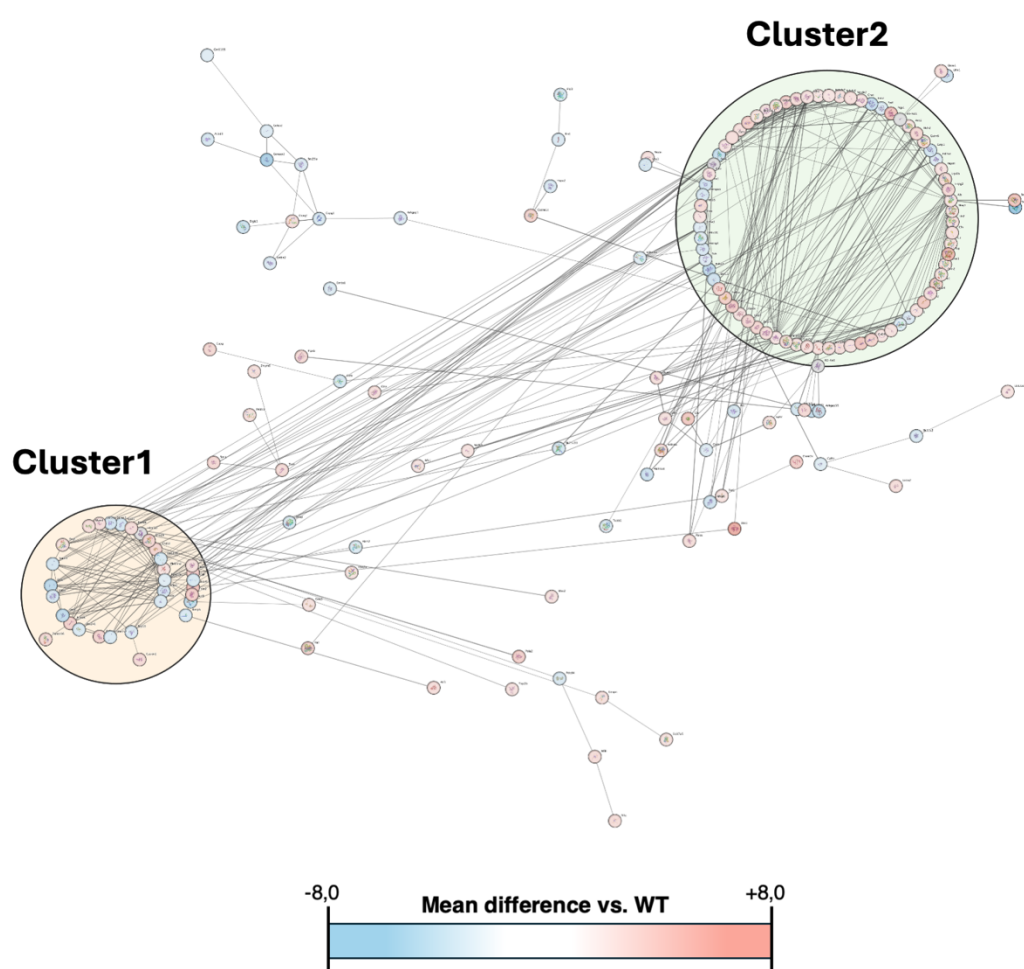


Figure 3.3.5 Pancreatic WTPTC124 off-label-related PPI network and significant DEPs. a) PPI network of DEPs in WTPTC124 compared to WT, generated using the STRING database. Topological clusters within the network were identified using the MCODE algorithm. Significantly upregulated and downregulated proteins (FDR < 0.01) are shown in red and blue, respectively.

We then proceeded to evaluate the efficacy of NV848 in restoring CFTR expression and functionality in nonsense-mutated mice models. To this end, we compared the pancreatic proteome of untreated mutant mice (Mut) with that of WT controls to identify significant CFTR-related differences, mutant mice treated with NV848 (MutNV848) and with PTC124 (MutPTC124), the latter serving as a comparative benchmark for translational readthrough activity.

Compared with untreated Mut pancreas, 332 DEPs were found in the WT group, 633 in the MutNV848 group and 103 in the MutPTC124 group. The observed differences were consistent across biological replicates for each condition, as illustrated by the heatmap in Fig 3.3.6. In this case, 158 differentially expressed proteins were shared across conditions, pointing to a common proteomic core that may be directly or indirectly associated with CFTR dysfunction (Fig 3.3.7a). This shared signature provides a valuable starting point for identifying pathways consistently altered in nonsense mutation related CF and potentially responsive to readthrough therapy.

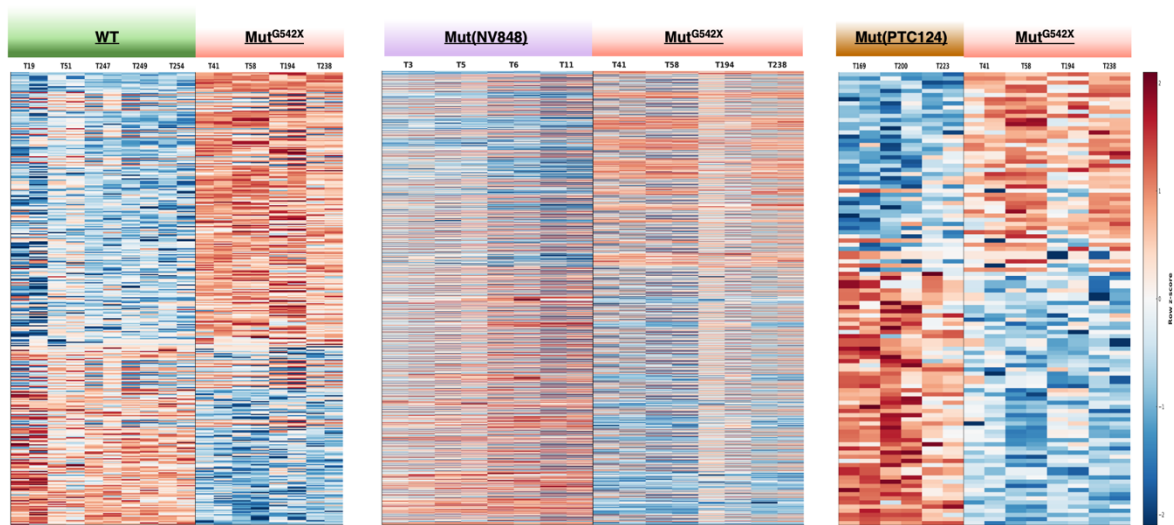


Figure 3.3.6 Comparison of Pancreatic CFTR related DEPs. Normalized (Z-score) abundance heatmap of DEPs (t -test, $FDR < 1\%$, $S_0 = 1\%$) in WT and Mut mice treated with NV848 (MutNV848) or PTC124 (MutPTC124), compared with untreated Mut.

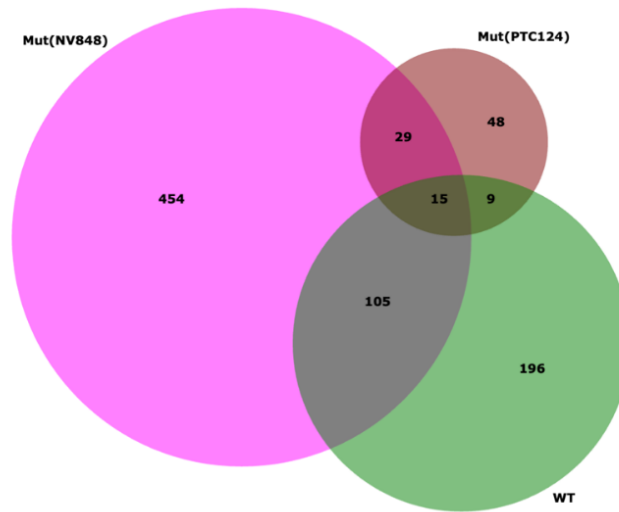
Given the large overlap of DEPs across conditions and the minimal topological differences observed in condition-specific PPI networks, we integrated all significant DEPs into a single network (CFTR related network) for functional and topological analysis. This strategy allowed us to identify a common core proteomic signature and highlight central pathways consistently associated with CFTR dysfunction, while minimizing redundancy between conditions.

To better understand the CFTR in the PPI network built also CFTR and its close known interactor were taken in consideration for network generation.

The resulting CFTR-related proteomic network formed a large, extended, and relatively homogeneous structure. To specifically investigate CFTR's function in the pancreatic context, we filtered the dataset to include only proteins expressed in the pancreas and considered only high-confidence interactions (confidence score > 0.7), thus retaining high-confidence interactions. Remarkably, even proteins not detected in all three conditions were well connected within the network and did not form isolated clusters. Given the large extent a PPI network a stratified analysis was needed. To this aim we performed a topological analysis further identifying eight distinct clusters, each containing differentially expressed proteins observed in various combinations of conditions, as illustrated in Fig 3.3.7b. On each cluster a biological pathway enrichment was performed using the GO biological process database (Appendix B). The enrichment in terms like “regulation of calcium ion transport”, “inflammatory response” and “vesicle transport” directly linked to CFTR expression and functionality, and more in general with pancreas health and secretory capacities involving DEPs not only observed in WT but also in MutNV848 and MutPTC124, could suggest a realignment of mutated mice proteome after TRIDs treatment with the wild-type one.

Moreover, other terms such as “gene expression,” “glutathione metabolic process,” “detoxification,” and “generation of precursor metabolites and energy” were also widely enriched. This pattern is consistent with, and mechanistically linked to, CFTR functionality, since the loss of CFTR function is known to induce ER stress through multiple mechanisms. First, aberrant CFTR protein, as reported for Phe508del and potentially for truncated G542X, accumulates in the ER, saturating the folding machinery and triggering the unfolded protein response (UPR). Second, altered ion transport and ER Ca^{2+} homeostasis compromise chaperone activity, further exacerbating proteostatic imbalance. Finally, ER stress is reinforced by cross-talk with mitochondria and oxidative stress: uncontrolled Ca^{2+} release from the ER to mitochondria leads to mitochondrial membrane depolarization, reduced oxidative phosphorylation (OXPHOS), and increased production of reactive oxygen species (ROS)²¹¹. Collectively, these events chronically activate the UPR and drive inflammatory cascades, resulting in altered expression of genes involved in inflammation, oxidative stress response, and proteostasis. The coordinated enrichment of RNA processing and energy-related pathways in our data suggests that TRIDs act not only to restore CFTR synthesis, but also to remodel post-transcriptional and bioenergetic networks that are frequently impaired by CFTR dysfunction and ER stress^{212,213}.

a)



b)

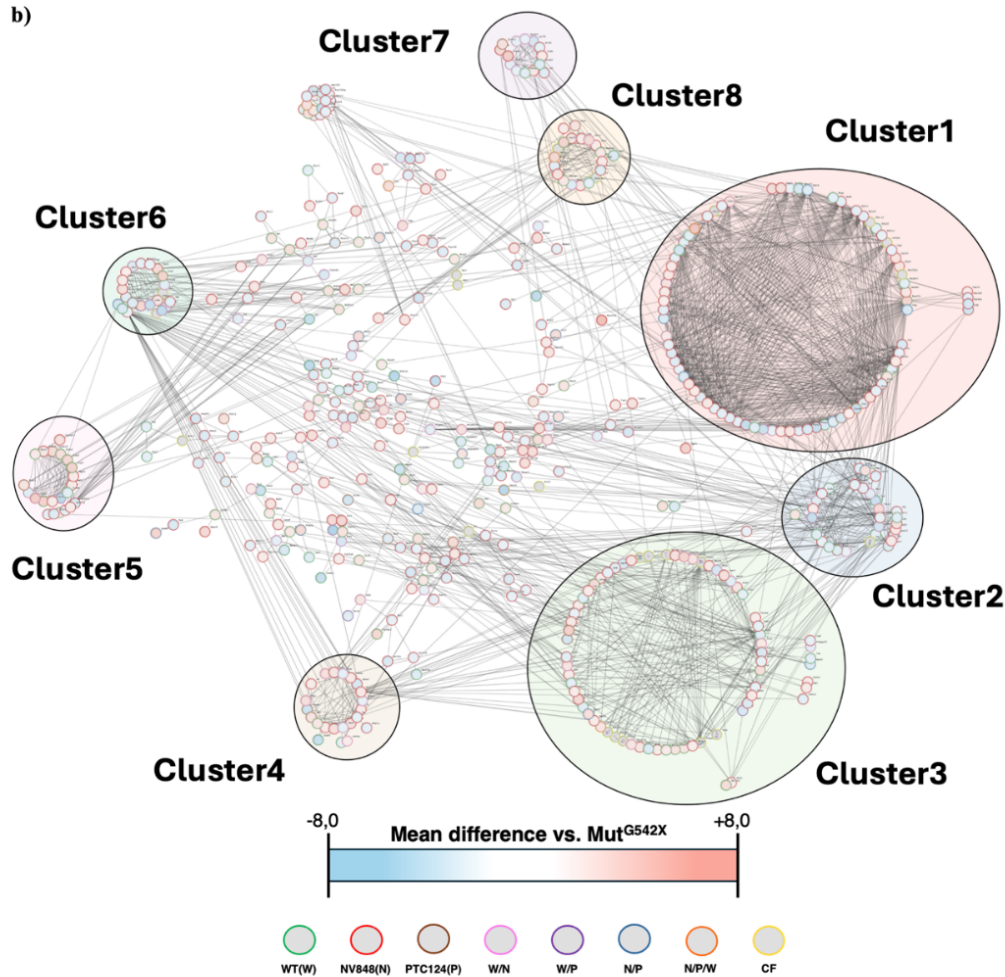


Figure 3.3.7 Pancreatic CFTR-related PPI network. a) Venn diagram of observed DEPs in WT, MutNV848 and MutPTC124 compared to Mut; b) PPI network of DEPs in WT, MutNV848 and MutPTC124 compared to Mut, generated using the STRING database. Significantly upregulated and downregulated proteins ($FDR < 0.01$) are shown in red and blue, respectively. Topological clusters were identified using the MCODE algorithm, border color of each node indicates the condition in which the corresponding DEP was observed.

These findings were further supported by proteomic analysis (Fig 3.3.8), which revealed decreased expression of Eif4b in Mut compared with WT, alongside increased expression of inflammatory markers such as Lta4h²¹⁴, Tmbim6²¹⁵, Gstt2²¹⁶, and Psma3²¹⁷ and Reg2²¹⁸ as well as the ER stress marker Edem1²¹⁹. Treatment with NV848 effectively realigned the expression of these proteins with WT levels in all cases, whereas PTC124 failed to produce a significant reduction in inflammatory markers. These data suggest that NV848 can restore CFTR function in the pancreas and performs better than PTC124 in this context. Notably, we identify for the first time an association between CFTR and eIF4B, Tmbim6, and Reg2, uncovering previously unrecognized metabolic dimensions of CFTR regulation.

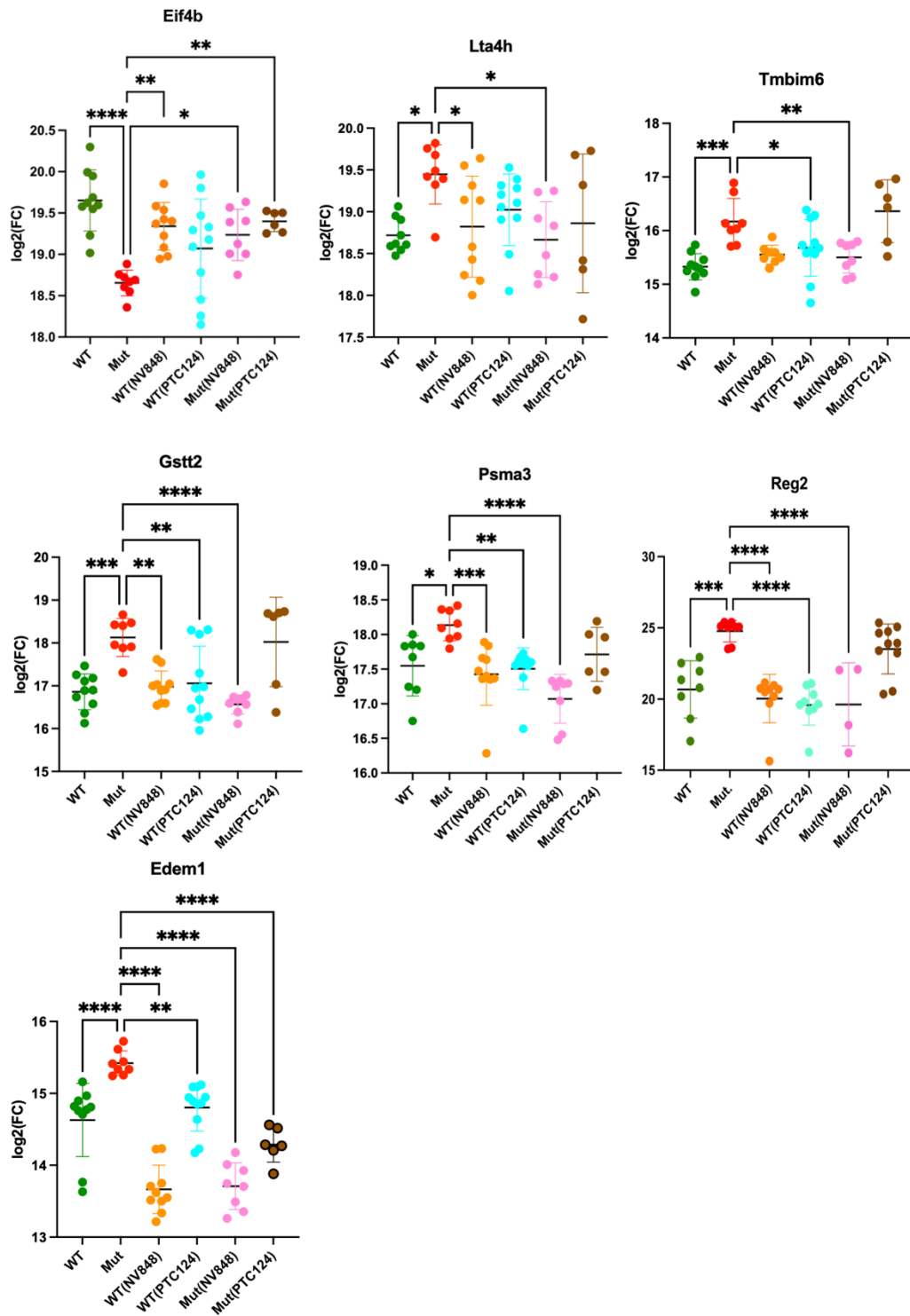


Figure 3.3.8 Pancreatic LFQ comparison of CFTR-related DEPs. Data were analyzed with GraphPad Prism 6 and are expressed as mean $\pm$ SEM. Statistical significance relative to Mut is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (ANOVA with Dunnett's post hoc test).

Liver

CF-associated liver disease (CFLD) represents the third leading cause of death in CF after pulmonary pathologies and transplant complications, accounting for 3.4% of CF patient deaths yearly²²⁰. CFTR is expressed specifically on the luminal membrane of biliary tree cells, namely cholangiocytes. Cholangiocytes regulate the fluidity and alkalinity of bile through the expression of an array of transporters, including CFTR, located on their apical membrane, where it plays a crucial role in proper bile duct secretion. As a result of a meal, the duodenum releases the hormone secretin, which, by binding to its receptor on the basolateral membrane of cholangiocytes, leads to an increase in intracellular cAMP that activates PKA-mediated chloride efflux through CFTR. The Cl^- gradient across the membrane drives HCO_3^- secretion into the lumen via activation of anion exchange protein 2, a $\text{Cl}^-/\text{HCO}_3^-$ exchanger, and water efflux through aquaporins, thereby ensuring the correct fluidity and alkalinity of bile. Loss of CFTR functionality leads to impaired HCO_3^- and water secretion across the luminal membrane, resulting in reduced bile flow and toxin accumulation, which contribute to the onset of pro-inflammatory conditions²²¹. Moreover, impairment of bile secretion alone is not sufficient to trigger the inflammatory state that leads to the classical manifestations of CFLD. CFTR loss of functionality also causes hyperphosphorylation of Toll-like receptor 4 (TLR4) due to unregulated Src kinase activity, resulting in dysregulated activation of NF- κ B and a strong reduction in endotoxin tolerance, which drives the establishment of an inflammatory state²²². It has also been reported that CFTR interacts with β -catenin, modulating the inflammatory response, and that loss of this interaction amplifies NF- κ B activation²²³.

These combined alterations orchestrate the classical pathological outcomes observed in CFLD, including microgallbladder, steatosis, cirrhosis, and portal hypertension, which occur in approximately 30% of patients, with more than 10% developing severe complications²²⁴. Moreover, this number is tragically expected to rise as the population of adult CF patients increases and life expectancy continues to improve, further pointing out the urgent need for targeted therapies.

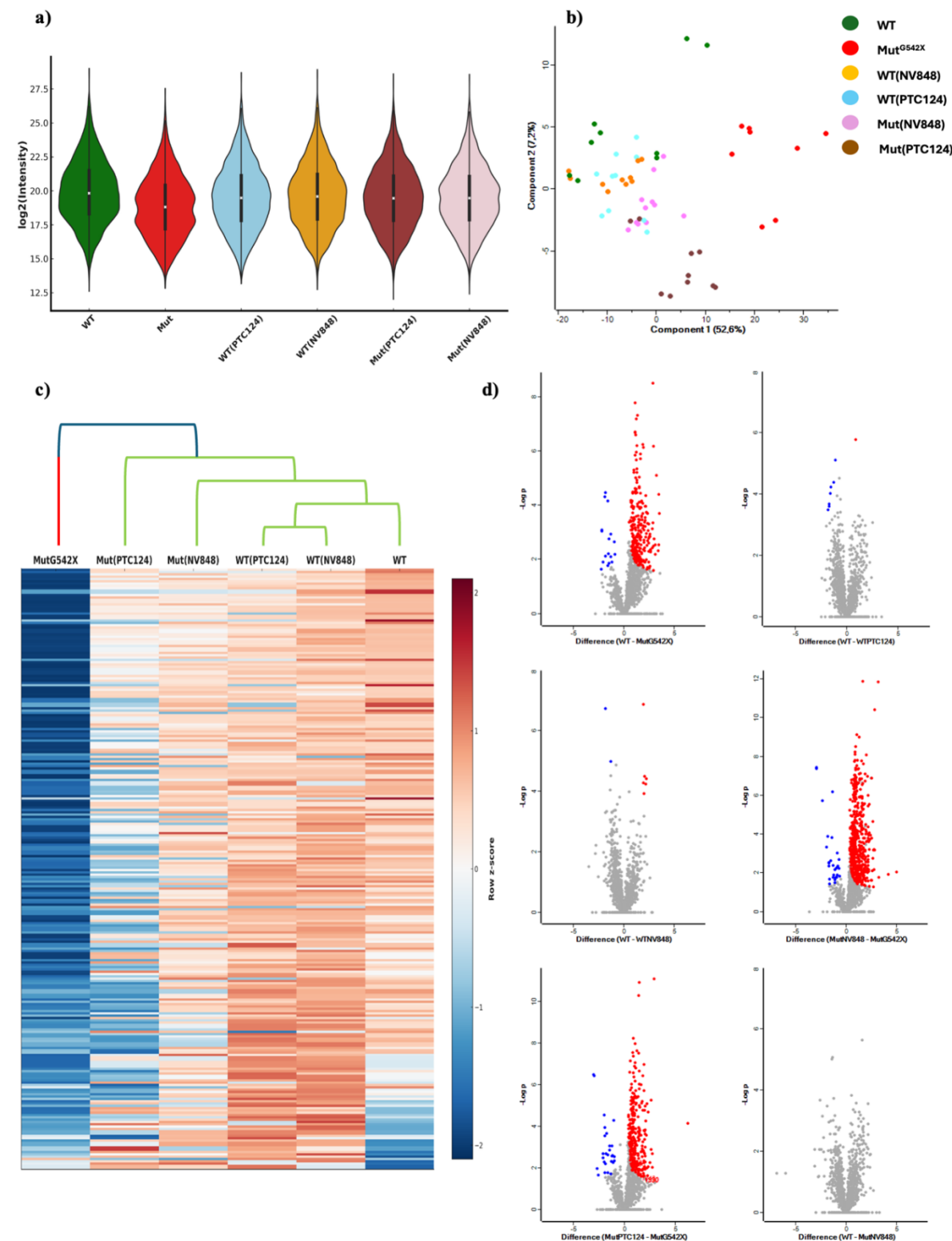
As for pancreas, we evaluated the recovery of CFTR in the liver through proteomic analysis to validate the TR activity of NV848 and to identify novel protein interactions associated with CFTR loss of function. For this purpose, we examined liver tissue from both WT and G542X mice, untreated and treated with NV848, using PTC124 as a comparative control for TR for a total of six conditions.

In murine CF models, CFTR is also localized to the apical membrane of cholangiocytes; however, these mice typically do not develop spontaneous liver pathology unless aged or exposed to toxic agents. This is likely due to a higher contribution of CFTR-independent calcium-activated chloride conductance (CaCC), which can partially mediate chloride efflux when CFTR is nonfunctional. Nevertheless, despite the absence of a strong spontaneous phenotype, several physiological features parallel those observed in human disease like increased faecal bile acid loss, similar to what is seen clinically in CF patients. These similarities make mouse models suitable for evaluating CFTR recovery in nonsense-mutated backgrounds²²⁵. We applied the same technical procedures and statistical analysis strategies previously used for pancreatic tissue samples. The Pearson correlation coefficients, ranging between 0.75 and 0.90 across replicates, confirmed both the reproducibility and homogeneity of the analyzed samples as it's possible to appreciate from normal distribution of $\log_2$ of intensities (Fig. 3.3.9a).

A total of 5520 were identified across different conditions, of which only 1195 present in the 60% of the samples of at least one condition, that were taken in consideration for statical analysis to ensure robustness of the results.

Unlike pancreatic tissue analysis, PCA of liver samples revealed a clear separation and homogeneous clustering of WT and Mut proteomes along principal component 1 (PC1). WTNV848 and WTPTC124 overlapped with the WT cluster, whereas MutNV848 and MutPTC124 occupied intermediate positions between the two groups. Remarkably, MutNV848 samples clustered closer to WT than to Mut, and noticeably more so than MutPTC124 (fig. 3.3.9b). PCA findings were further supported by hierarchical clustering (fig. 3.3.9c), which showed MutNV848 and MutPTC124 clustering with WT, WTNV848 and WTPTC124 rather than with Mut indicating a proteomic profile “correct” and not simply “intermediate”. Overall, these results suggest that loss of CFTR expression due to nonsense mutations induces significant proteomic shifts in the liver, and that TRID-based treatments partially realign the hepatic proteome toward a WT-like profile. Importantly, both PCA and hierarchical clustering consistently indicate that NV848 drives the mutant proteome closer to WT than PTC124 does. Pairwise comparisons, assessed by two-sample t-test ($FDR < 1\%$ and $S_0 = 1\%$) revealed robust proteomic differences between Mut and WT, MutNV848 and MutPTC124, consistent with the rescue signatures highlighted by PCA and hierarchical clustering. In contrast, comparisons of WTNV848 and WT and WTPTC124 and WT showed no significant proteomic alterations, indicating that TRID administration in wild-type mice does not perturb basal proteomic or transcriptional liver programs.

Notably, no significant differences were observed in the comparison between WT and MutNV848, pointing to a complete recovery of the wild-type proteome profile in nonsense-mutated CFTR mice after NV848 treatment (Fig 3.3.9d).



(See caption on next page)

Figure 3.3.9 Statistical analysis of liver tissue. a) Violin plot of log2 intensities distribution across conditions; b) Principal component analysis (PCA) of WT, Mut, WTNV848, WTPTC124, MutNV848, and MutPTC124 samples; c) Hierarchical clustering of DEPs across conditions identified by multi-sample t-test ($FDR < 1\%$, $S_0 = 1\%$); d) Volcano plot highlighting significant differences in protein expression (t-test, $FDR < 1\%$, $S_0 = 1\%$) against WT for off-label DEPs and against Mut for CFTR-related DEPs.

Since no significant change were observed in WT liver proteome after NV848 and PTC124 administration, we proceeded to evaluate the efficacy of NV848 in restoring CFTR expression and functionality. As in pancreas, we compared the hepatic proteome of untreated Mut with that of WT controls to identify significant CFTR-related differences, MutNV848 and MutPTC124, as a positive benchmark for TR activity.

Compared with untreated Mut liver, 279 DEPs were found in the WT group, 491 in the MutNV848 group and 303 in the MutPTC124 group. The observed differences were consistent across biological replicates for each condition, as illustrated by the heatmap in Fig. 3.3.10.

A set of 336 DEPs was shared across conditions (Fig 3.3.11a), defining a common proteomic core, also observed in pancreas, likely linked to CFTR dysfunction and potentially responsive to readthrough therapy.

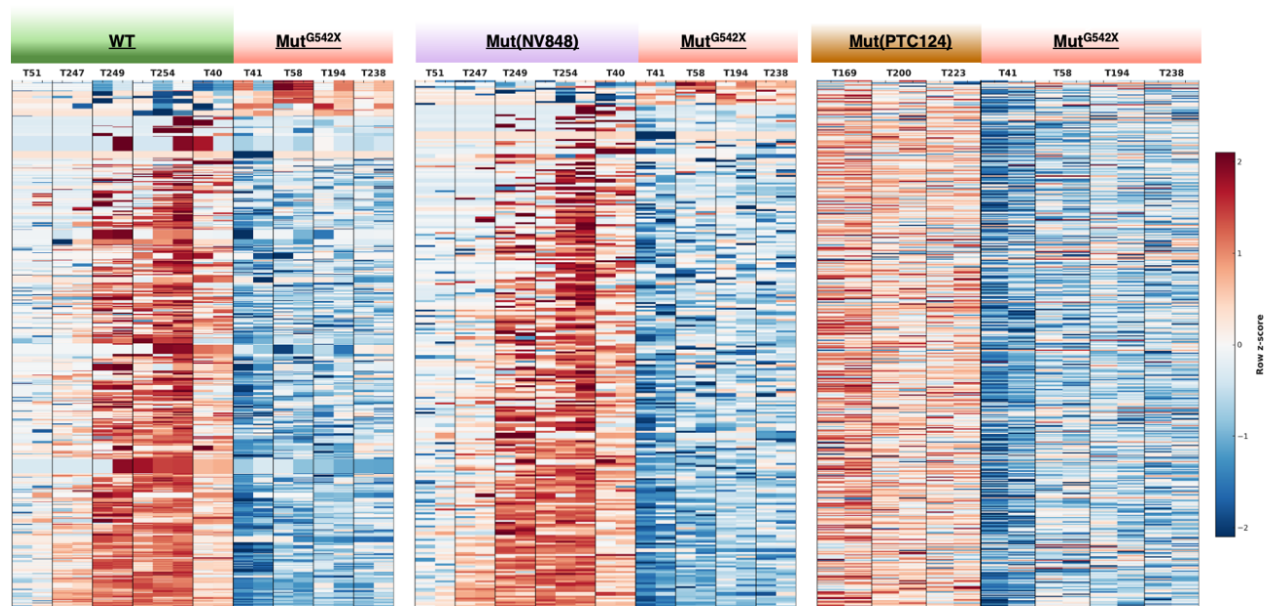


Figure 3.3.10 Comparison of hepatic CFTR related DEPs. Normalized (Z-score) abundance heatmap of DEPs (t-test, $FDR < 1\%$, $S_0 = 1\%$) in WT and Mut mice treated with NV848 (MutNV848) or PTC124 (MutPTC124), compared with untreated Mut.

Given the large overlap of DEPs across conditions and the minimal topological differences in condition-specific PPI networks, we used the same network analysis strategies applied to pancreatic tissues. We integrated all significant DEPs into a single CFTR-related network for functional and topological analysis, incorporating CFTR itself and its closest known interactors.

This allowed the identification of a common proteomic core and highlighted central pathways consistently linked to CFTR dysfunction, while reducing redundancy across conditions.

The CFTR-related proteomic network (Fig 3.3.11b) formed a large, homogeneous structure where only high-confidence edges (score >0.7) were considered. Notably, even proteins absent in some conditions were well integrated into the network and did not form isolated clusters. Given the large extent of the PPI network we performed a network stratified analysis. We identified seven distinct topological clusters, and for each cluster performed biological pathway enrichment using the GO Biological Process database (Appendix B).

From functional analysis, the terms “negative regulation of inflammatory response to antigenic stimulus,” “negative regulation of oxidative stress-induced intrinsic apoptotic signalling pathway,” and “cellular detoxification” emerged, which may be due to a decrease in hepatic inflammation and, more generally, to reduced oxidative stress linked to correct CFTR expression and functionality.

Enrichment in terms such as “response to glucose,” “carbohydrate metabolic process,” “glycogen metabolic process,” and “cellular amino acid metabolic process” suggests an altered energetic and catabolic equilibrium in Mut, probably due to mitochondrial stress linked to the loss of CFTR expression and impairment of pancreatic hormone secretion, as reported in the literature and indirectly observed in pancreatic tissue. Recovery of CFTR functionality and re-establishment of pancreatic functionality could lead to a metabolic reset toward healthy conditions.

The enrichment for “protein localization to lipid droplet” and “glycogen metabolic process” suggests that correct CFTR functionality could reset hepatic energy storage. Lipid dysfunction in cystic fibrosis has been described in other tissues, such as intestine and systemic metabolism, but a direct liver proteomic link between glycogen and lipid droplets is rarely reported²²⁶. Glycogen drives glucose-derived acetyl-CoA, lipogenesis, and lipid droplet formation, whereas its depletion disrupts this axis, leading to abnormal lipid accumulation (steatosis), ROS production, and hepatic stress. Our data indicate that CFTR restoration may reprogram droplet biogenesis and localization, thereby re-aligning glycogen-lipid cross-talk^{227,228}.

DEPs present in each enriched biological pathway, were observed not only in WT, as expected, but also in MutNV848 and MutPTC124, with a similar pattern of expression compared to Mut, suggesting that TRID treatment could lead to a realignment of expression pathways in mutated mice with those of healthy mice due to the recovery of CFTR expression and functionality.

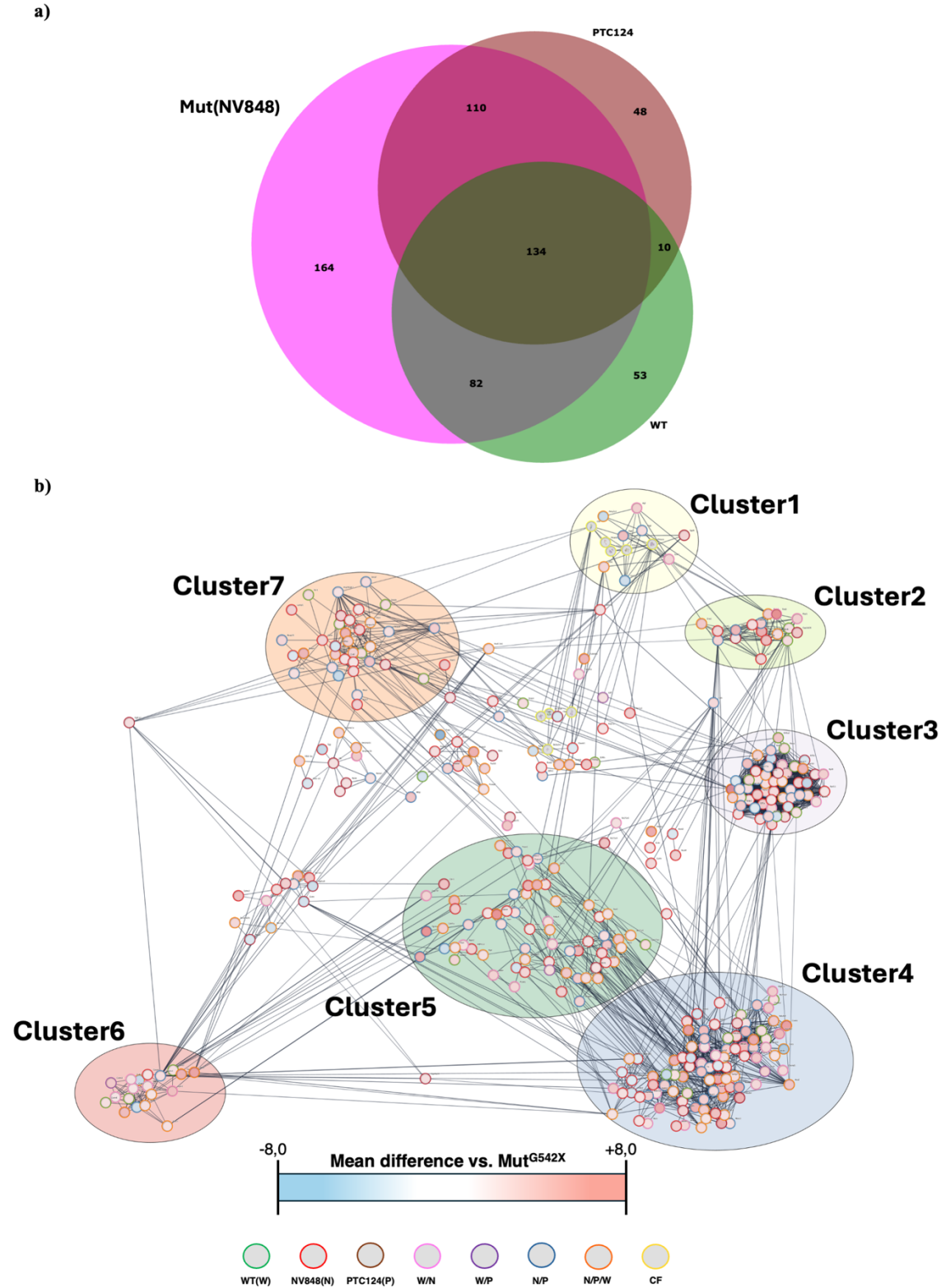


Figure 3.3.11 Hepatic CFTR-related PPI network. a) Venn diagram of observed DEPs in WT, MutNV848 and MutPTC124 compared to Mut; b) PPI network of DEPs in WT, MutNV848 and MutPTC124 compared to Mut, generated using the STRING database. Significantly upregulated and downregulated proteins ($FDR < 0.01$) are shown in red and blue, respectively. Topological clusters were identified using the MCODE algorithm, border color of each node indicates the condition in which the corresponding DEP was observed.

These findings were further supported by proteomic analysis (Fig 3.3.12), where we observed downregulation in Mut compared to WT of Sod1, Cat, and Gpx2²²⁹, key antioxidant enzymes, along with Glud1²³⁰ and Etfa1²³¹, regulators of mitochondrial activity. In parallel, reduced levels of Hspd1, Psma3, and Eef1a²³² indicated impaired proteostasis and altered protein synthesis. This coordinated repression underscores defects in ROS defence, mitochondrial bioenergetics, and protein quality control, collectively predisposing the liver to inflammation and fibrogenesis, as commonly observed in patients with CFLD. Treatment with NV848 and PTC124 effectively realigned the expression of these proteins with WT levels in all cases. Altogether these data suggest that loss of CFTR expression in liver tissue leads to the onset of an inflammatory state through multiple pathways such as suppression of antioxidant enzyme expression, altered mitochondrial functionality and disrupted glycogen-lipid coordination. NV848 restored CFTR expression and function more effectively than PTC124, both in liver and pancreas, confirming a general trend across tissues. Notably, we report for the first time an association between CFTR and Glud1, Etfa, Psma3, and Eef1a1, uncovering previously unrecognized metabolic relationships influenced by CFTR regulation.

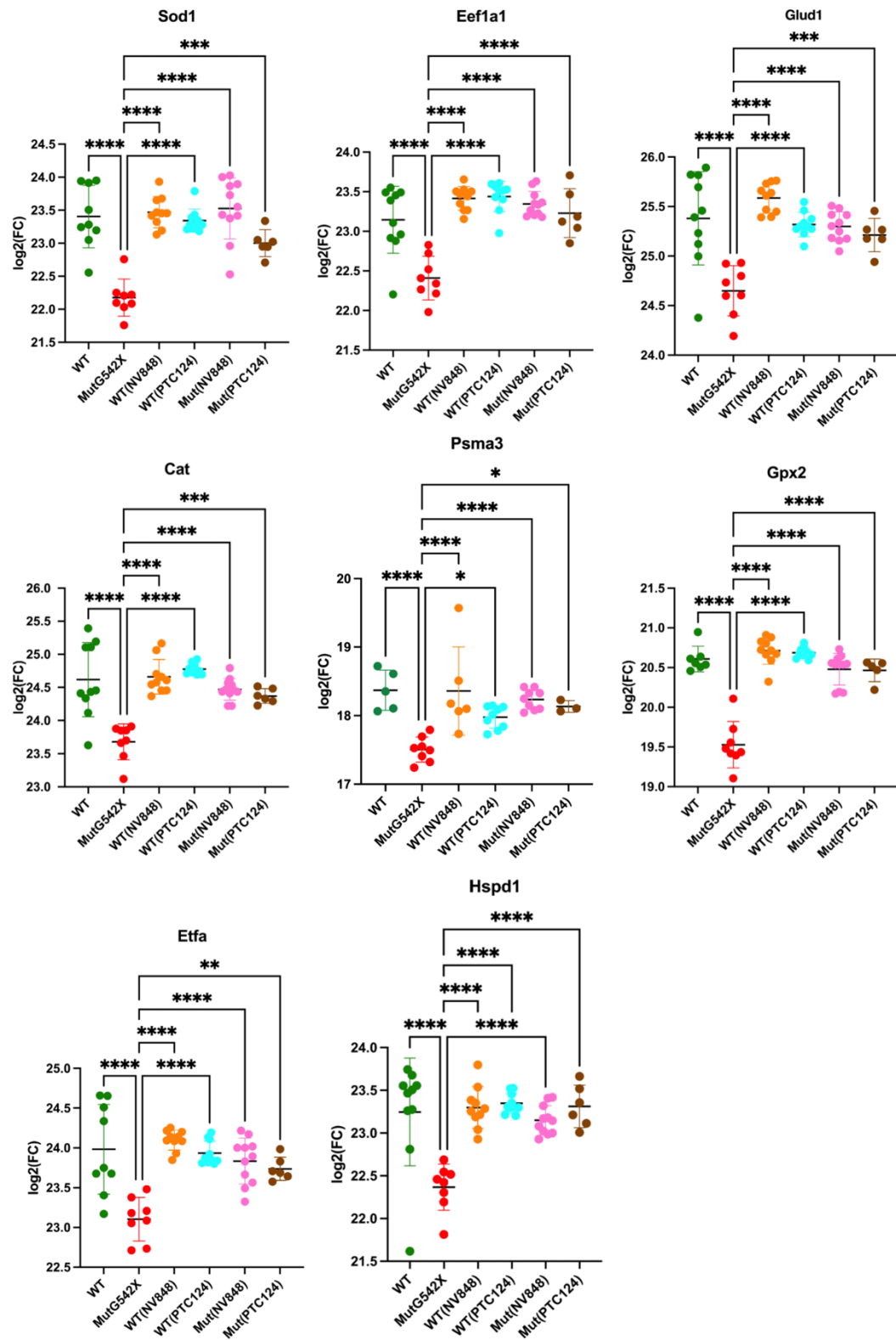


Figure 3.3.12 Hepatic LFQ comparison of CFTR-related DEPs. Data were analyzed with GraphPad Prism 6 and are expressed as mean $\pm$ SEM. Statistical significance relative to Mut is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (ANOVA with Dunnett's post hoc test).

Brain

While the role of CFTR and the consequences of its loss of function are well documented in the pancreas and liver, relatively little is known about its importance in brain function and physiology. In humans, CFTR is expressed in several brain regions, including the cerebral cortex, particularly the frontal, temporal, and parietal cortices, as well as in pyramidal neurons, hippocampus, hypothalamus, cerebellum, amygdala, brainstem, choroid plexus, and the ependymal lining. CFTR expression has also been detected in multiple brain cell types, including neurons, astrocytes, and microglia. More broadly, significant CFTR expression has been reported not only in the brain but also in the peripheral nervous system, underscoring the potential importance of this protein in neural physiology²³³. With increasing life expectancy in CF patients, attention has shifted toward the role of CFTR in non-canonical target organs such as the brain, where age-related manifestations, including neurological complications, are becoming increasingly relevant to patient quality of life²³⁴.

CFTR loss of function, in patients with clinically stable severe lung disease, has been linked to impaired neurocognitive functions, including cognitive and mood deficits, autonomic issues, and daytime sleepiness, with evidence of an early onset of cognitive decline in the context of CF. Moreover, high rates of anxiety and depression were revealed in CF patients that can compromise health outcomes and quality of life in general²³⁵.

Brain magnetic resonance imaging studies, using T2-relaxometry to detect subtle tissue changes and voxel-based morphometry (VBM) to assess grey matter density, in a cohort of 5 CF patients (mean age 29.7 ± 3.7 years) compared with 15 healthy controls, revealed marked alterations in the cerebellum, hippocampus, amygdala, superior temporal cortices, basal forebrain, insula, parietal cortices, and frontal and prefrontal cortices proving that cognitive and behavioural changes are driven by biological cause²³⁴.

In light of the above, we identified an intriguing challenge in exploring the impact of CFTR loss of function on the proteome of nonsense-mutated CFTR mouse models, as well as assessing the effects of TR-based therapy on its recovery.

In CFTR^{G542X} mice, progressive cognitive decline, brain pathology, and reduced hippocampal function have been observed, together with increased phosphorylated Tau protein, likely due to microtubule instability already documented in CF cells^{236,237}.

We evaluated the impact and recovery of CFTR in the brain through proteomic analysis to validate the TR activity of NV848. For this purpose, we examined brain tissue from both WT and G542X mice, untreated and treated with NV848, using PTC124 as a comparative control for TR for a total of six conditions.

We adopted the same technical and statistical analysis strategies as previously described for pancreatic and liver tissue samples. Pearson correlation coefficient ranged between 0,84 and 0,93 across each replicate ensuring the reproducibility and the homogeneity of the samples analysed as it's possible to appreciate from normal distribution of $\log_2$ of intensities (Fig 3.13a). A total of 2664 proteins were identified across different conditions, of which 2636 present in the 60% of the samples of at least one condition, that were taken in consideration for statical analysis to ensure robustness of the results.

From PCA analysis, a clear separation between WT, treated WT, Mut, and treated Mut samples emerges (Fig 3.3.13b). In particular, WT and Mut are well separated along PC1, indicating that CFTR loss of function causes a proteome shift, while WT and treated WT (WTNV848 and WTPTC124) are separated along PC2, suggesting a treatment-induced proteomic effect independent of CFTR recovery. Notably, the point cloud associated to MutNV848 and MutPTC124 clusters is significantly closer to the WT region than to the Mut, consistent with a recovery of the WT proteome phenotype. However, treatment with NV848 or PTC124 did not produce a complete overlap with WT, indicating that, in addition to recovery, treatment-specific molecular effects must also be considered.

Hierarchical clustering (Fig 3.3.13c) confirms a neat separation between WT and Mut with distinctive expression patterns. WTNV848 and WTPTC124 co-clustered but formed a subcluster within the WT group, reflecting a partial reshaping of the proteome in response to treatment. Similarly, MutNV848 and MutPTC124 co-clustered more closely with WT than with Mut, indicating a partial realignment with the healthy proteome, while still retaining hybrid characteristics.

Pairwise comparisons (Fig 3.3.13d), assessed by two-sample t-test ($FDR < 1\%$ and $S_0 = 1\%$) revealed robust proteomic differences between WT and WTNV848 and WTPTC124 indicating that TRID administration in wild-type mice produce changes in basal proteomic or transcriptional brain programs.

Robust proteomic differences were also observed for Mut and WT, MutNV848 and MutPTC124, consistent with the rescue signatures highlighted by PCA and hierarchical clustering.

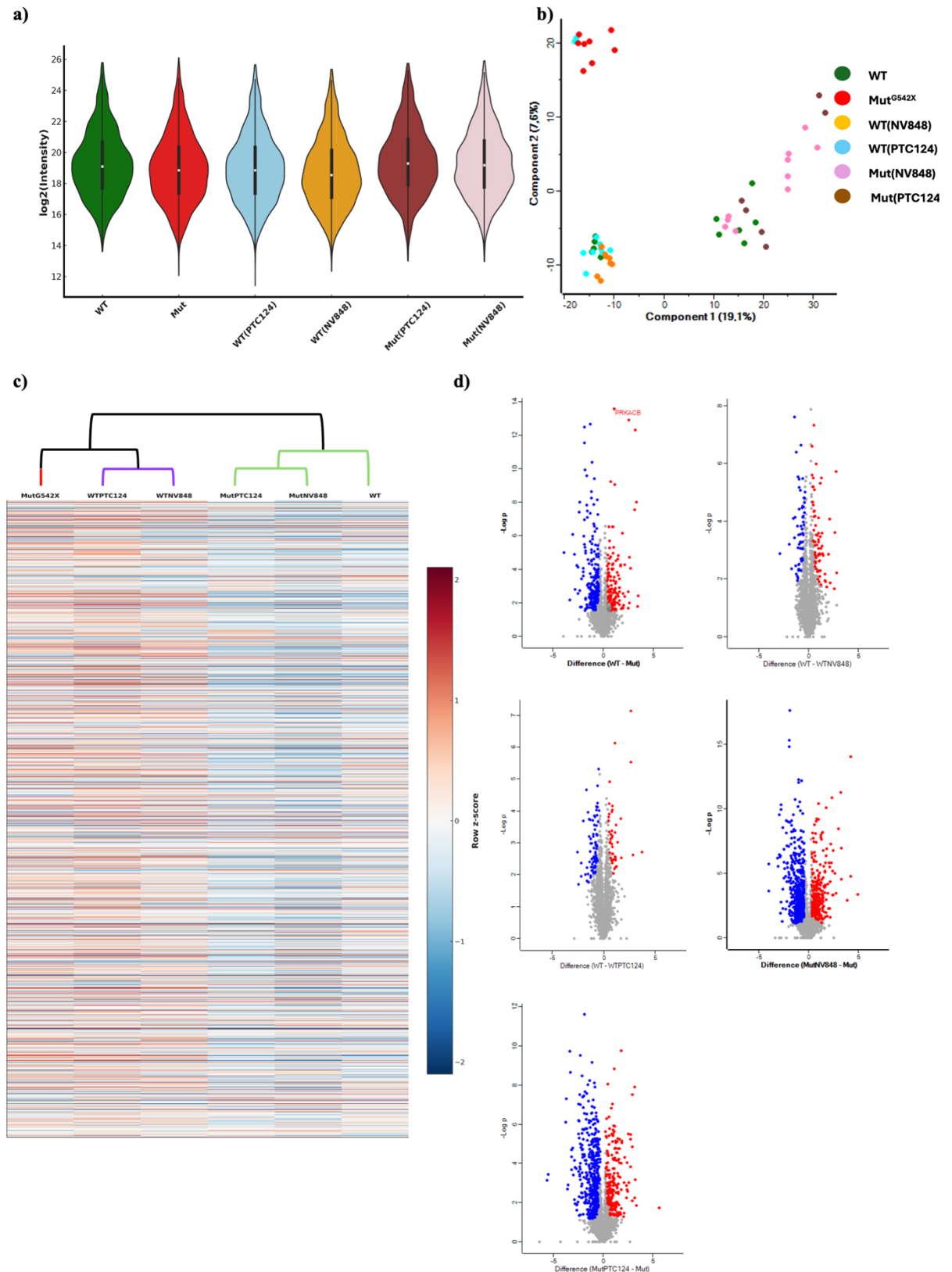


Figure 3.3.13 Statistical analysis of brain tissue. a) Violin plot of $\log_2$ intensities distribution across conditions; b) Principal component analysis (PCA) of WT, Mut, WTNV848, WTPTC124, MutNV848, and MutPTC124 samples; c) Hierarchical clustering of DEPs across conditions identified by multi-sample t-test (FDR < 1%, $S_0 = 1\%$); d) Volcano plot highlighting significant differences in protein expression (t-test, FDR < 1%, $S_0 = 1\%$) against WT for off-label DEPs and against Mut for CFTR-related DEPs.

Overall, these findings suggest an off-target effect of NV848 and PTC124 on the brain proteome, significant differences between Mut and WT profiles, and a partial realignment of the Mut proteome toward WT after TRID treatment.

We first evaluated the effects of NV848 and treatment on wild-type mice brain tissue to highlight potential off-target effects. Compared with untreated WT brains 323 DEPs were found in the WTNV848 group and 252 in the WTPTC124 group. The observed differences were consistent across biological replicates for each condition, as illustrated by the heatmap in Fig. 3.3.14. Notably, only 78 proteins were shared between the two treatment conditions, underscoring the distinct proteomic signatures induced by NV848 and PTC124, once again suggesting different mechanism of TR for the two molecules.

Network-based functional analysis was performed using STRING on DEPs, with protein-protein association data integrated into Cytoscape to identify functional clusters and enriched biological pathways using Go Biological process (Appendix B).

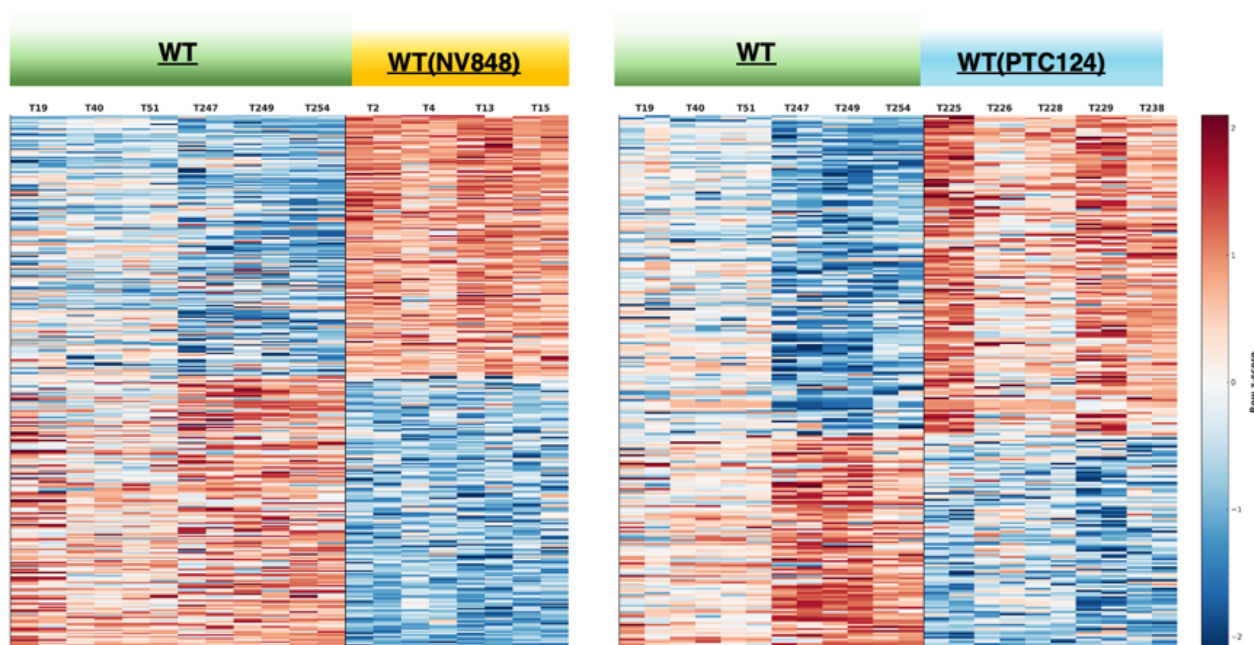


Figure 2.3.14 Comparison of brain DEPs for off-label treatments effect. Normalized (Z-score) abundance heatmap of DEPs (*t*-test, *FDR* < 1%, *S₀* = 1%) in WT mice treated with NV848 (WTNV848) or PTC124 (WTPTC124), compared with untreated WT.

WT NV848-related networks (Fig 3.3.15a) did a homogeneous PPI structure with two distinct topological cluster.

Cluster 1 was enriched in general terms “protein metabolic process”, “positive regulation of cellular component organization” and “regulation of neural precursor cell proliferation and

response to stress”, whereas Cluster 2 was associated with “positive regulation of biological process” and “nervous system development”.

Proteomic analysis revealed a marked upregulation of stomatin-like protein 2 (STOML2) following NV848 treatment (Fig 3.3.15b). STOML2, an inner mitochondrial membrane protein, is a key regulator of mitochondrial biogenesis, cristae organization, and respiratory chain stability. Its induction is consistent with the enrichment of NADH metabolism and proteostasis-related processes observed in our dataset and likely reflects an adaptive mitochondrial remodelling response to NV848 exposure. Beyond structural and metabolic remodelling, STOML2 upregulation has been associated with neuroprotective functions and enhanced antioxidative capacity²³⁸, suggesting that TRID treatment may engage compensatory mitochondrial pathways that support resilience against oxidative stress in the CF brain.

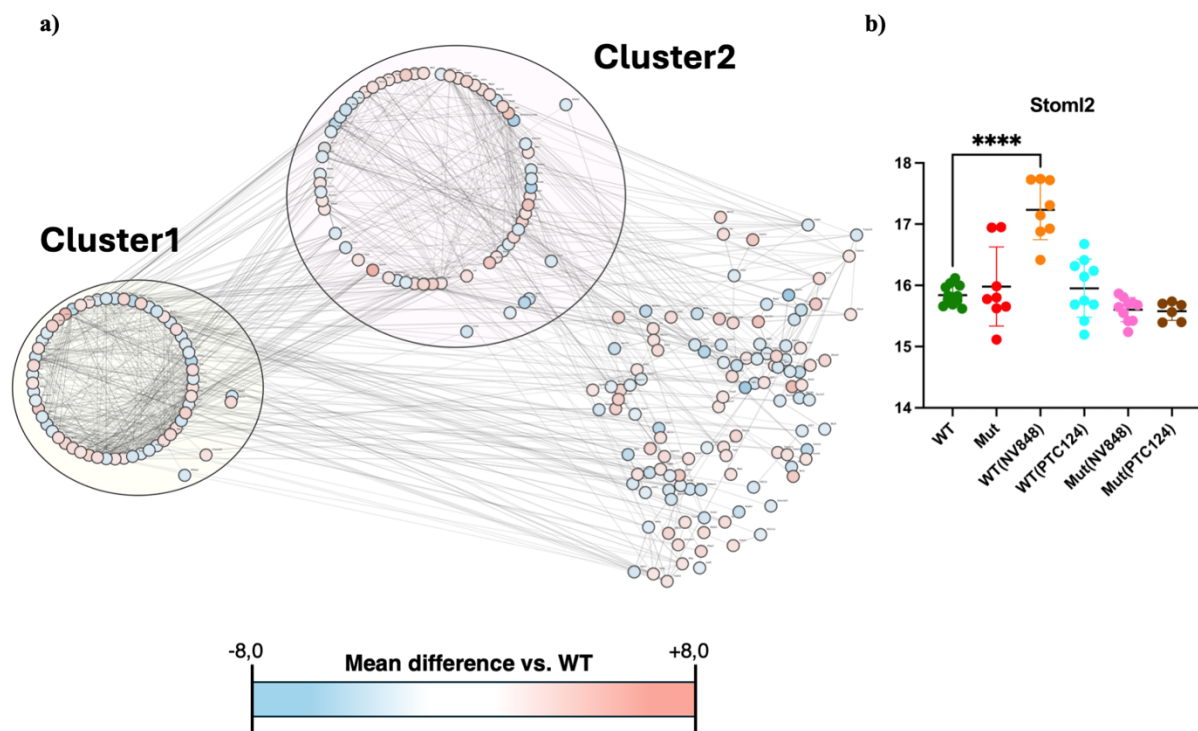


Figure 3.3.15 Brain WTNV848 off-label-related PPI network and significant DEPs. a) PPI network of DEPs in WTNV848 compared to WT, generated using the STRING database. Significantly upregulated and downregulated proteins ($FDR < 0.01$) are shown in red and blue, respectively. b) LFQ comparison of WTNV848 DEPs. Data were analyzed with GraphPad Prism 6 and are expressed as mean $\pm$ SEM. Statistical significance relative to WT is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (ANOVA with Dunnett's post hoc test).

WTPTC124-related networks (Fig 3.3.16) formed a coherent PPI structure with three distinct topological clusters. Cluster 1 was enriched in general terms related to gene expression, such as “positive regulation of gene expression.” Cluster 2 was associated with vesicular protein

transport, including “vesicle-mediated transport” and “protein localization,” while Cluster 3 was linked to metabolic processes such as “generation of precursor metabolites and energy.” These enrichment terms represent shared “core drug-response pathways” commonly observed in brain proteomic analyses of unrelated compounds. Their presence here suggests that NV848 and PTC124 trigger cellular stress and compensatory responses in WT mice brain beyond CFTR-specific activity, highlighting a novel aspect for CFTR readthrough agents and underscoring their potential central nervous system off-target effects.

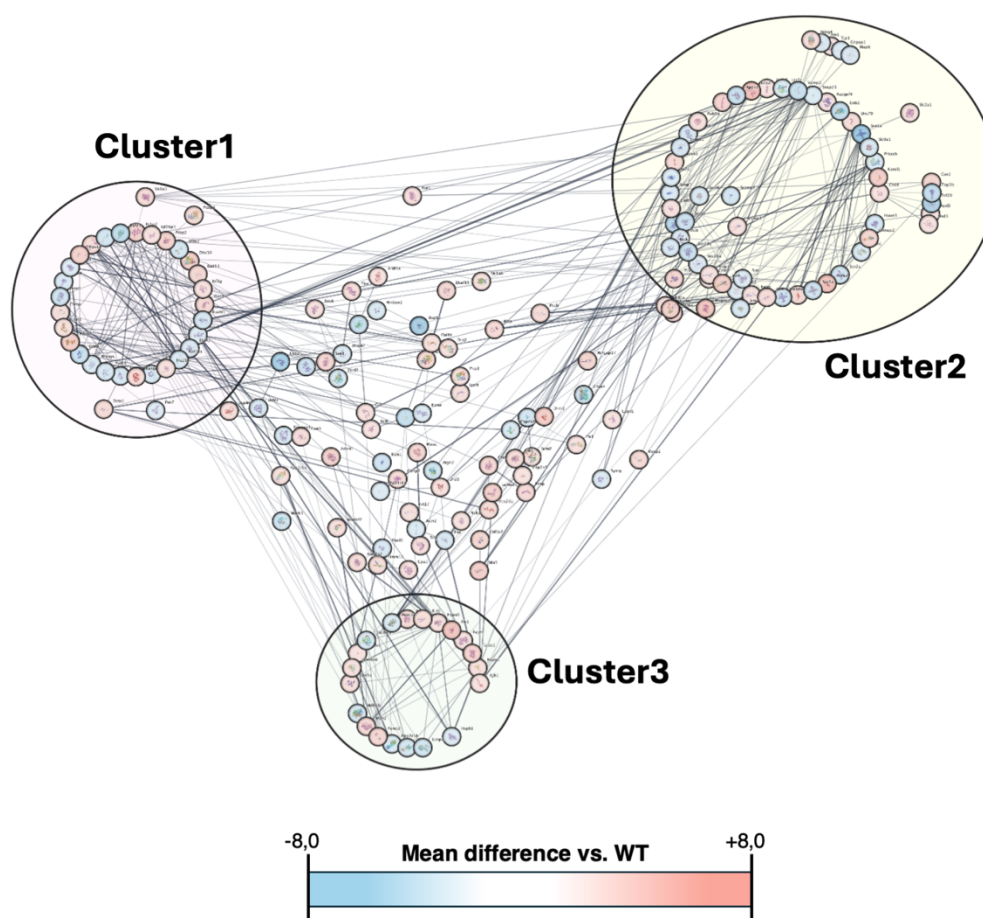


Figure 3.3.16 Brain WTPTC124 off-label-related PPI network and significant DEPs. a) PPI network of DEPs in WTPTC124 compared to WT, generated using the STRING database. Topological clusters within the network were identified using the MCODE algorithm. Significantly upregulated and downregulated proteins ($FDR < 0.01$) are shown in red and blue, respectively.

We then proceeded to evaluate the impact of CFTR loss of expression and efficacy of NV848 in TR activity. To this aim we compared the brain proteome of untreated mutant mice (Mut) with that of WT controls to identify significant CFTR-related differences, MutNV848 and MutPTC124. Compared with untreated Mut brain 470 DEPs were found in the WT group, 1179

in the MutNV848 group and 845 in the MutPTC124 group. The observed differences were consistent across biological replicates for each condition, as illustrated by the heatmap in Fig 3.3.17.

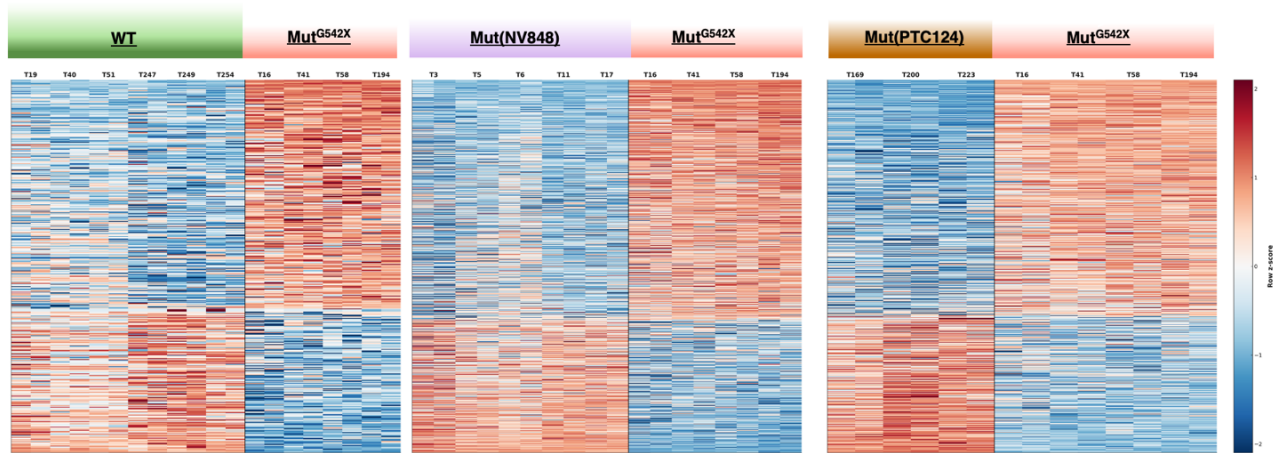


Figure 3.3.17 Comparison of brain CFTR related DEPs. Normalized (Z-score) abundance heatmap of DEPs (*t*-test, FDR < 1%, $S_0 = 1\%$) in WT and Mut mice treated with NV848 (MutNV848) or PTC124 (MutPTC124), compared with untreated Mut.

In this case, 752 differentially expressed proteins were shared across conditions, pointing to a common proteomic core that may be directly or indirectly associated with CFTR dysfunction (Fig 3.3.18a). This shared signature provides a valuable starting point for identifying pathways consistently altered in nonsense mutation related CF and potentially responsive to readthrough therapy.

Given the large overlap of DEPs across conditions and the minimal topological differences in condition-specific PPI networks, we used the same network analysis strategies applied in our previous analysis. We integrated all significant DEPs into a single CFTR-related network for functional and topological analysis, incorporating CFTR itself and its closest known interactors. This allowed the identification of a common proteomic core and highlighted central pathways consistently linked to CFTR dysfunction, while reducing redundancy across conditions.

The CFTR-related proteomic network formed a large, homogeneous structure where only high-confidence edges (score >0.7) were considered (Fig 3.3.18b). Notably, even proteins absent in some conditions were well integrated into the network without forming isolated or disconnected clusters. Given the extensive nature of the PPI, we proceeded with a stratified network analysis to better characterize the specific contributions and functional associations of different protein subsets within the context of CFTR-related pathways.. We identified six distinct topological clusters, and for each cluster performed biological pathway enrichment using the GO Biological Process database (Appendix B).

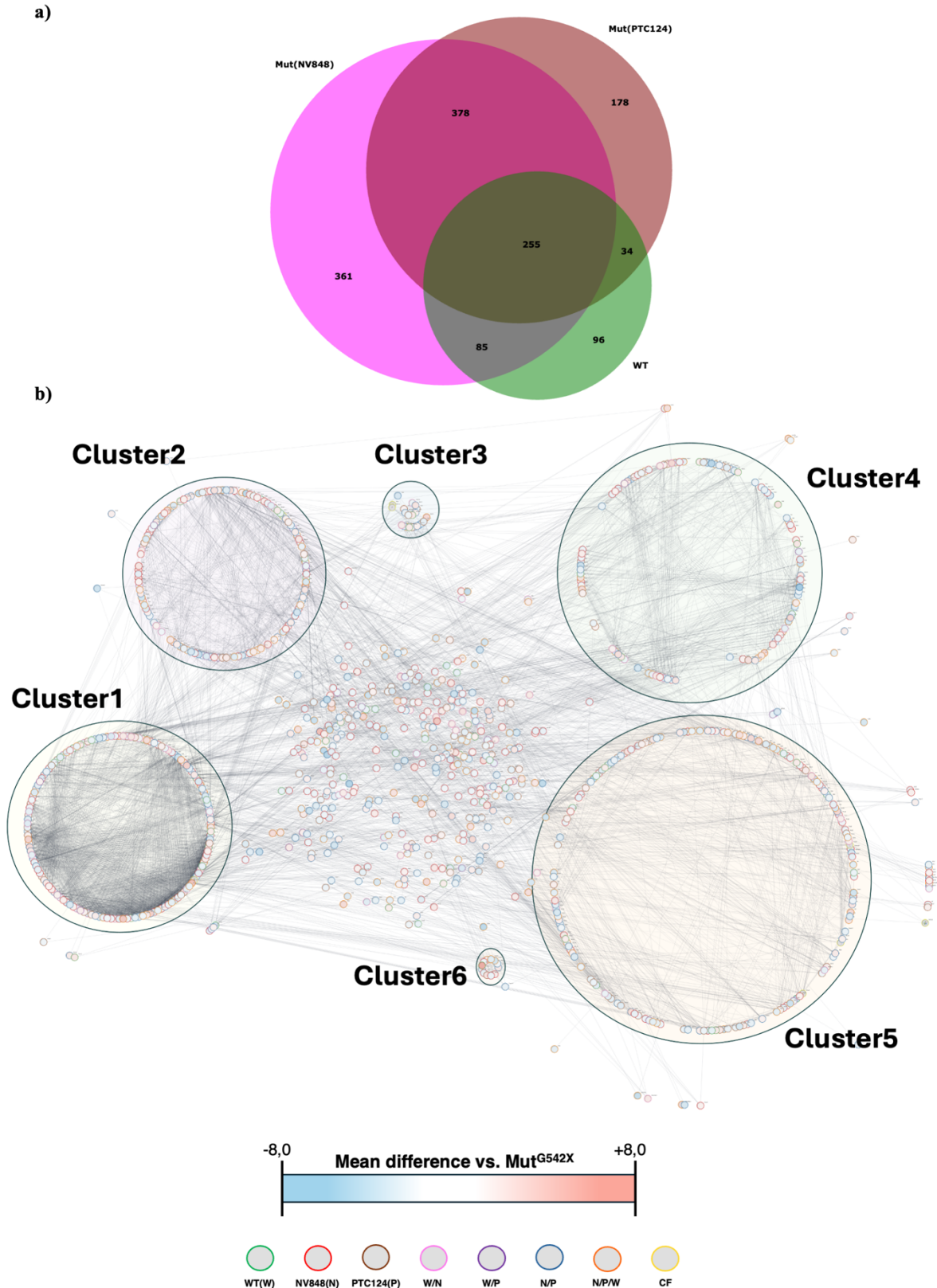


Figure 3.3.18 Brain CFTR-related PPI network. a) Venn diagram of observed DEPs in WT, MutNV848 and MutPTC124 compared to Mut; b) PPI network of DEPs in WT, MutNV848 and MutPTC124 compared to Mut, generated using the STRING database. Significantly upregulated and downregulated proteins ($FDR < 0.01$) are shown in red and blue, respectively. Topological clusters were identified using the MCODE algorithm, border color of each node indicates the condition in which the corresponding DEP was observed.

Functional analysis highlighted “signalling,” “developmental process,” and “transport” as prominently enriched categories. The “signalling” term is consistent with altered neuronal activity, in line with reports of neuronal CFTR expression and chloride channel-dependent excitability. In contrast, “developmental process” and “transport” have been only sparsely linked to CFTR dysfunction, but likely reflect stress-adaptive remodelling of vesicular pathways. Together, these findings suggest a previously unrecognized role for CFTR in vesicle trafficking and synaptic sorting, broadening its functional impact beyond classical epithelial contexts. Importantly, DEPs within these enriched pathways were observed not only in WT, but also in MutNV848 and MutPTC124, showing a trend toward WT-like expression, which suggests that TRID treatment partially realigns altered pathways in mutant mice through recovery of CFTR expression and functionality.

These findings were further supported by proteomic analysis (Fig 3.3.19), where we observe upregulation of Mapre2 and downregulation of Adam22, Msn, Prkacb, and Mapk9 in Mut compared to WT controls. These proteins represent key functional nodes within the CFTR-related network modules coherent with enrichment in “signalling,” “developmental process,” and “transport”. Specifically, Adam22²³⁹, a postsynaptic receptor essential for stabilizing glutamatergic synapses, and Mapre2²⁴⁰, a regulator of microtubule dynamics and intracellular transport, suggest that CFTR dysfunction leads to impaired synaptic maturation and microtubule instability. The reduction of Msn, which links the actin cytoskeleton to membrane proteins and supports vesicular trafficking, further indicates a weakening of endo-exocytic pathways. In parallel, decreased levels of Prkacb²⁴¹, the catalytic subunit of PKA directly involved in CFTR phosphorylation and activation, and Mapk9 (JNK2), a kinase of the MAPK pathway²⁴², point to a disruption of cAMP-dependent phosphorylation cascades and intracellular signalling networks. This combination of proteins expression alterations suggests that the loss of CFTR not only impairs ion conductance but also influences synaptic connectivity, microtubule dynamics, and neuronal signaling circuits, thereby expanding its functional impact well beyond the classical epithelial context. Treatment with NV848 effectively realigned the expression of these proteins to WT levels in all cases confirming its recovery ability. Notably, we report for the first time an association between CFTR, Adam22, Mapre2, Msn, Prkacb, and Mapk9, uncovering previously unrecognized metabolic relationships influenced by CFTR regulation.

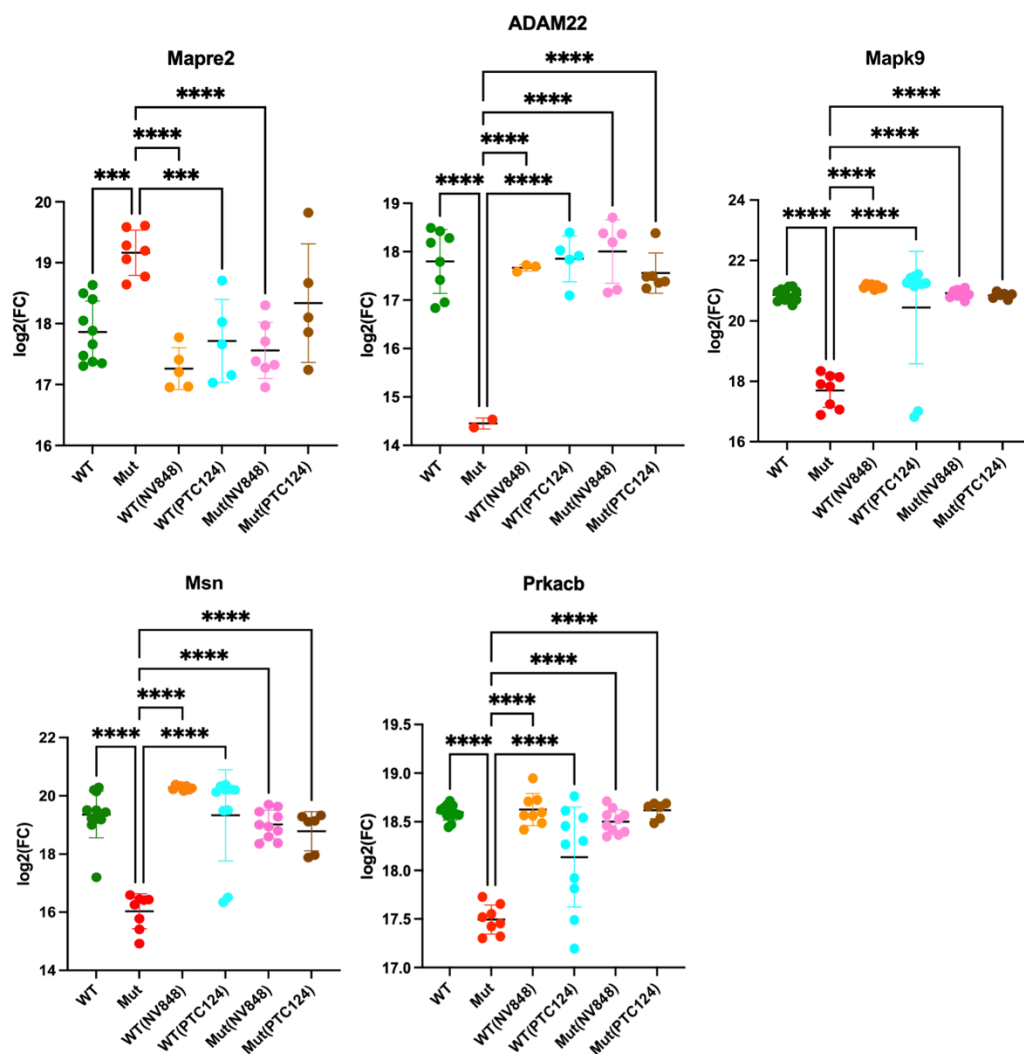


Figure 3.3.19 Pancreatic LFQ comparison of CFTR-related DEPs. Data were analyzed with GraphPad Prism 6 and are expressed as mean $\pm$ SEM. Statistical significance relative to Mut is indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (ANOVA with Dunnett's post hoc test).

Cross tissue consideration.

Overall, our findings highlight the crucial role of CFTR in cellular metabolism, particularly in bioenergetics and protein homeostasis in mice. We demonstrate a strong link between CFTR functionality and mitochondrial activity, where its disruption may promote a basal inflammatory state. In addition, we uncover tissue-specific mechanisms, including disrupted glycogen–lipid coordination in the liver and impaired neuronal signalling in the brain. In this context, both NV848 and PTC124 were able to remodel the proteomic profiles of mutant tissues, partially in brain and pancreas and more extensively in liver, realigning them toward WT patterns. Importantly, NV848 induced deeper proteomic remodelling and achieved closer alignment with WT than PTC124, confirming its superior efficacy in the examined CF mouse model.

Notably, differences in the expression of selected markers, as well as the broader proteomic shifts observed between MutNV848 and MutPTC124, suggest that in mice, the tissue proteome responds proportionally to the extent of CFTR recovery, consistent with findings recently reported by Pibiri's group. This raises the possibility that even partial loss of CFTR function, such as in monoallelic mutation, could lead to significant proteomic alterations that predispose to inflammatory conditions and possibly neurodegenerative disease. Such evidence redefines the impact of CFTR mutations, positioning them not only as the cause of a genetic autosomal recessive disease but also as a potential risk factor relevant to approximately 1 in 25 individuals in the general population. These results broaden the clinical significance of CFTR mutations beyond inherited disease, underscoring the need to investigate heterozygous carriers as a potentially at-risk population for inflammatory and neurodegenerative disease.

CHAPTER IV

CONCLUSIONS

Nonsense mutations account for approximately 11% of genetic diseases and 12% of cancer cases, where they are frequently associated with poor prognosis. More than 7,000 rare genetic disorders are known to affect an estimated 350 million people worldwide.

Moreover, data on TP53 (“the guardian of the genome”)—a pivotal tumour-suppressor gene—indicate that it is mutated in around 50% of all human cancers.

Approximately 10% of these TP53 mutations are nonsense variants (i.e., premature termination codons). This means that tens to hundreds of millions of patients could potentially benefit from therapies specifically targeting nonsense mutations, highlighting the urgency for new TRID-discovery efforts.

Among genetic diseases, cystic fibrosis (CF) is the most common life-limiting autosomal recessive disease in Caucasians, with an incidence of approximately 1 in 2500-3500 newborn in Europe. It is caused by mutations in the CFTR gene, which encodes the cystic fibrosis transmembrane conductance regulator protein. Since the identification of the link between CF onset and CFTR mutations, more than 2,000 variants have been registered, of which around 300 have been functionally characterized. Among these, nonsense mutations account for approximately 10% of cases.

nmCF is typically associated with a more severe clinical phenotype and patients with nonsense mutations currently rely only on high cost symptomatic management and risk mitigation.

On the other side, TP53, which encodes the p53 transcription factor, a pivotal regulator of genomic integrity, is mutated in more than 50% of human cancers, with approximately 10% of these mutations being nonsense variants. TP53 nonsense mutations not only drive tumorigenesis but are also increasingly implicated in a spectrum of emerging p53-related diseases, underscoring the urgent need for targeted therapeutic strategies⁵⁹.

Translational readthrough (TR) therapy represents a targeted precision medicine strategy that restores protein expression disrupted by nonsense mutations through a pharmacological, non-invasive approach, thereby enabling the treatment of NRDs

In this context, the non-renewal of PTC124 and the absence of alternative FDA-approved pharmacological therapies underscore the urgent need for novel TRIDs. The importance of discovering new TRIDs is further highlighted by the fact that nonsense mutations represent a common pathogenic cause across numerous NRDs. Newly identified TRIDs could potentially be repurposed for the treatment of heterogeneous diseases, including cancer, thereby extending the applicability of TR-based therapies to the oncologic field.

However, the discovery of new TRIDs remains a challenging task, as their precise MoA is still unclear and current TRID scaffolds are limited to only a few chemical structures.

Optimized drug development strategies, via extensively integrating CADD, provide a way to overcome the limitations posed by unknown molecular targets while optimizing both time and resources. Moreover, the introduction of advanced analytical methods such as proteomics in NRD research enables a more comprehensive characterization of the biological impact and effectiveness of TRIDs. Proteomics not only supports the validation of TRID efficacy but also serves as a unique tool for identifying novel proteins and biomarkers linked either to specific diseases, thereby offering new insights into the MoA of these promising molecules.

Building on the work developed by my research group and aiming to address these gaps, my doctoral project pursued three main objectives: (i) pharmacophore modelling SAR analysis for the identification of novel TR inducers and their synthesis; (ii) evaluation of biological activity *in vitro* using validated reporter systems to recover TP53 expression carrying a nonsense mutation; and (iii) *in vivo* proteomic evaluation of the impact of translational readthrough therapy with NV848 and PTC124 for CFTR recovery in mice model non-canonical target organs: pancreas, brain, and liver.

To address the first objective, we implemented an *in silico* drug discovery workflow that enables the rapid identification and characterization of candidate molecules while minimizing time and material consumption. Specifically, a ligand-based pharmacophore model was developed and validated as a predictive tool for the identification of novel translational readthrough-inducing drugs (TRIDs). The model, supported by high enrichment factors and robust ROC curve performance, successfully guided the selection of candidate scaffolds from both in-house collections and virtual screening libraries.

From the in-house library screening, we identified and synthesized eleven 1H-1,2,4-triazole derivatives and six diaryl urea derivatives, to which two additional compounds (IP28 and IP29) were added as byproducts of the reactions, as detailed in the synthesis and characterization section.

Interestingly, some 1H-1,2,4-triazole derivatives have been reported, in preliminary studies exploiting reporter system assay, to induce TR. This heterocyclic scaffold confers metabolic stability, structural rigidity, and the capacity to engage in diverse non-covalent interactions, making it attractive across pharmaceuticals, agriculture, and materials science. Its properties can be further optimized through carbon functionalization, improving solubility and pharmacokinetic behaviour. Compared with 1,2,4-oxadiazoles (e.g., ataluren), triazoles are less prone to light- or heat-induced rearrangements that generate regioisomers, thereby providing superior long-term stability. Notably Diaryl ureas have never been reported to display TR activity nevertheless, their importance in medicinal chemistry is well established. More than a

century has passed since the clinical introduction of suramin—the first chemotherapeutic agent developed through a medicinal chemistry project—and today several diaryl urea derivatives are approved anticancer drugs. Among the most clinically relevant examples are sorafenib, approved in 2005 for the treatment of renal cell carcinoma, and lenvatinib, approved in 2015 for recurrent and metastatic thyroid cancer, both of which underscore the therapeutic success of the diaryl urea scaffold in the therapeutic field. The broad utility of diaryl ureas derives from their ability to form stable, highly directional hydrogen bonds that synergize with π -interactions, particularly CH- π and π - π stacking, resulting in high binding specificity and affinity for their molecular targets.

Upon evaluation with the FLuc assay to assess TR activity, five 1H-1,2,4-triazole derivatives and three diaryl urea derivatives were found to induce readthrough.

Among the synthesized compounds, 1H-1,2,4-triazole derivatives demonstrated measurable readthrough activity, particularly when bearing bulky aromatic substituents at C-5 combined with hydrogen bond acceptor groups at C-3. Diaryl urea derivatives, although previously unreported in this context, also exhibited activity, with SAR analysis highlighting the superior performance of 2-naphthyl over 1-naphthyl substitution, the favorable impact of meta- over para-substitution on the phenyl ring, and the importance of scaffold asymmetry and hydrogen-bonding functionality.

Taken together, these findings underscore the recurring leitmotif of heteroatom-rich cores, such as triazole, urea, and oxadiazole, capable of forming strong directional interactions as a fundamental requirement for TR-inducing activity. Importantly, these SAR trends are consistent with the pharmacophore model, which highlighted the critical role of multiple hydrogen bond acceptors and a donor feature, thereby reinforcing the relevance of these interactions. The identification of FDA-approved pharmacophore hits further points to opportunities for drug repurposing. Overall, this work establishes a rational framework for TRID discovery, highlights two new chemotypes with promising activity, and sets the stage for further optimization, mechanistic investigation, and evaluation in disease-relevant models.

Subsequently to address the second objective, the potential of newly synthesized 1H-1,2,4-triazole derivatives (IP14, IP15, IP17, and IP18) in TR-based therapy for the recovery of nonsense-mutated p53. Four 1H-1,2,4-triazole derivatives were selected for their synthetic accessibility and to enable a systematic comparison of the triazole core with the 1,2,4-oxadiazole scaffold of PTC124, the reference compound and the only EMA-approved TRID. This approach was designed to assess the impact of core substitution on translational readthrough (TR) activity.

Although being limited to the use of H1299 p53^{R213X} cells, in which the cDNA-based reporter construct is not fully subject to the classical NMD pathway and may alter p53 expression dynamics relative to physiological controls, the use of multiple complementary techniques consistently support p53 recovery and function following IP14, IP15, IP17, and IP18 treatment. The predicted ADMET profiles of IP14, IP15, IP17, and IP18 further support their viability as drug candidates, highlighting favorable bioavailability, metabolic stability, and low predicted toxicity.

These compounds offer a potential strategy for addressing a common genetic cause of cancer, responsible for approximately one million new cancer cases globally each year.

Notably, this findings provides the first evidence that 1,2,4-triazole-based compounds can function as low-toxicity TRIDs with therapeutic potential in targeting nonsense mutations. These findings are relevant not only to cancer therapy but also to emerging p53-associated conditions, including cardiovascular and neurodegenerative disorders, where chronic treatment would be incompatible with the basal oxidative stress induced by G418, an effect not observed with IP compounds, as well as to other diseases driven by nonsense mutations.

Future studies will further validate the restoration of p53 functionality using additional reporter systems harboring constitutive nonsense-mutated p53, as well as in more complex *in vivo* models. With their favorable ADMET profiles and ability in restoring p53 functionality, these compounds hold promise as candidates for future preclinical development and potential clinical translation, offering a compelling treatment strategy for cancers driven by nonsense mutations. Finally, to address the last objective, during my visiting period at the NOVA University of Lisbon, under the supervision of Prof. L. Capelo-Martínez and Prof. H.M. Santos, we investigated the *in-vivo* impact of TR-based therapy for CFTR recovery in non-canonical target organs (pancreas, brain, and liver) through proteomic analysis WT and Mut mice untreated and treated with NV848, a drug prone to clinical trial developed by Prof. Pibiri group, and PTC124 used as comparative benchmark.

NV848 has emerged as a particularly promising candidate based on its robust preclinical performance, restoring full-length, functional CFTR expression both *in vitro* and *in vivo* with favourable tolerability profile and a good pharmacokinetic profiling

Proteomic analysis of NV848 treatment in WT and CF nonsense mutant mice represents the first proteomic application to TR-based therapy in a mammalian CF model. This approach not only confirmed CFTR recovery but also revealed how NV848 remodels protein biosynthesis, trafficking, redox balance, and inflammatory signaling, while uncovering new CFTR-related pathways and TR-linked proteins.

By extending investigation to pancreas, liver, and brain, organs less explored than lungs and intestines, this study provides novel insights into CF pathology in non-canonical tissues and complements previous Western blotting and immunohistochemistry findings. The organs were kindly provided by the ATEN Center Animal Facility Lab at the University of Palermo, where, in collaboration with Prof. Zizzo, *in vivo* studies were conducted on G542X mice. In these models, CFTR recovery in the lungs had already been demonstrated, as reported in previous studies

Comprehensive proteomic profiling of pancreas, liver, and brain tissues from WT and G542X CF mice highlighted widespread CFTR-related alterations and the corrective effects of NV848. In the pancreas, analysis identified ~3,295 proteins, with 3,205 included in statistical evaluation. NV848 modulated 633 proteins compared with only 103 for PTC124, with a shared core of 158 DEPs defining a CFTR-related proteomic signature. Importantly, NV848 was more effective in realigning mutant expression patterns to WT levels, particularly by reducing inflammatory and ER stress markers. PPI analyses showed enrichment in calcium transport, energy metabolism, and inflammatory pathways, fully consistent with CFTR's physiological role. Furthermore, novel associations with proteins such as eIF4B, LMOD2, and SCN8A were revealed, suggesting new regulatory and metabolic mechanisms linked to CFTR.

In the liver, over 5,500 proteins were identified, of which ~1,200 were used for statistical analysis. NV848 treatment shifted the mutant proteome much closer to the WT profile than PTC124, correcting 491 DEPs compared to 303. Importantly, NV848 restored expression of antioxidant enzymes (Sod1, Cat, Gpx2), mitochondrial regulators (Glud1, Etfa), and proteostasis components (Hspd1, Psma3, Eef1a1), all impaired in untreated mutants. The corrected pathways included regulation of inflammation, oxidative stress balance, and mitochondrial metabolism. Novel CFTR associations with metabolic regulators such as Glud1 and Etfa expand the understanding of CFTR's role in hepatic homeostasis. Importantly, no off-target alterations were detected in WT livers, underscoring the favorable tolerability of NV848. In the brain, ~2,664 proteins were identified, confirming profound proteomic changes associated with CFTR loss, including alterations in neuronal signaling, synaptic development, and vesicular trafficking. NV848 outperformed PTC124 in normalizing these alterations, rescuing the expression of key neuronal proteins such as Adam22, Mapre2, Msn, Prkacb, and Mapk9. Notably, NV848 also upregulated STOML2, a mitochondrial protein linked to biogenesis, respiratory chain stabilization, and neuroprotection, indicating compensatory mechanisms that may mitigate neuronal stress. While some proteomic shifts were also observed in treated WT brains, no evidence of toxicity was found, further supporting the safety of NV848.

Collectively, our results highlight the crucial role of CFTR in cellular metabolism, particularly in bioenergetics and proteostasis. We confirmed strong links between CFTR function and mitochondrial activity, with its impairment promoting basal inflammation. Tissue-specific mechanisms were revealed, including glycogen–lipid coordination defects in the liver and neuronal signaling disruption in the brain.

Both NV848 and PTC124 remodeled mutant proteomes toward WT profiles, with NV848 consistently showing deeper remodeling and closer alignment. This superior efficacy underscores its therapeutic promise.

Notably, the differential responses observed between NV848 and PTC124 treatments suggest that tissue proteomes adjust in proportion to the degree of CFTR recovery. This raises the intriguing possibility that even partial CFTR deficiency such as that seen in heterozygous carriers, may contribute to increased susceptibility to inflammatory or neurodegenerative conditions. These findings extend the clinical relevance of CFTR mutations beyond classical recessive rare diseases, suggesting they may act as broader risk factors within the general population. (~1 in 25 carriers).

In conclusion, my doctoral research helps to give a contribute to advances the field of nonsense mutation therapeutics by integrating rational drug design, *in vitro* validation, and *in vivo* proteomic investigation to uncover novel TRIDs and evaluate their translational potential. From the discovery of diaryl urea– and triazole-based chemotypes with p53-restoring activity, to the in-depth proteomic characterization of NV848 across non-canonical CF target organs, this work establishes a comprehensive framework for both innovation and mechanistic insight. The results not only reinforce NV848 as a superior candidate compared with ataluren but also expand the understanding of CFTR’s systemic role, linking its dysfunction to broader metabolic and neurological pathways. By bridging drug discovery, functional recovery, and systems-level biology, this thesis underscores the therapeutic promise of TR-based strategies for rare genetic disorders and cancer, while opening new perspectives on the clinical significance of CFTR mutations, even in heterozygous carriers. These findings provide a strong foundation for the continued development of NV848 toward clinical application and for the broader application of TRIDs in precision medicine.

Beyond its scientific and translational relevance, this research also holds significant economic and social implications. The development of effective TRIDs such as NV848 offers a promising route to reduce the long-term healthcare costs associated with chronic management of genetic diseases like cystic fibrosis, by addressing the molecular cause rather than merely alleviating symptoms. For example, the Socio-economic burden of cystic fibrosis in Europe study reports

that the annual per-patient cost ranges from approximately €14,000 to €53,000, depending on disease severity and country, with pediatric cases reaching €60,000 per year²⁴³. Moreover, an analysis covering 23 rare diseases estimated an average yearly cost per patient of about €107,000, nearly 15 times higher than that for high-prevalence diseases ($\approx$ €7,000 per year)^{244,245}.

In addition to these direct healthcare costs, the economic impact on caregivers represents a substantial yet often underrecognized burden. Studies have shown that families caring for patients with cystic fibrosis or other rare genetic diseases experience a loss of income ranging from €5,000 to €20,000 per year, primarily due to reduced working hours or job discontinuation to provide daily care²⁴³.

Caregivers also face significant indirect costs, including increased healthcare use, psychosocial stress, and reduced productivity, which can cumulatively exceed 30–40% of the total economic burden of rare diseases²⁴⁶.

By potentially reducing hospitalization rates, improving treatment efficacy, and enhancing patients' quality of life, TRID-based therapies could significantly alleviate both direct healthcare costs and indirect socio-economic burdens such as loss of productivity and family caregiving demands. On a broader scale, the methodological framework developed in this work promotes sustainable and efficient drug discovery, optimizing early-stage validation to accelerate clinical translation. Ultimately, this research not only contributes to scientific innovation but also supports a more equitable and economically sustainable healthcare paradigm, reinforcing the societal value of investment in precision medicine and rare disease research.

CHAPTER V

MATERIALS AND METHODS

Ligand-based Pharmacophore model design

The pharmacophore model was developed through a ligand-based approach exploiting available experimental data on our previously identified compounds and compounds present in literature^{169,182,183,184,185,186,187,188} with proven TR-inducing activity reported in Appendix A, using LigandScout 4.4 software.

The operation of conformer generation and alignment on the library's structure was first performed before the pharmacophore model generation. Subsequently, we proceed with the identification of a common core of shared features. These shared features served as the basis for merging additional features, thereby enriching the pharmacophore model. During the generation process, certain features were excluded to maintain the optimal features of the pharmacophoric model.

The obtained pharmacophore model has undergone a validation process to assess its capability to discern between active and inactive molecules and ensure the trustfulness and accuracy of the pharmacophore analysis.

The validation was conducted via screening of libraries formed by 61 known active molecules and 2651 decoys generated using the online enhanced Databases of Useful Decoys (DUDe) and a Receiver Operating Characteristic (ROC) curve was produced.

After validation, the pharmacophore model was used to screen new “*in house*” libraries of molecule candidates for synthesis to find new hits compounds.

Synthesis

All reagents and solvents were purchased from commercial sources. All reagents for synthesis were supplied from Merck KGaA (Darmstadt, DE), deuterated solvent and not employees for synthesis, purification, and characterization were bought from Carlo Erba (Milano, IT). All the compounds were characterized using ¹H NMR and HPLC-MS ensuring the purity (>95%). ¹H NMR spectra were recorded on Bruker 400 MHz and 300 MHz spectrometers and reported in Table A2 of Appendix A. HR-MS analysis was conducted on a 6540 UHD Accurate-Mass Q-TOF LC/MS (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a Dual AJS ESI source recording spectra on 10 ppm solution for each sample reported in Table A3 of Appendix A.

IP8 (5-methyl-3-acetamido-1H-1,2,4-triazole) synthesis: IP8 was obtained from 100 mg (0,735 mmol) of aminoguanidine bicarbonate (**1**) and 66 mg (1,10 mmol) of acetic acid reacting in toluene at reflux for 12h. Later, after cooling the reaction mixture, the solvent was removed for

evaporation under vacuum and the products were purified by chromatography on silica gel using a mixture of Ethyl acetate (AcEt) and Methanol (MetOH) (8:1); Scheme 2.1.1.

IP8: yield 12%, ^1H NMR (400MHz, DMSO- d_6) δ (ppm): 2,05 (s, 3H), 2,18 (s, 3H), 11,31 (s, 1H), 12,86 (s, 1H); HRMS for $\text{C}_5\text{H}_8\text{N}_4\text{O}$ expected ($\text{M}+\text{H}^+=141,0771$), observed ($\text{M}+\text{H}^+=141,0851$)

IP10 (5-trifluoromethyl-3-ammino-1H-1,2,4-triazole) synthesis: IP10 was obtained from 100 mg (0,735 mmol) of aminoguanidine bicarbonate and 125 mg (1,10 mmol) of trifluoroacetic acid reacting in toluene at reflux for 12h. Later, after cooling the reaction mixture, the solvent was removed for evaporation under vacuum and the product was purified by chromatography on silica gel using a mixture of AcEt/MetOH (10:1) ; Scheme 2.1.1.

IP10: yield 96%, ^1H NMR (400MHz, DMSO- d_6) δ (ppm): 6,83 (s, 2H), 12,70 (s, 1H); HRMS for $\text{C}_3\text{H}_3\text{N}_4\text{F}_3$ expected ($\text{M}+\text{H}^+=153,0382$), observed ($\text{M}+\text{H}^+=153,0389$).

IP11 (N-(5-(trifluoromethyl)-1H-1,2,4-triazol-3-yl)acetamide) synthesis: IP 11 was obtained from 100 mg (0.735 mmol) of aminoguanidine bicarbonate and 125 mg (1,10 mmol) of trifluoroacetic acid reacting in toluene at reflux for 12h. Later, after cooling the reaction mixture, the solvent was removed for evaporation under vacuum and the product was purified by chromatography on silica gel using a mixture of AcEt/MetOH (10:1).

Subsequently, 50 mg (0.32 mmol) of 5-trifluoromethyl-3-ammino-1H-1,2,4-triazole were then reacted with 49 mg (0.47 mmol) of acetic anhydride in acetic acid at 40 °C for 12 h. The reaction mixture was cooled, concentrated under vacuum, and the final product was purified by silica gel chromatography using AcOEt as eluent.

IP11: yield 80%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 14.14 (s, 1H), 7.91 (s, 1H), 2.54 (s, 3H).

IP12 (5-fenyl-3-benzamido-1H-1,2,4-triazole) and IP13 (5-fenyl-3-ammino-1H-1,2,4-triazole) synthesis: IP12 and IP13 were obtained from 100 mg (0,735 mmol) of aminoguanidine bicarbonate (1) and 66 mg (1,10 mmol) of benzoic acid reacting in toluene at reflux for 12h. Later, after cooling the reaction mixture, the solvent was removed for evaporation under vacuum and the products were purified by chromatography on silica gel using a mixture of AcEt/MetOH (10:1).

IP12: yield 20%, ^1H NMR (400MHz, DMSO- d_6) δ (ppm): 7,53-7,47 (m, 2H), 7,65-7,59 (m, 2H), 7,94 (d, 2H), 7,97 (d, 2H), 12,92 (s, 2H); HRMS for $\text{C}_{15}\text{H}_{14}\text{N}_4\text{O}$ expected ($\text{M}+\text{H}^+=265,1083$), observed ($\text{M}+\text{H}^+=265,1208$); Scheme 2.1.1.

IP13: yield 79%: ^1H NMR (400MHz, DMSO- d_6) δ (ppm): 5,95 (s, 2H), 7,32 (t, 1H), 7,39 (t, 2H), 7,87 (d, 1H), 7,89 (d, 1H), 12,13 (s, 1H); HRMS for $\text{C}_8\text{H}_8\text{N}_4$ expected ($\text{M}+\text{H}^+=161,08217$), observed ($\text{M}+\text{H}^+=161,1067$).

IP14 (5-phenyl-3-acetamido-1H-1,2,4-triazole) synthesis: IP 14 was obtained from 100 mg (0.735 mmol) of aminoguanidine bicarbonate (**1**) and 66 mg (1,10 mmol) of benzoic acid reacting in toluene under reflux for 12 h (Scheme 1). Then, after cooling the reaction mixture, the solvent was removed under vacuum and the 5-phenyl-1H-1,2,4-triazol-3-amine was purified by chromatography on silica gel using a mixture of AcOEt/MeOH (10:1).

Subsequently, 50 mg (0.31 mmol) of 5-phenyl-1H-1,2,4-triazol-3-amine were then reacted with 49 mg (0.47 mmol) of acetic anhydride in acetic acid at 40 °C for 12 h. The reaction mixture was cooled, concentrated under vacuum, and the final product was purified by silica gel chromatography using AcOEt as eluent); Scheme 2.1.2.

IP14: yield 80%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 2.12 (s, 3H), 7.41 (t, $J = 7.1$ Hz, 1H), 7.49 (t, $J = 7.4$ Hz, 2H), 7.95 (d, $J = 6.8$ Hz, 2H), 11.63 (s, 1H), 13.43 (s, 1H); HR-MS for $\text{C}_{10}\text{H}_{10}\text{N}_4\text{O}$ calculated ($\text{M}+\text{H}^+=203.0927$), found ($\text{M}+\text{H}^+=203.0930$).

IP15 (2,3,4,5,6-pentafluoro-N-(5-(perfluoro phenyl)-1H-1,2,4-triazol-3-yl)benzamide) synthesis: IP15 was obtained from 100 mg (0.735 mmol) of aminoguanidine bicarbonate and 269 mg (1.10 mmol) of pentafluorobenzoyl chloride reacting in toluene under reflux for 12 h (Scheme 2). Then, after cooling the reaction mixture, the solvent was removed under vacuum and the product was purified by chromatography on silica gel using AcOEt as eluent; Scheme 2.1.1.

IP15: yield 43%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 13.01 (s, 1H), 14.52 (s, 1H); HR-MS per $\text{C}_{15}\text{H}_2\text{N}_4\text{OF}_{10}$ calculated ($\text{M}+\text{H}^+=445.0141$), found ($\text{M}+\text{H}^+=445.0171$).

IP17 (3-(3-(2-fluorophenyl)-1H-1,2,4-triazol-5-yl)benzoic acid) synthesis: IP17 was obtained from 200 mg (1.36 mmol) of 3-cyanobenzoic acid firstly converted into the corresponding ester through reaction with 287 mg (2.04 mmol) of methyl iodide and 187 mg (1.36 mmol) of potassium carbonate in 5 ml of DMF for 2 h at room temperature (Scheme 3). The 3-cyano-methyl benzoate was quantitative isolated after liquid/liquid extraction (H_2O / AcOEt).

The 3-cyano-methyl benzoate (**5**) was converted in the corresponding Pinner salt (**6**) by reaction with acidic ethanolic solution (EtOH/HCl), isolated for evaporation under vacuum, and used to obtain the methyl 3-(hydrazineyl(imino))benzoate (**7**) by reaction of the latter with 217 mg (6.8 mmol) of hydrazine monohydrate in 5 ml of pyridine for 2 h.

Finally, 100 mg of methyl 3-(hydrazineyl(imino))benzoate (0.56 mmol) and 132 mg of 2-fluorobenzoyl chloride (0.84 mmol) were set to react in toluene under reflux for 24 h.

Then, after cooling the reaction mixture, the solvent was removed for evaporation under vacuum and the products were purified by chromatography on silica gel using a mixture of AcOEt/Petroleum Ether (2:1); Scheme 2.1.5.

IP17: yield 42%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 7.38-7.32 (m, 2H), 7.64-7.58 (m, 1H), 7.68 (t, $J = 7.82$ Hz, 1H), 7.77 (t, $J = 7.8$ Hz, 1H), 8.09 (d, $J = 7.8$ Hz, 1H), 8.23 (d, $J = 8.1$ Hz, 1H), 8.32 (s, 1H), 10.47 (s, 1H), 10.83 (s, 1H); HR-MS for $\text{C}_{15}\text{H}_{10}\text{N}_3\text{O}_2\text{F}$ calculated ($\text{M}+\text{H}^+=284.0829$), found ($\text{M}+\text{H}^+=284.0829$).

IP18 (3-methoxy-5-(p-tolyl)-1H-1,2,4-triazole) synthesis: IP 18 was obtained following the method reported in literature from Mali et al. 2017 [43]. In particular, 100 mg of semicarbazide (**9**) (1.33 mmol), 169 mg (1.33 mmol) of 4-methylbenzaldehyde, and 178 mg (1.45 mmol) of 4-dimethylaminopyridine (DMAP) were set to react in methanol under reflux for 24 h (Scheme 4).

Then, after cooling the reaction mixture, the solvent was removed for evaporation under vacuum and the products were purified by chromatography on silica gel using a mixture of dichloromethane (DCM) and MeOH (2:1); Scheme 2.1.3.

IP18: yield 70%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 2.24 (s, 3H), 3.93 (s, 3H), 7.29 (d, $J = 7.8$ Hz, 2H), 7.80 (d, $J = 8.1$ Hz, 2H), 13.54 (s, 1H); HR-MS for $\text{C}_{10}\text{H}_{11}\text{N}_3\text{O}$ calculated ($\text{M}+\text{H}^+=190.0974$), found ($\text{M}+\text{H}^+=190.1233$).

IP19 (5-(p-tolyl)-1H-1,2,4-triazol-3-amine) synthesis: IP19 was obtained starting from 50 mg of 3-chloro-5-(p-tolyl)-1,2,4-oxadiazole (**10**) (0.25 mmol) and 19 mg of hydrazine monohydrate (0.38mmol) in DMF at r.t for 2h.

After completion, the reaction mixture was subjected to liquid-liquid extraction ($\text{H}_2\text{O}/\text{EtOAc}$) with a basic aqueous phase. The organic layer was dried, concentrated, and the crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (2:1) as eluent; Scheme 2.1.4.

IP19: yield 80%, ^1H NMR (300 MHz, DMSO- d_6) δ (ppm): 12.06 (s, 1H), 7.83 (d, J = 8.26 Hz, 2H), 7.25 (d, J = 7.50 Hz, 2H), 6.12 (s, 1H), 2.34 (s, 1H); HR-MS for $\text{C}_9\text{H}_{10}\text{N}_4$ calculated ($M+\text{H}^+=175.0905$), found ($M+\text{H}^+=175.1275$).

IP20 (5-(furan-2-yl)-1H-1,2,4-triazol-3-amine) synthesis: IP20 was obtained starting from 50 mg of 3-chloro-5-(furan-2-yl)-1,2,4-oxadiazole (**11**) (0.29 mmol) and 33 mg of hydrazine monohydrate (0.66 mmol) in DMF at r.t for 2h.

After completion, the reaction mixture was subjected to liquid–liquid extraction ($\text{H}_2\text{O}/\text{EtOAc}$) with a basic aqueous phase. The organic layer was dried, concentrated, and the crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (2:1) as eluent; Scheme 2.1.4.

IP19: yield 80%, ^1H NMR (300 MHz, DMSO- d_6) δ (ppm): 12.08 (s, 1H), 7.66 (s, 1H), 6.66 (s, 1H), 6.52 (s, 1H), 6.08 (s, 1H); HR-MS for $\text{C}_6\text{H}_6\text{N}_4\text{O}$ calculated ($M+\text{H}^+=151.0614$), found ($M+\text{H}^+=151.0778$).

Methyl 3-aminobenzoate synthesis: Methyl 3-aminobenzoate was prepared starting from 151 mg of 3-nitrobenzaldehyde (**12**) (1.0 mmol), which was reacted with 254 mg of silver nitrate (AgNO_3 , 1.5 mmol) in aqueous potassium hydroxide (7% w/v) at 60 °C for 1 h. After cooling, the reaction mixture was filtered through filter paper. The filtrate was collected and acidified to $\text{pH} < 2$ in an ice bath, affording the precipitation of 3-nitrobenzoic acid (**13**). The solid was collected by Büchner filtration, washed with acidic water ($\text{pH} < 5$), and isolated quantitatively. The obtained 3-nitrobenzoic acid was dissolved in methanol containing 1% H_2SO_4 and refluxed for 16 h. The reaction mixture was then cooled to 4 °C, leading to crystallization of methyl 3-nitrobenzoate (**14**), which was isolated by Hirsch filtration.

Finally, 181 mg of methyl 3-nitrobenzoate (1.0 mmol) was reacted with 87 mg of sodium borohydride (NaBH_4 , 2.3 mmol) and 6 mg of nickel boride (0.05 mmol) in water at room temperature for 2 h. The mixture was filtered, basified to $\text{pH} > 8$, and the product methyl 3-aminobenzoate (**15**) was quantitatively isolated by liquid–liquid extraction ($\text{H}_2\text{O}/\text{EtOAc}$) ; Scheme 2.1.6.

Methyl 3-aminobenzoate: yield 96%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): δ 7.94 (d J = 7.8, 1H), 7.40 (t, J = 7.8 Hz), 7.28 (d, J = 7.8, 1H), 6.56 (s, 1H), 5.25 (s, 2H) 3.82 (s, 3H).

Methyl 4-aminobenzoate synthesis: Methyl 4-aminobenzoate was prepared starting from 151 mg of 4-nitrobenzaldehyde (**16**) (1.0 mmol), which was reacted with 254 mg of silver nitrate

(AgNO₃, 1.5 mmol) in aqueous potassium hydroxide (7% w/v) at 60 °C for 1 h. After cooling, the reaction mixture was filtered through filter paper. The filtrate was collected and acidified to pH < 2 in an ice bath, affording the precipitation of 4-nitrobenzoic acid (**17**). The solid was collected by Büchner filtration, washed with acidic water (pH < 5), and isolated quantitatively. The obtained 4-nitrobenzoic acid was dissolved in methanol containing 1% H₂SO₄ and refluxed for 16 h. The reaction mixture was then cooled to 4 °C, leading to crystallization of methyl 3-nitrobenzoate (**18**), which was isolated by Hirsch filtration.

Finally, 181 mg of methyl 4-nitrobenzoate (1.0 mmol) was reacted with 87 mg of sodium borohydride (NaBH₄, 2.3 mmol) and 6 mg of nickel boride (0.05 mmol) in water at r.t for 2 h. The mixture was filtered, basified to pH > 8, and the product methyl 4-aminobenzoate (**19**) was quantitatively isolated by liquid–liquid extraction (H₂O/EtOAc); Scheme 2.1.6.

Methyl 4-aminobenzoate: yield 96%, ¹H NMR (300 MHz, DMSO-d₆) δ (ppm): 7.63 (d, J = 8.8 Hz, 2H), 6.56 (d, J = 8.9 Hz, 2H), 5.95 (s, 2H) 3.73 (s, 3H).

IP21 ((4-(3-(naphthalen-1-yl)ureido)benzoic acid) synthesis: IP21 was prepared from 127 mg (0.75 mmol) of 1-naphthyl isocyanate (**20**) and 75 mg (0.50 mmol) of methyl 4-aminobenzoate in dry tetrahydrofuran (THF). The mixture was stirred at 0 °C and allowed to warm to room temperature over 48 h. The solvent was removed under reduced pressure, and the residue was treated with a 1:1 mixture of methanol and aqueous lithium hydroxide (1 M) at 60 °C for 24 h. After completion, the reaction mixture was subjected to liquid–liquid extraction (H₂O/EtOAc). The basic aqueous layer was acidified (pH < 2), affording precipitation of the target compound. The solid 4-(3-(naphthalen-1-yl)ureido)benzoic acid (IP21) was collected and purified by filtration; Scheme 2.1.7.

IP 21: yield 40%, ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 12.61 (s), 9.99 (s, 1H), 9.48 (s, 1H), 8.21 (d, J = 8.1 Hz, 1H), 7.98 (d, J = 7.6 Hz, 1H), 7.92 (t, J = 7.9 Hz, 3H), 7.68 – 7.43 (m, 6H); HR-MS for C₁₈H₁₄N₂O₃ calculated (M+H⁺=307.107), found (M+H⁺=307.1073).

IP22 ((3-(3-(naphthalen-1-yl)ureido)benzoic acid) synthesis: IP22 was prepared from 127 mg (0.75 mmol) of 1-naphthyl isocyanate (**20**) and 75 mg (0.50 mmol) of methyl 3-aminobenzoate in dry tetrahydrofuran (THF). The mixture was stirred at 0 °C and allowed to warm to room temperature over 48 h. The solvent was removed under reduced pressure, and the residue was treated with a 1:1 mixture of methanol and aqueous lithium hydroxide (1 M) at 60 °C for 24 h. After completion, the reaction mixture was subjected to liquid–liquid extraction (H₂O/EtOAc). The basic aqueous layer was acidified (pH < 2), affording precipitation of the target compound.

The solid 3-(3-(naphthalen-1-yl)ureido)benzoic acid (IP21) was collected and purified by filtration; Scheme 2.1.7.

IP 22: yield 28%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 12.92 (s, 1H), 9.76 (s, 1H), 9.35 (s, 1H), 8.45 (d, J = 28.9 Hz, 1H), 8.18 (t, J = 2.0 Hz, 1H), 8.07 (t, J = 8.1 Hz, 1H), 7.96 – 7.88 (m, 1H), 7.74 (d, J = 7.7 Hz, 1H), 7.66 – 7.38 (m, 6H). HR-MS for $\text{C}_{18}\text{H}_{14}\text{N}_2\text{O}_3$ calculated ($\text{M}+\text{H}^+=307.107$), found ($\text{M}+\text{H}^+=307.1072$).

IP6 (methyl 3-(3-(naphthalen-1-yl)ureido)benzoate) synthesis: IP6 was prepared from 127 mg (0.75 mmol) of 1-naphthyl isocyanate (**20**) and 75 mg (0.50 mmol) of methyl 3-aminobenzoate in dry tetrahydrofuran (THF). The mixture was stirred at 0 °C and allowed to warm to room temperature over 48 h. The solvent was removed under reduced pressure, and the residue was treated with a 1:1 mixture of methanol and aqueous lithium hydroxide (1 M) at 60 °C for 24 h. After completion, the reaction mixture was subjected to liquid–liquid extraction ($\text{H}_2\text{O}/\text{EtOAc}$). The basic aqueous layer was acidified ($\text{pH} < 2$), affording precipitation of the target compound. The solid 3-(3-(naphthalen-1-yl)ureido)benzoic acid was collected and purified by filtration. The obtained 3-(3-(naphthalen-1-yl)ureido)benzoic acid was dissolved in methanol containing 1% H_2SO_4 and refluxed for 16 h. After cooling, the solvent was removed under reduced pressure. The residue was subjected to liquid–liquid extraction ($\text{H}_2\text{O}/\text{EtOAc}$) with the aqueous phase basified prior to extraction. The combined organic extracts were dried and evaporated, and the product isolated; Scheme 2.1.7.

IP6: yield 38%, ^1H NMR (400 MHz, DMSO- d_6) δ (ppm): 9.29 (s, 1H), 8.78 (s, 1H), 8.25 (s, 1H), 8.10 (s, 1H), 7.98 (d, J = 21.5 Hz, 1H), 7.71 – 7.41 (m, 1H), 3.86 (s, 3H); HR-MS for $\text{C}_{19}\text{H}_{16}\text{N}_2\text{O}_3$ calculated ($\text{M}+\text{H}^+=321.1233$), found ($\text{M}+\text{H}^+=321.1220$).

2-naphtoic acid synthesis: 2-naphtoic acid (**21**) was prepared starting from 100 mg of 2-naphthylaldehyde (0.64 mmol), which was reacted with 135 mg of silver nitrate (AgNO_3 , 0.80 mmol) in aqueous potassium hydroxide (7% w/v) at 60 °C for 1 h. After cooling, the reaction mixture was filtered through filter paper. The filtrate was collected and acidified to $\text{pH} < 2$ in an ice bath, affording the precipitation of 2-naphtoic acid. The solid was collected by Büchner filtration, washed with acidic water ($\text{pH} < 5$), and isolated quantitatively.

2-naphtoic acid: yield 98%, ^1H NMR (300 MHz, DMSO- d_6) δ (ppm): 9.29 (s, 1H), 8.78 (s, 1H), 8.26 (s, 1H), 8.12 (d, J = 8.6 Hz, 2H), 7.99 (d, J = 7.6 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.71 – 7.41 (m, 7H).

IP23 (4-(3-(naphthalen-2-yl)ureido)benzoic acid) synthesis: IP23 was prepared from 50 mg of 2-naphthoic acid (**21**) (0.33 mmol), 30 mg of methyl 4-aminobenzoate (0.20 mmol), 81 mg of diphenylphosphoryl azide (DPPA, 0.30 mmol), and 60 mg of triethylamine (TEA, 0.60 mmol) in toluene. The reaction mixture was heated under microwave irradiation at 100 °C for 10 min. After completion, the mixture was subjected to liquid–liquid extraction (H₂O/EtOAc). The basic aqueous phase was acidified (pH < 2), leading to precipitation of the target compound. The solid 4-(3-(naphthalen-2-yl)ureido)benzoic acid (IP23) was collected by filtration and purified; Scheme 2.1.8.

IP23: yield 29%, ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 12.65 (s, 1H), 9.18 (s, 1H), 9.05 (s, 1H), 8.11 (s, 2H), 7.89 – 7.78 (m, 6H), 7.64–7.56 (m, 3H); HR-MS for C₁₈H₁₄N₂O₃ calculated (M+H⁺=307.107), found (M+H⁺=307.1072).

IP24 (3-(3-(naphthalen-2-yl)ureido)benzoic acid) synthesis: IP24 was prepared from 50 mg of 2-naphthoic acid (**21**) (0.33 mmol), 30 mg of methyl 3-aminobenzoate (0.20 mmol), 81 mg of diphenylphosphoryl azide (DPPA, 0.30 mmol), and 60 mg of triethylamine (TEA, 0.60 mmol) in toluene. The reaction mixture was heated under microwave irradiation at 100 °C for 10 min. After completion, the mixture was subjected to liquid–liquid extraction (H₂O/EtOAc). The basic aqueous phase was acidified (pH < 2), leading to precipitation of the target compound. The solid 3-(3-(naphthalen-2-yl)ureido)benzoic acid (IP23) was collected by filtration and purified; Scheme 2.1.8.

IP24: yield 29%, ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 12.93 (s, 1H), 9.37 (d, J = 13.3 Hz, 2H), 8.19 (s, 1H), 8.16 (s, 1H), 7.87 – 7.75 (m, 3H), 7.68 (d, J = 10.4 Hz, 1H), 7.60 – 7.50 (m, 2H), 7.48 – 7.31 (m, 3H); HR-MS for C₁₈H₁₄N₂O₃ calculated (M+H⁺=307.107), found (M+H⁺=307.1068).

IP27 (methyl 3-(3-(naphthalen-2-yl)ureido)benzoate) synthesis: IP27 was prepared from 50 mg of 2-naphthoic acid (**21**) (0.33 mmol), 30 mg of methyl 3-aminobenzoate (0.20 mmol), 81 mg of diphenylphosphoryl azide (DPPA, 0.30 mmol), and 60 mg of triethylamine (TEA, 0.60 mmol) in toluene. The reaction mixture was heated under microwave irradiation at 100 °C for 10 min.

After completion, the reaction mixture was subjected to liquid–liquid extraction (H₂O/EtOAc). The basic aqueous layer was acidified (pH < 2), affording precipitation of the

target compound. The solid 3-(3-(naphthalen-1-yl)ureido)benzoic acid was collected and purified by filtration.

The obtained 3-(3-(naphthalen-2-yl)ureido)benzoic acid was dissolved in methanol containing 1% H₂SO₄ and refluxed for 16 h. After cooling, the solvent was removed under reduced pressure. The residue was subjected to liquid–liquid extraction (H₂O/EtOAc) with the aqueous phase basified prior to extraction. The combined organic extracts were dried and evaporated, and the product isolated; Scheme 2.1.8.

IP27: yield 57%, ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 9.17 (s, 1H), 9.07 (s, 1H), 8.27 (s, 1H), 8.13 (s, 1H), 7.88 – 7.73 (m, 3H), 7.65 (ddd, J = 8.1, 2.3, 1.1 Hz, 1H), 7.58 (dt, J = 7.9, 1.3 Hz, 1H), 7.53 – 7.40 (m, 3H), 7.35 (ddd, J = 8.1, 6.9, 1.3 Hz, 1H), 3.82 (s, 3H); HR-MS for C₁₉H₁₆N₂O₃ calculated (M+H⁺ = 321.1233), found (M+H⁺ = 321.1221).

IP28 1,3-di(naphthalen-2-yl)urea synthesis: IP28 was obtained as a byproduct from the dimerization of 2-naphthyl isocyanate, which was generated via Curtius rearrangement during the reaction of 2-naphthoic acid with DPPA in the syntheses of IP23, IP24, and IP27. After hydrolysis of the target products, IP28 was separated by liquid–liquid extraction (H₂O/EtOAc), remaining in the organic phase. Evaporation of the solvent afforded isolated IP28; Scheme 2.1.8.

IP28: yield 60%, ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 9.48 (s, 2H), 8.19 (d, J = 2.2 Hz, 2H), 7.80 (dd, J = 17.5, 8.1 Hz, 2H), 7.52 (dd, J = 8.8, 2.2 Hz, 2H), 7.47 – 7.42 (m, 2H), 7.39 – 7.30 (m, 2H), 7.28 – 7.12 (m, 2H), 6.97 (t, J = 7.2 Hz, 2H); HR-MS for C₂₁H₁₆N₂O₃ calculated (M+H⁺ = 313.1484), found (M+H⁺ = 313.1323).

IP29 1,3-di(naphthalen-1-yl)urea synthesis: IP29 was obtained as a byproduct from the dimerization of 1-naphthyl isocyanate in the syntheses of IP21, IP22, and IP6. After hydrolysis of the target products, IP29 was separated by liquid–liquid extraction (H₂O/EtOAc), remaining in the organic phase. Evaporation of the solvent afforded isolated IP28; Scheme 2.1.7.

IP29: yield 60%, ¹H NMR (400 MHz, DMSO-d₆) δ (ppm): 9.43 (s, 1H), 8.30 (d, J = 8.3 Hz, 1H), 8.07 (d, J = 7.3 Hz, 1H), 7.94 (d, J = 9.3 Hz, 1H), 7.68 – 7.43 (m, 4H); HR-MS for C₂₁H₁₆N₂O₃ calculated (M+H⁺ = 313.1484), found (M+H⁺ = 313.1335).

NV848 (3-amino-5-methyl-1,2,4-oxadiazole) synthesis: NV848 was obtained starting from 2.0 g of cyanamide (**22**) (47.56 mmol), which was reacted with 6.24 g of acetic anhydride (71.34

mmol) and 7.0 g of potassium acetate (71.34 mmol) in acetone for 24 h at r.t.. The reaction mixture was then filtered, and acetone was removed by evaporation under vacuum.

Subsequently, 4.96 g of hydroxylamine hydrochloride (71.34 mmol) and 5.45 g of pyridine (71.34 mmol) were added, and the mixture was allowed to react at r.t. for 24 h. The solvent was then evaporated under reduced pressure.

The crude reaction mixture was extracted by liquid–liquid extraction (H₂O/AcOEt), and the organic phase was purified by chromatography on silica gel using AcOEt/petroleum ether (2:1) to isolate 3-amino-5-methyl-1,2,4-oxadiazole (**23**).

Finally, 3-amino-5-methyl-1,2,4-oxadiazole was reacted with 7.24 g of acetic anhydride (71.34 mmol) and 7.0 g of potassium acetate (71.34 mmol) at 40 °C for 24 h under solvent-free conditions. After cooling, the reaction mixture was purified by chromatography on silica gel using AcOEt/petroleum ether (2:1); Scheme 2.3.1.

NV848: yield 46%. ¹H NMR (DMSO-d₆) δ (ppm): 2.08 (s, 3H), 2.52 (s, 3H), 10.94 (s, 1H). HRMS for C₅H₇N₃O₂ calculated (M+H⁺=142.0611), found (M+H⁺=142.0625).

PTC124 (3-[5-(2-Fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid) synthesis: PTC124 was obtained starting from 1.0 g of 3-cyanobenzoyl chloride (**24**)(6.04 mmol), reacted with 1.68 mL of triethylamine (TEA, 12.08 mmol) and 5 mL of MeOH in a round-bottom flask at r.t. for 24 h. After 24 h, the solvent was removed by evaporation under vacuum, and liquid–liquid extraction with water and diethyl ether (H₂O/Et₂O) was performed. The dried organic phase was evaporated under vacuum, and methyl 3-cyanobenzoate (**25**) was isolated by column chromatography.

Then, 0.61 g of hydroxylamine hydrochloride (8.84 mmol) was neutralized with 0.74 g of sodium bicarbonate (8.84 mmol) in 2 mL of water for 10 min. Subsequently, 0.95 g of methyl 3-cyanobenzoate (**25**) (5.9 mmol) and 10 mL of absolute ethanol were added, and the mixture was allowed to react at r.t. for 16 h. The solvent was then removed under vacuum, and methyl 3-(N'-hydroxycarbamimidoyl)benzoate (**26**) was isolated after liquid–liquid extraction (H₂O/AcOEt). The organic phase was purified by chromatography on silica gel using AcOEt/petroleum ether (2:1).

Next, 1.06 g of methyl 3-(N'-hydroxycarbamimidoyl)benzoate (5.46 mmol) was reacted with 1.28 g of 2-fluorobenzoyl chloride (8.19 mmol) and 1.52 mL of TEA (10.92 mmol) in THF (dry) at r.t. for 1 h. The reaction mixture was filtered, and the precipitate was washed with THF. The filtrate was evaporated under vacuum, and the crude 3-(N'-((2-

fluorobenzoyl)oxy)carbamimidoyl)benzoate (**27**) was subjected to liquid–liquid extraction (H₂O/AcOEt). The organic phase was purified by chromatography on silica gel using AcOEt/petroleum ether (2:1).

Then, 1.36 g of methyl 3-(N'-((2-fluorobenzoyl)oxy)carbamimidoyl)benzoate (4.3 mmol) was dissolved in toluene and cyclized under reflux for 1 h obtaining the methyl 3-(5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl)benzoate (**28**). Finally, the crude reaction mixture was reacted with 5 mL of aqueous NaOH (1.5 M) in 5 mL of THF at r.t. for 16 h. The pH was then adjusted to 1 using hydrochloric acid (HCl, 1 M), leading to precipitation of the product. Crude Ataluren was recovered by vacuum filtration and recrystallized from ethanol; Scheme 2.3.2.

PTC124: yield 34%. ¹H NMR (DMSO-d₆) δ (ppm): 8.16 (d, 2H), 7.80 – 7.71 (m, 1H), 7.66 (t, 2H), 4.45 (q, 2H), 1.36 (t, 3H). HRMS for C₁₅H₉FN₂O₃ calculated (M+H⁺=285.067), found (M+H⁺=285.0682).

Cell Culture Conditions

To perform assays for evaluating newly synthesized compounds' biological activity, HCT116 colon cancer cells constitutively expressing a p53 wild-type, H1299 lung adenocarcinoma p53^{-/-} cells, and H1299 lung adenocarcinoma p53^{R213X} cells stably transfected, vector bearing a premature stop codon UGA in the TP53 cDNA were used (a kindly gift of Prof. K. Wiman, Karolinska Institute, Stockholm) [44].

Cells were cultured in 1X DMEM medium with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Corning, Mediatech, Manassas, USA), to which 10% FBS (yourSial, Rome, Italy) was added. Cells were maintained in an incubator under optimal growth conditions at 37°C and 5% CO₂.

Luciferase Reporter Assay

HeLa cells were transfected with Fluc-WT and Fluc-opal plasmids using Lipofectamine 2000 (Invitrogen) in 6-well plates at a density of 2×10^5 cells/mL. After 24 h of incubation, the newly synthesised molecules and PTC124, as positive control, at a concentration of 12 μM were added and cells were further incubated for 24 h. Following PBS washing, cells were treated with the Steady-Glo luciferase reagent (Promega, Madison, WI, USA). Subsequently, 200 μL of the cell suspension was transferred in triplicate into 96-well plates, and luciferase activity was measured using a luminometer (Promega, Madison, WI, USA).

Cytotoxic assay

a. Trypan Blue exclusion assay.

200.000 HCT 116 cells were seeded in 60 mm dishes and then incubated at 37 °C for 24 h. The cells were then treated with the newly synthesized compounds dissolved in DMSO at a concentration of 12 μ M for 24 h, 48 h, and 72 h. Cells treated with diluent (0.001% DMSO) were included as controls.

After treatments, treated and untreated cells were harvested by trypsinization, incubated in 0.04% Trypan Blue (Gibco™, Thermo Fisher, Waltham, USA) for 4 min, and were counted under a hemocytometer for visual counting. The total number of cells that retained the dye was noted.

b. MTT assay.

5,000 HCT 116 cells per well were seeded in a 96-well plate in 100 μ L of medium and then incubated at 37 °C for 24h. The cells were then treated with the newly synthesized compounds dissolved in DMSO at a concentration of 12 μ M for 24h, 48h, and 72h. Cells treated with diluent (0.001% DMSO) were included as controls.

After treatments, 10 μ L of MTT solution in PBS (5 mg/ml) was added to the cells and incubated for 4h in the dark. Then, the medium was removed, and cells were washed with PBS before 100 μ L of fresh DMSO was added to dissolve the formazan crystal, and a spectrophotometric measure was realized.

The absorbance of each well was measured at 560 nm and 570 nm by means of an ELISA plate reader.

Immunofluorescence Microscopy

Cells (200 000 per well) were seeded in 12-well plates and treated the following day with either newly synthesized triazole compounds (12 μ M; non-toxic for Ataluren derivatives as reported by Pibiri et al., 2020), Ataluren (12 μ M), or G418 (300 μ g/mL), alone or in combination with doxorubicin (0.2 μ g/mL).

After 24 hours, cell fixation was performed on coverslips with frozen methanol for 1 minute at room temperature, followed by membrane permeabilization with 200 μ L of Triton X-100 0,01% (Sigma-Aldrich, Darmstadt, Germany) for 10 minutes. Finally, blocking was carried out to eliminate the presence of non-specific signal using 200 μ L of albumin from chicken egg white 0.1% (Sigma-Aldrich, Darmstadt, Germany) for 1 h at room temperature. Then, it was performed the incubation with 20 μ L of primary antibody in-mouse α -p53 DO-1 1:500, (Santa

Cruz, Dallas, USA) diluted in albumin 0.1%, O.N. at 4 °C. After 24 hours, it was performed the incubation with 20 µL of secondary antibody α -mouse Alexa Fluor 488 1:500, (Abcam, Waltham, USA) diluted in albumin 0.1%, at 37 °C for 1 h. To visualize the nuclei, 10 µL of SlowFade® Diamond Antifade Mountant with DAPI (Invitrogen™, Thermo Fisher, Waltham, USA) was used. Cells were visualized by mean of a fluorescence microscope (Axiovision, Zeiss) equipped with a 63x magnification oil immersion objective and collected image were analyzed using Anovision 4.8 and ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997–2018).

Protein extraction and quantification

Cells (200,000 per well) were seeded in 60-mm dishes and treated the following day with either newly synthesized triazole compounds (12 µM), or with the positive controls G418 (300 µg/mL) and Ataluren (12 µM), alone or in combination with doxorubicin (0.2 µg/mL).

After 24 hours of treatment, proteins were extracted by adding 500 µL of Pierce™ RIPA Buffer (Thermo Scientific™, Waltham, MA, USA) supplemented with 1% Halt™ Protease Inhibitor Cocktail (100X, Thermo Scientific™). Cells were detached using a cell scraper (Thermo Scientific™) and transferred to 1.5 mL Eppendorf tubes. Lysates were incubated on ice at 4 °C for 1 hour and subsequently centrifuged at 12,000 rpm for 20 minutes at 4 °C to collect the supernatant containing soluble proteins.

Protein concentration was determined using the Bradford colorimetric assay, with a standard curve generated using chicken egg white albumin (Sigma-Aldrich, Darmstadt, Germany) at a concentration of 1 mg/mL. Absorbance was measured at 595 nm, and protein concentrations were calculated by interpolation from the standard curve. Samples were stored at -80 °C for subsequent analysis.

Western Blotting

A total of 30 µg of protein from each sample was mixed with Milli-Q water, 4X (Bolt™, Invitrogen™, Thermo Fisher, Waltham, USA), and 10X Sample Reducing Agent (Bolt™, Invitrogen™, Thermo Fisher, Waltham, USA) to a final volume of 15 µL. Samples were denatured at 70 °C for 10 minutes using a thermoblock. Subsequently, the samples were loaded onto 10% Bis-Tris Plus gels (Bolt™, Invitrogen™, Thermo Fisher, Waltham, USA) and subjected to electrophoresis in 1X MES SDS Running Buffer (Bolt™, Invitrogen™, Thermo Fisher, Waltham, USA) alongside the Precision Plus Protein™ Kaleidoscope™ Prestained

Protein Standards (Bio-Rad, Hercules, CA, USA). Electrophoretic separation was performed at 150 V and 100 mA for approximately 1 hour.

Following electrophoresis, proteins were transferred onto PVDF membranes (Thermo Fisher Scientific) by overnight electroblotting at 4 °C. Membranes were probed with mouse anti-p53 (DO-1, Santa Cruz Biotechnology) and rabbit anti-vinculin (N3C1, GeneTex, Irvine, CA, USA) primary antibodies to detect p53 and vinculin levels, respectively. Protein bands were visualized using the SuperSignalTM West Femto Maximum Sensitivity Substrate (Thermo ScientificTM, Waltham, MA, USA). Image acquisition was performed using the ChemiDoc XRS system (Bio-Rad), and band intensities were quantified using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997–2018).

Real-Time RT-PCR

i. RNA Extraction from Cell Cultures

Cells (500 000 per well) were treated with IP molecules (12 µM) or G418 (300 µg/mL), alone or in combination with doxorubicin at 0.2 µg/mL for DNA damage induction. After 24 hours, cells were detached with 0.05% trypsin (Corning, Mediatech, Manassas, USA), centrifuged at 1000 rpm for 10 minutes to obtain the cellular pellet and RNA extraction was performed using the RNeasy® Mini Kit (Qiagen, Hilden, Germany).

The RNA samples were quantified using the NanoDropTM 2000 spectrophotometer (Thermo ScientificTM, Waltham, USA) and RNA integrity was assessed by gel electrophoresis on a 1% agarose gel (UltraPureTM Agarose, InvitrogenTM, Thermo Fisher, Waltham, USA) in TAE 1X buffer. Afterward, samples were stored at -80°C for further analysis.

ii. Reverse Transcription and Real-Time RT-PCR

Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA), with 1 µg of total RNA in a final reaction volume of 10 µL. Synthesized cDNA was stored at -20 °C for subsequent analyses. Quantitative real-time PCR (qRT-PCR) was carried out using PowerUpTM SYBRTM Green Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Two separate reaction mixtures were prepared: one for the target genes *GADD45A* and *CDKN1A* (*p21*), and another for the reference gene *GAPDH*. cDNA was added to the respective reaction mixes, and each sample was analyzed in technical triplicates. Amplification and quantification were performed on a 7300 Real-Time PCR System (Applied

Biosystems), and data were analyzed using the 7300 System Software. Primer sequences used for qRT-PCR are listed in Supplementary Table 1.

In-silico ADMET properties prediction

To evaluate the ADMET properties online tools SwissADME (Swiss Institute of Bioinformatics, Switzerland) and ProTox 3.0 were deployed.

The estimated pharmacokinetic parameter with SwissADME includes lipophilicity, water-soluble, gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeation, and Liepinski law violation.

The endpoint estimated with ProTox 3.0 [45] includes: LD50, Toxicity Class, Organ toxicity, and Toxicity Endpoint.

Animals

Adult wild-type (WT) C57BL/6 mice were purchased from ENVIGO Srl (San Pietro al Natisone, UD, Italy). Both sexes were included to evaluate potential sex-dependent differences in response to the test molecules. C57BL/6 CFTR^{G542X/G542X} mice were kindly provided by Professor C. Hodges (Case Western Reserve University, Cleveland, USA).

Animals were housed in temperature-controlled rooms (22–25 °C, 50–60% humidity) under a 12 h light/dark cycle, with free access to standard pellet chow and water until the start of the experimental protocol.

All experimental procedures were conducted in accordance with Italian law (D.Lgs 26/2014) and EU Directive 2010/63/EU on the protection of animals used for scientific purposes. Protocols were approved by the University of Palermo Animal Care and Use Ethics Committee and authorized by the Italian Ministry of Health (Authorization n. 1235/2020; Authorization n. 2/2022-PR).

Treatment Protocol and Tissue Collection

In order to evaluate the proteomic impact of NV848 and PTC124 in a CF mouse model, wild-type (CFTR^{+/+}) and homozygous CFTR^{G542X/G542X} mice were analyzed in both untreated and treated conditions. Treatments with NV848 (60 mg/kg) or PTC124 (60 mg/kg) began on postnatal day 21, following genotyping, and were administered by intragastric gavage once daily for 15 consecutive days.

Both male and female mice were used, with $n = 5$ animals per group initially considered; however, due to the severe phenotype associated with cystic fibrosis in the CFTR^{G542X/G542X} model, some animals did not survive until the end of the study.

On the 15th day of treatment, all surviving animals were anesthetized with 2% isoflurane and euthanized by cervical dislocation. The brain, liver, and pancreas were excised, weighed, and stored at -80°C . These organs were subsequently used for proteomic analysis.

Tissue homogenization and protein extraction

Freshly frozen tissue samples (~ 10 mg per mouse) were excised using a sterile bistoury after an initial wash with 1 mL of phosphate-buffered saline (PBS). Each tissue fragment was rinsed again with 1 mL of PBS and transferred into vials containing 100 μL of extraction buffer (2% SDS, 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 50 mM DTT) per 10 mg tissue. Samples were mechanically homogenized, heated at 100°C for 5 min, and subjected to ultrasonication assisted protein extraction using a UP50H ultrasonic processor (50 W, 30 kHz, 1 mm probe tip) at 50% amplitude for 2 min in pulsed mode (30 s on, 10 s off).

Then, 200 μL of protein extracts were diluted to 1 mL with 8 M urea in 0.1 M Tris-HCl (pH 8). Protein concentrations were determined using the tryptophan fluorescence emission assay.

Tryptophan fluorescence emission assay

Proteome and proteome digest concentrations were determined using the tryptophan fluorescence emission assay. A standard calibration curve was prepared with 0.0102 $\mu\text{g}/\mu\text{L}$ tryptophan dissolved in 8 M urea in 0.1 M Tris-HCl (pH 8), covering a linear detection range from 0 to 5.1×10^{-3} $\mu\text{g}/\mu\text{L}$. For the assay, 75 μL of calibration solutions were transferred into a quartz-bottom 96-well plate. To ensure measurements were within the linear range, a dilution protocol was applied: 5 μL of each unknown sample was added in duplicate to the wells, followed by 70 μL of 8 M urea in 0.1 M Tris-HCl (pH 8). Fluorescence measurements were performed using an excitation wavelength of 280 nm, a long-pass dichroic mirror at 309 nm, and an emission wavelength of 350 nm with a 20 nm bandwidth. Proteome and digest concentrations were quantified from fluorescence intensity values, assuming an average tryptophan content of 1.16% in mice protein²⁴⁷.

Protein reduction/alkylation and digestion

Thirty micrograms of extracted protein were loaded onto a FASP membrane (molecular weight cut-off 30 kDa; Sartorius, Göttingen, Germany), diluted to 200 μL with 8 M urea in 0.1 M Tris-

HCl (pH 8), and centrifuged at 14,000 g for 15 min at 25 °C. Reduction and alkylation were then performed using 100 µL of 8 M urea in 0.1 M Tris-HCl (pH 8) containing 40 mM chloroacetamide (CAA) and 10 mM tris(2-carboxyethyl)phosphine (TCEP), with incubation for 20 min at 37 °C. The membrane was centrifuged at 14,000 g for 15 min at 25 °C and washed twice with 200 µL of 8 M urea in 0.1 M Tris-HCl (pH 8), followed by two washes with 200 µL of 70 mM tetraethylammonium bromide (TEAB), each wash step including centrifugation at 14,000 g for 15 min at 25 °C.

Proteins were digested on-membrane with 100 µL Lys-C/Trypsin (10 µg/mL) and incubated overnight at 37 °C. The membrane was then centrifuged at 14,000 g for 15 min at 25 °C, followed by two washes with 200 µL of 3% (v/v) acetonitrile (ACN) in 0.1% (v/v) aqueous formic acid (FA_{aq}), each with centrifugation at 14,000 g for 15 min at 25 °C, to collect the peptides. The peptide solution was evaporated in a speed vacuum concentrator at 18 °C and resuspended in 100 µL of 3% (v/v) ACN in 0.1% (v/v) FA_{aq}, followed by ultrasonication for 10 min in an ultrasonic bath.

Peptide concentration was determined and quantified using the tryptophan fluorescence emission assay.

Nano-LC-ESI-MS/MS Analysis

The LC-MS/MS analysis was performed using an Ultimate 3000 nLC system coupled to a UHR-QqTOF IMPACT HD mass spectrometer (Bruker Daltonics) equipped with a CaptiveSpray ion source (Bruker Daltonics). Protein digests were reconstituted to a final concentration of 0.25 µg/µL in 3% acetonitrile (ACN)/0.1% (v/v) aqueous formic acid (FA). For each run, 5 µL of sample was loaded onto an Acclaim PepMap100 trap column (5 µm, 100 Å, 300 µm i.d. × 5 mm) and desalted for 5 min with 3% mobile phase B (90% ACN/0.1% FA) at a flow rate of 15 µL/min. Chromatographic separation was achieved on an Acclaim PepMap 100 C18 analytical column (2 µm, 0.075 mm i.d. × 150 mm) at 35 °C with a linear gradient at 300 nL/min. The mobile phases were: A, 0.1% (v/v) aqueous FA; and B, 90% (v/v) ACN with 0.08% (v/v) FA. The gradient was as follows: 0–5 min, 3% B; 5–95 min, 3–35% B; 95–105 min, 35–95% B; and 105–115 min, 95% B. The total run time was 130 min, and two replicate injections were performed per sample.

MS acquisition consisted of cycles of full MS scans (2 Hz), followed by data-dependent MS/MS scans (8–32 Hz), with a total cycle time of 3.0 s and active exclusion enabled. Precursors were excluded from selection for 0.5 min after one MS/MS spectrum was acquired, with an intensity

threshold for fragmentation of 2,500 counts. Active exclusion was set to 1; however, precursors were reconsidered if their intensity increased by a factor of three, ensuring re-fragmentation near the peak maximum. All spectra were acquired over a mass range of 150–2200 m/z.

Data analysis

Raw LC–MS/MS spectra were analyzed using PEAKS Studio v12.0 (Bioinformatics Solutions Inc.). Database searches and *de novo* sequencing were performed with the built-in PEAKS search engine against the UniProt-SwissProt Mus musculus reference proteome (Proteome ID UP000000589, latest release at the time of analysis). Search parameters were set to default values, with precursor and fragment mass tolerances automatically optimized by PEAKS. Lys-C/Trypsin was specified as the proteolytic enzyme, allowing up to two missed cleavages. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation and N-terminal acetylation were considered variable modifications. Label-free quantification (LFQ) was carried out within PEAKS using relative quantification based on extracted ion chromatograms (XICs). A false discovery rate (FDR) of 5% was applied at both peptide-spectrum match (PSM) and protein levels. Data normalization and statistical analyses of protein abundances were performed with Perseus v2.1.5.0, using default settings. Network visualization and analysis were performed in Cytoscape (v3.10.3), Protein–protein interaction (PPI) networks were generated using the STRING app (Search Tool for the Retrieval of Interacting Genes/Proteins) within Cytoscape, applying the Mus musculus database with a medium confidence cutoff of 0.4 and the full STRING network option and highly interconnected clusters were identified using the MCODE plugin to define topological modules of functional relevance.

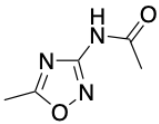
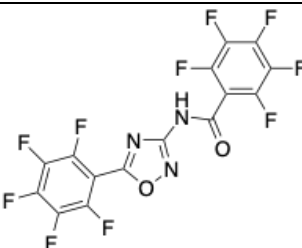
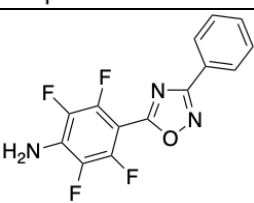
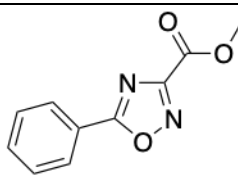
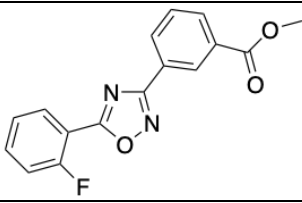
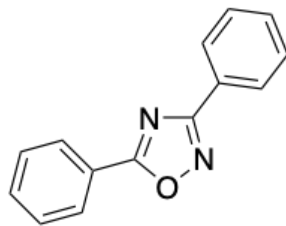
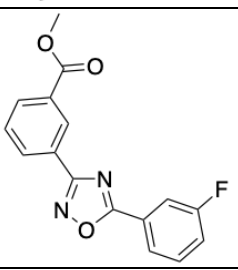
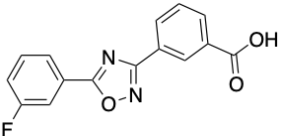
Statistics

All data are expressed as mean values $\pm$ standard error of the mean (SEM). Statistical analysis was performed by Student's t-test and one-way ANOVA when appropriate by GraphPad Prism software (Inc., La Jolla, CA, USA) version 10.1.1 for MacOS. A probability value (p) of less than 0.05 was regarded as significant and indicated in relevant graphs as one symbol (*) for $p < 0.05$, two symbols (**) for $p < 0.01$, three symbols (***) for $p < 0.001$, and four symbols (****) for $p < 0.0001$.

APPENDIX

APPENDIX A

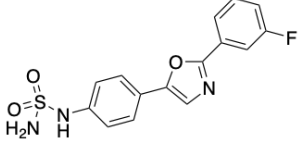
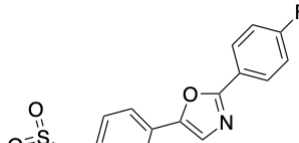
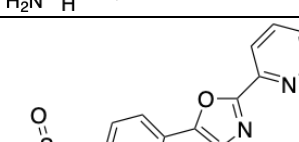
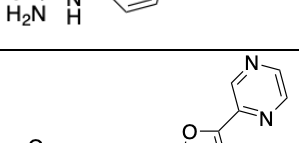
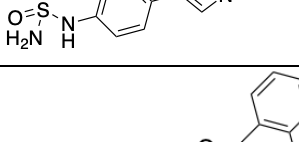
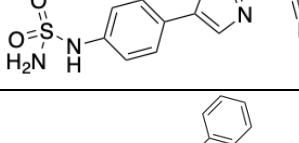
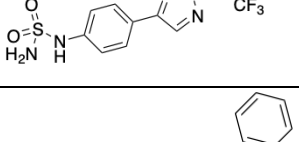
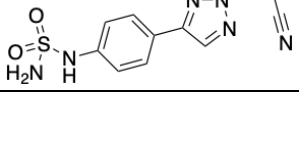
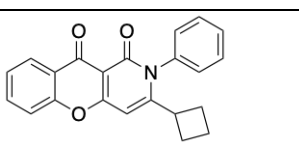
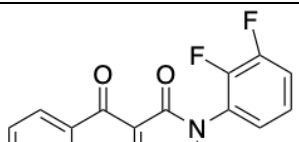
Table A1. Training set for Pharmacophore model building and validation

Name	Structure	Smiles ID	Pfluc activity	Chemical families
NV848		<chem>n1c(C)onc1NC(=O)C</chem>	3,57970	Ataluren like
NV914		<chem>Fc1c(F)c(F)c(F)c1c(n2)onc2NC(=O)c3c(F)c(F)c(F)c(F)c3F</chem>	3,62320	Ataluren like
NV928		<chem>c1ccccc1-c2noc(n2)-c3c(F)c(F)c(N)c(F)c3F</chem>	3,5648	Ataluren like
NV930		<chem>CCOC(=O)c1nc(on1)-c2ccccc2</chem>	3,5797	Ataluren like
NV1103		<chem>Fc1ccccc1-c(n2)onc2-c3cc(ccc3)C(=O)OC</chem>	3,5883	Ataluren like
NV1120		<chem>c1ccccc1-c2noc(n2)-c3ccccc3</chem>	3,5883	Ataluren like
NV1127		<chem>Fc1cccc(c1)-c(n2)onc2-c(c3)cccc3C(=O)OC</chem>	3,5328	Ataluren like
NV1133		<chem>c1ccc(cc1F)-c(n2)onc2-c3cccc(c3)C(=O)[O-]</chem>	3,6845	Ataluren like

NV1173		<chem>Fc1c(F)cc(F)c(c1)-c(n2)onc2-c3cccc(c3)C(=O)[O-]</chem>	3,8447	Ataluren like
NV1260		<chem>c1cc(N)ccc1-c(n2)onc2-c3cccc3</chem>	3,5017	Ataluren like
NV1898		<chem>c1ccc(cc1C)-c(n2)onc2-c3ccccn3</chem>	3,6073	Ataluren like
NV2367		<chem>c1cccc(C)c1-c(nn2)oc2-c3ccc(cc3)C(=O)NCCCOc</chem>	3,5259	Ataluren like
NV2396		<chem>c1cccc(C)c1-c(nn2)oc2c3ccc(cc3)C(=O)NCCc4ncccc4</chem>	3,5895	Ataluren like
NV2425		<chem>c1ccc(C(=O)[O-])cc1-c2noc(n2)-c3cccc(F)c3F</chem>	3,8161	Ataluren like
NV2426		<chem>c1ccc(C(=O)[O-])cc1-c2noc(n2)-c3cc(F)ccc3F</chem>	3,6657	Ataluren like
NV2428		<chem>c1ccc(C(=O)[O-])cc1-c2noc(n2)-c3ccc(F)c(F)c3</chem>	3,5608	Ataluren like
NV2429		<chem>c1ccc(C(=O)[O-])cc1-c2noc(n2)-c3ccc(F)cc3F</chem>	3,8205	Ataluren like
NV2439		<chem>c1ccc(C(=O)[O-])cc1-c2noc(n2)-c3c(F)cccc3F</chem>	3,9326	Ataluren like

NV2445		<chem>c1cc(C(=O)[O-])ccc1-c(o2)nnc2-c3ccccc3C</chem>	3,5797	Ataluren like
PTC124		<chem>c1ccc(C(=O)[O-])cc1-c2nc(on2)-c3ccccc3F</chem>	3,5986	Ataluren like
Amlexanox		<chem>CC(C)C1=CC2=C(OC3=NC(N)=C(C=C3C2=O)C(O)=O)C=C1</chem>		aminoglycoside
2,6-diaminopurine (DAP)		<chem>NC1=NC(N)=C2N=CNC2=N1</chem>		purine
RTC13		<chem>O=C1NC(SC1=CC2=CC=C(C3=C([N+])([O-])=O)C=CC=C3)O2)=N</chem>		RTC
RTC14		<chem>CC(C)(C)C1=CC(=C(C=C1)O)N=CC2=CC(=CC=C2)[N+](=O)[O-]</chem>		RTC
			Luciferase activity report as percentage of untreated cells	
Kawai 1		<chem>FC(C=CC=C1)=C1C2=NO C(C3=CC=CC(C(C)O)=C3)=N2</chem>	392	Ataluren like
Kawai 2		<chem>FC(C=CC=C1)=C1C2=NO C(C3=CC=CC(NS(=O)(C)=O)=C3)=N2</chem>	487	Ataluren like
Kawai 3		<chem>FC(C=CC=C1)=C1C2=NO C(C3=CC=CC(NS(N)(=O)=O)=C3)=N2</chem>	488	Ataluren like

Kawai 4		<chem>FC(C=CC=C1)=C1C2=NO C(C3=CC=C(NS(=O)(C)=O) C=C3)=N2</chem>	470	Ataluren like
Kawai 6		<chem>FC(C=CC=C1)=C1C2=NO C(C3=CC=C(NS(=O)(N)=O) C=C3)=N2</chem>	426	Ataluren like
Kawai 7		<chem>FC(C=CC=C1)=C1C(O2)= NN=C2C3=CC=C(NS(=O)(N)=O)C=C3</chem>	426	Ataluren like
Kawai8		<chem>FC(C=CC=C1)=C1N(N=N2)C=C2C3=CC=C(NS(=O)(N)=O)C=C3</chem>	439	Ataluren like
Kawai9		<chem>FC(C=CC=C1)=C1C2=NN(C3=CC=C(NS(=O)(N)=O)C =C3)C=N2</chem>	377	Ataluren like
Kawai10		<chem>FC(C=CC=C1)=C1N2N=C(C3=CC=C(NS(=O)(N)=O)C =C3)C=N2</chem>	405	Ataluren like
Kawai11		<chem>FC(C=CC=C1)=C1C2=NC(C3=CC=C(NS(=O)(N)=O)C =C3)=CO2</chem>	389	Ataluren like
Kawai12		<chem>FC(C=CC=C1)=C1C(O2)= CN=C2C3=CC=C(NS(=O)(N)=O)C=C3</chem>	428	Ataluren like
Kawai13		<chem>FC(C=CC=C1)=C1C(O2)= NC(C)=C2C3=CC=C(NS(= O)(N)=O)C=C3</chem>	406	Ataluren like
Kawai14		<chem>FC(C=CC=C1)=C1C(O2)= NC(C#N)=C2C3=CC=C(NS (=O)(N)=O)C=C3</chem>	413	Ataluren like

Kawai15		<chem>O=S(NC(C=C1)=CC=C1C2=CN=C(C3=CC(F)=CC=C3)O2)(N)=O</chem>	385	Ataluren like
Kawai17		<chem>O=S(NC(C=C1)=CC=C1C2=CN=C(C3=CC=C(F)C=C3)O2)(N)=O</chem>	401	Ataluren like
Kawai18		<chem>O=S(NC(C=C1)=CC=C1C2=CN=C(C3=NC=CC=C3)O2)(N)=O</chem>	430	Ataluren like
Kawai19		<chem>O=S(NC(C=C1)=CC=C1C2=CN=C(C3=NC=CN=C3)O2)(N)=O</chem>	398	Ataluren like
Kawai20		<chem>O=S(NC(C=C1)=CC=C1C2=CN=C(C3=C(C#N)C=CC=C3)O2)(N)=O</chem>	427	Ataluren like
Kawai21		<chem>O=S(NC(C=C1)=CC=C1C2=NN(C3=C(C(F)(F)F)C=CC=C3)N=C2)(N)=O</chem>	352	Ataluren like
KY-516		<chem>O=S(NC(C=C1)=CC=C1C2=NN(C3=C(C#N)C=CC=C3)N=C2)(N)=O</chem>	374	Ataluren like
			Luciferase activity report as ratio of treated and untreated cells (1uM-3uM)	
Kawai-2-1		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC=C4)OC5=CC=CC=C51</chem>	(x1,6-x7,4)	chromenopyridine derivatives
Kawai-2-2		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC(F)=C4F)OC5=CC=CC=C51</chem>	(x6,7-x10,2)	chromenopyridine derivatives
Kawai-2-3		<chem>O=C1C(C2=O)=C(C=C(N3CCC3)N2C4=CC=CC(F)=C4F)OC5=CC=CC=C51</chem>	(x1-x4,8)	chromenopyridine derivatives

Kawai-2-4		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC=C4)OC5=CC=C(C)C=C51</chem>	(x1,6-x5,2)	chromenopyridine derivatives
Kawai-2-5		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC=C4)OC5=C(C)C=CC=C51</chem>	(x0,92-x4,0)	chromenopyridine derivatives
Kawai-2-6		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC(F)=C4F)OC5=C(OC)C=CC=C51</chem>	(x1,2-x8,5)	chromenopyridine derivatives
Kawai-2-7		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC(F)=C4F)OC5=C(O)C=CC=C51</chem>	(x0,8-x4,3)	chromenopyridine derivatives
Kawai-2-8		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC(F)=C4F)OC5=C(CCN(C)C)C=CC=C51</chem>	(x14,1-x10,8)	chromenopyridine derivatives
Kawai-2-9		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC(F)=C4F)OC5=CC(CCN(C)C)=CC=C51</chem>	(x12,5-x11,2)	chromenopyridine derivatives
Kawai-2-10		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC(F)=C4F)OC5=C(CNCCC6)C6=CC=C51</chem>	(x3,8-)	chromenopyridine derivatives
Kawai-2-11		<chem>O=C1C(C2=O)=C(C=C(C3CCC3)N2C4=CC=CC(F)=C4F)OC5=C(CCNC6)C6=CC=C51</chem>	(x4,1-)	chromenopyridine derivatives
Morrill 1		<chem>N=C(NCCCN1CCCC1)NC2=NC(C3=COC=C3)=C(C)C(C)=N2</chem>		Morill

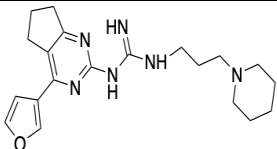
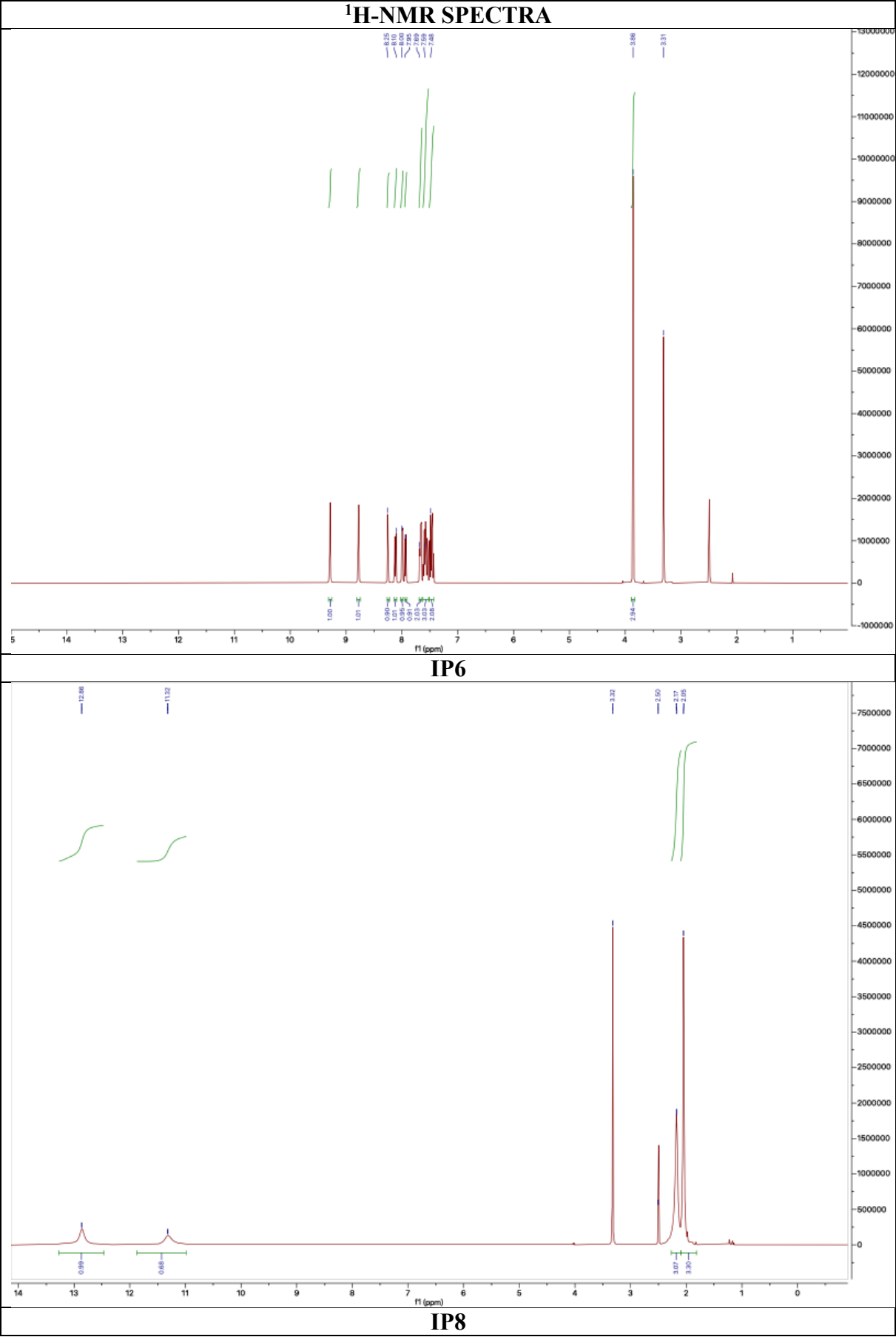
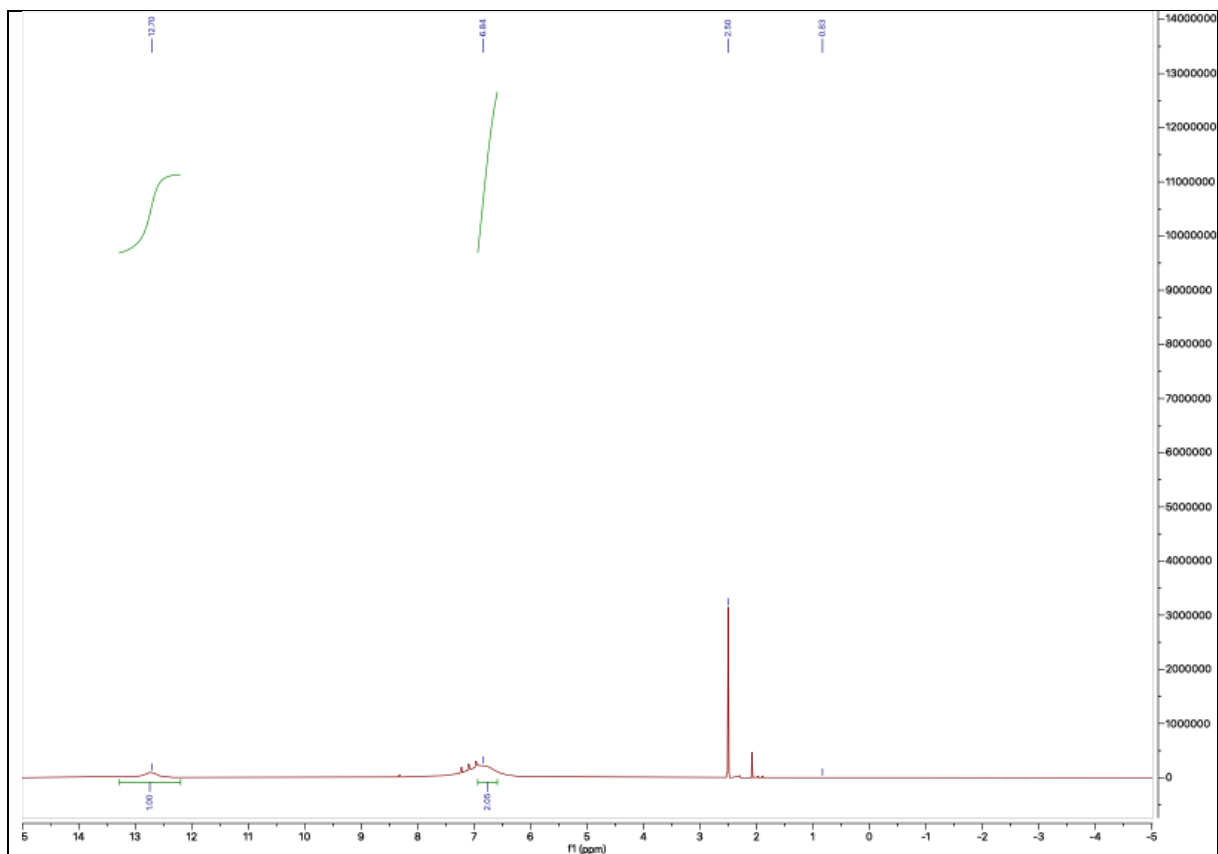
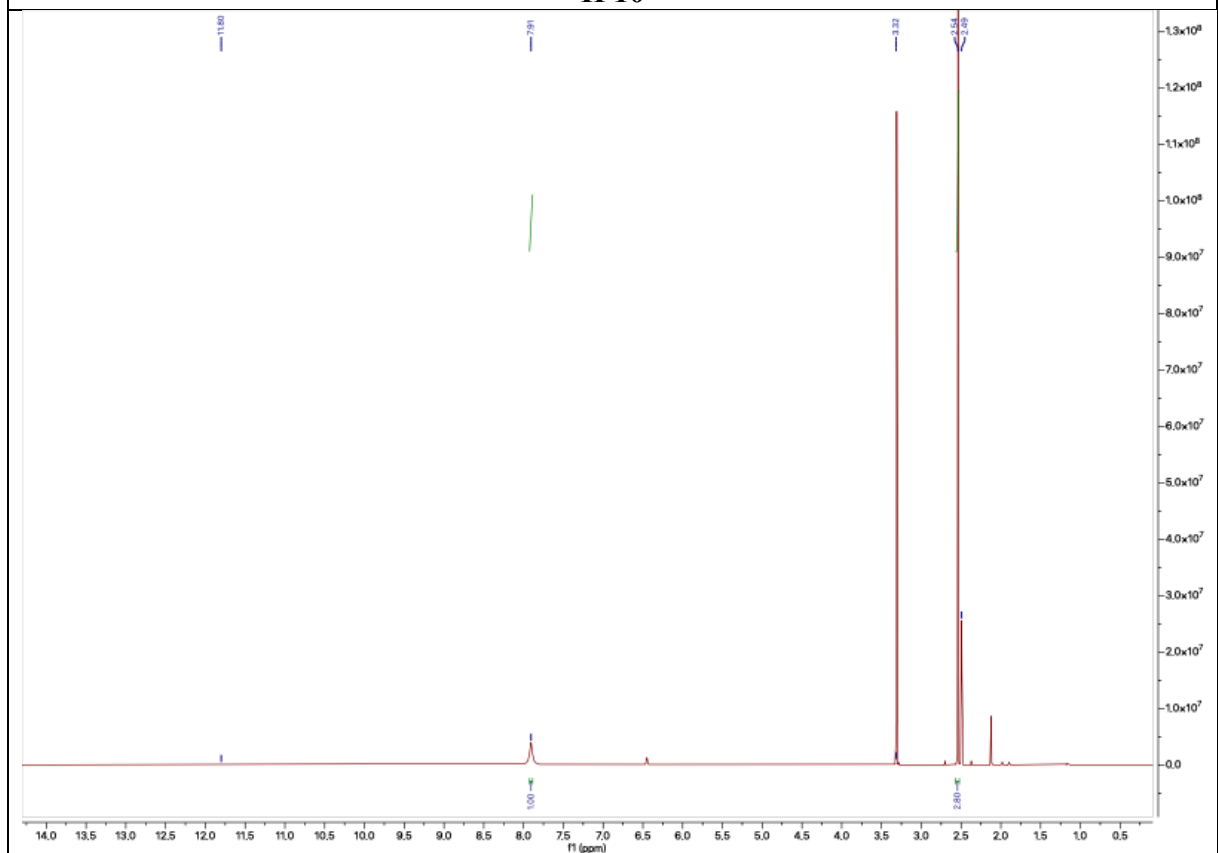
Morrill 2		<chem>N=C(NCCCN1CCCCC1)N</chem> <chem>C2=NC(C3=COC=C3)=C4</chem> <chem>C(CCC4)=N2</chem>		Morill
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Table A2. ¹H-NMR Data Section.

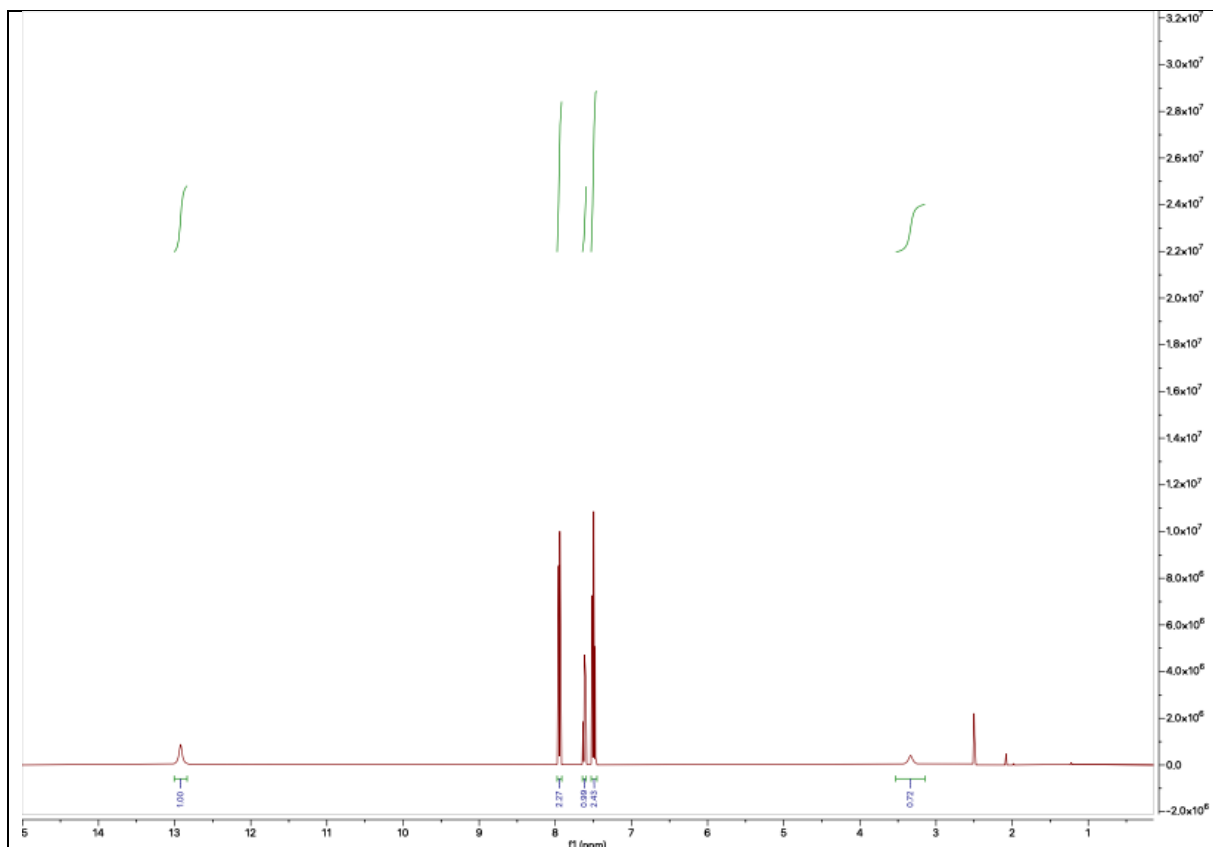




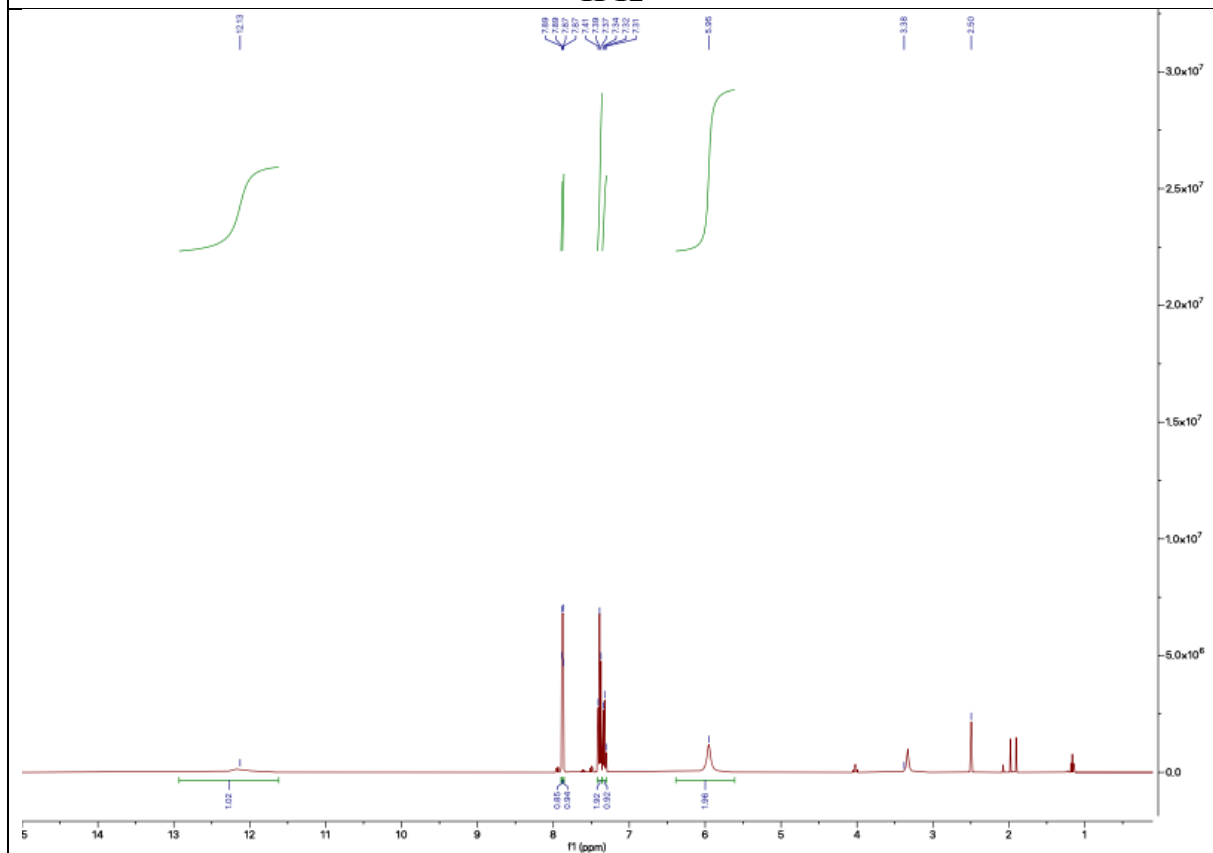
IP10



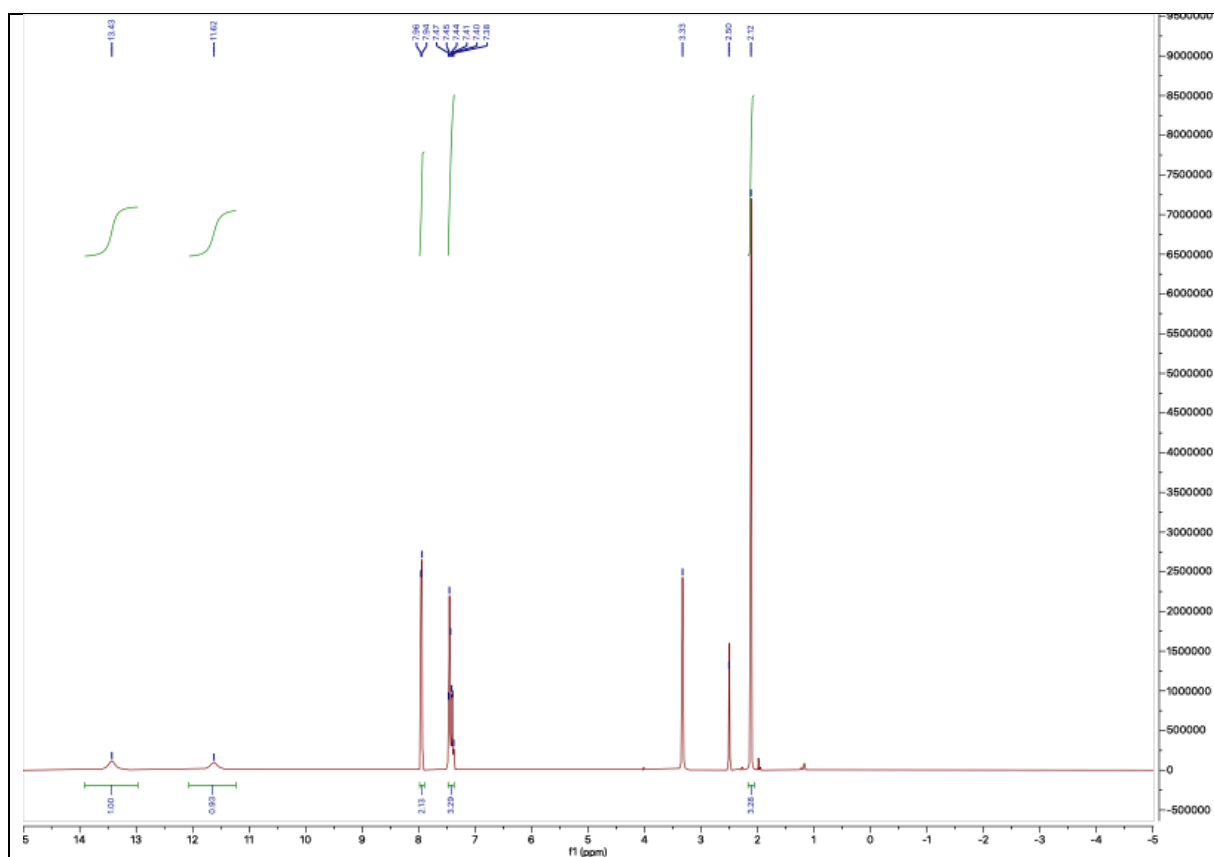
IP11



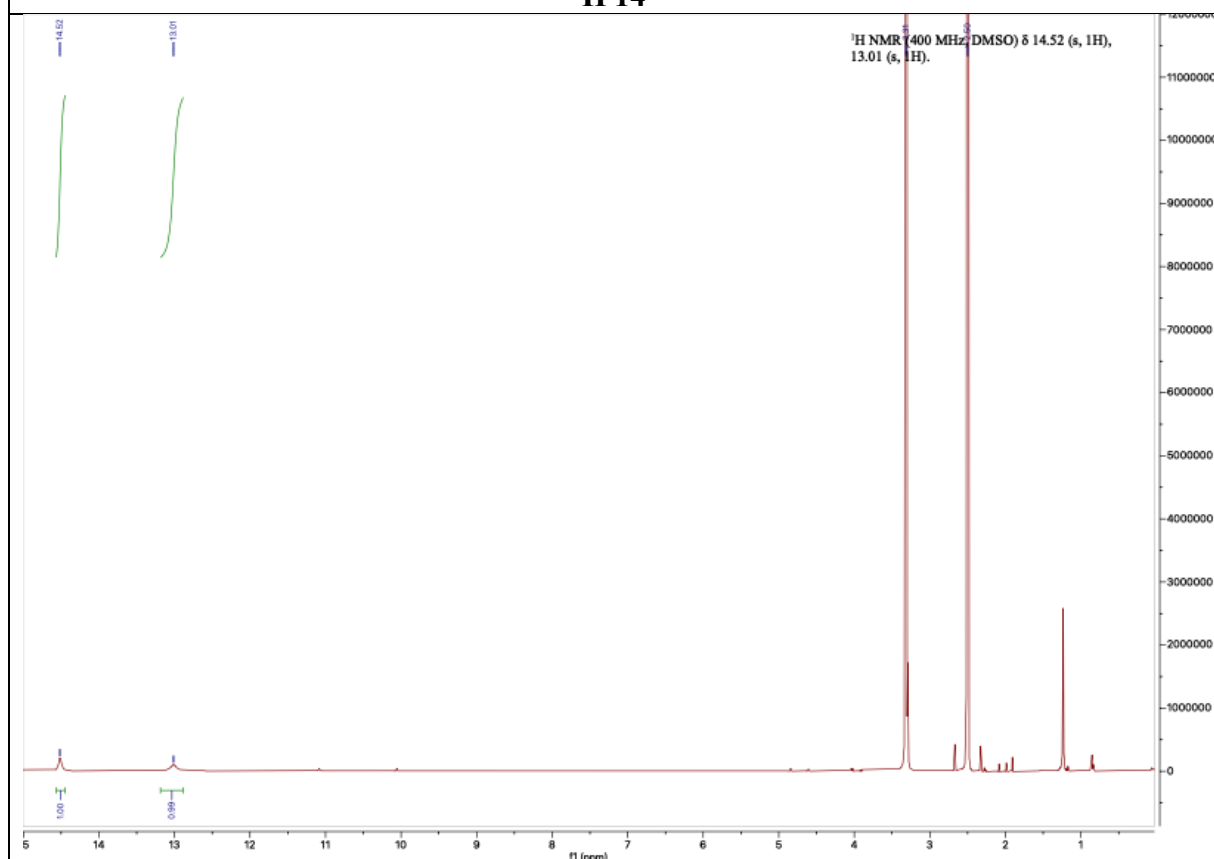
IP12



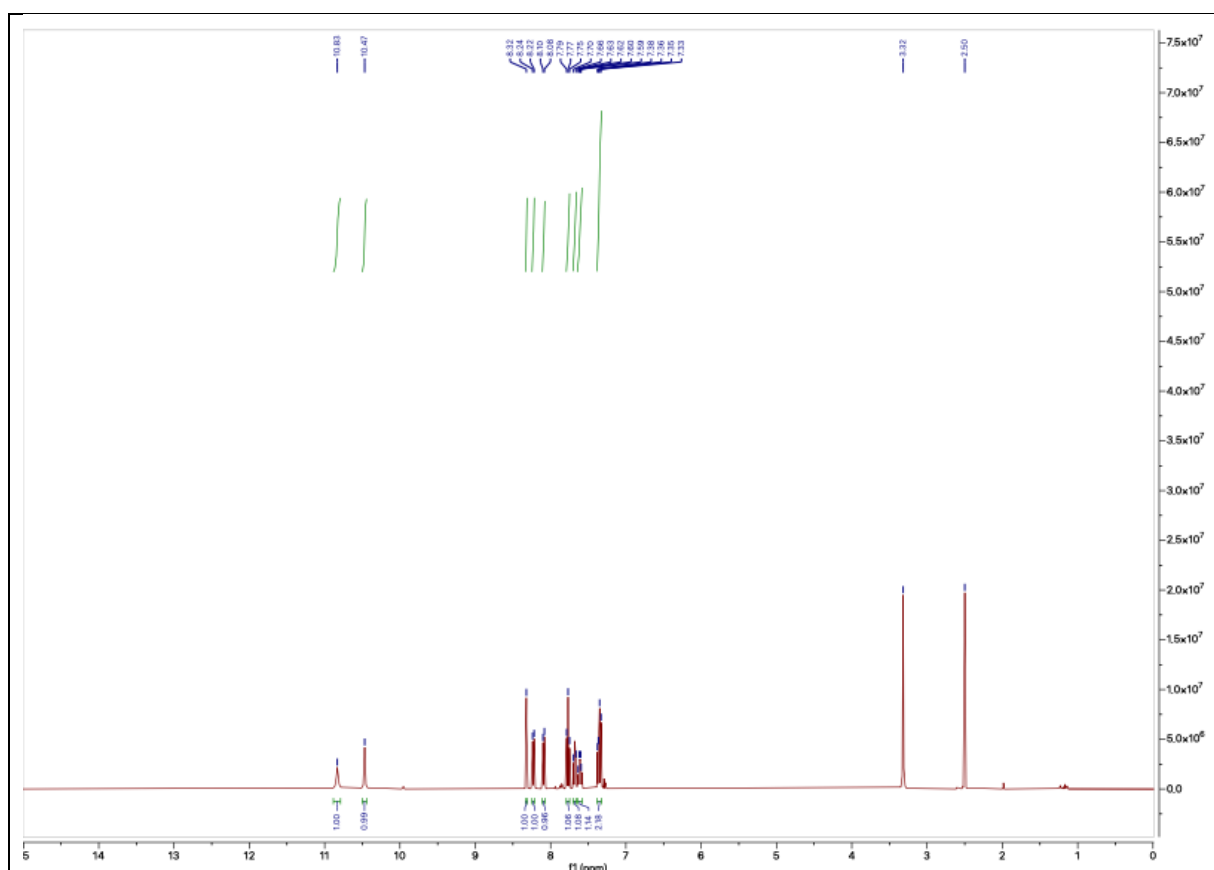
IP13



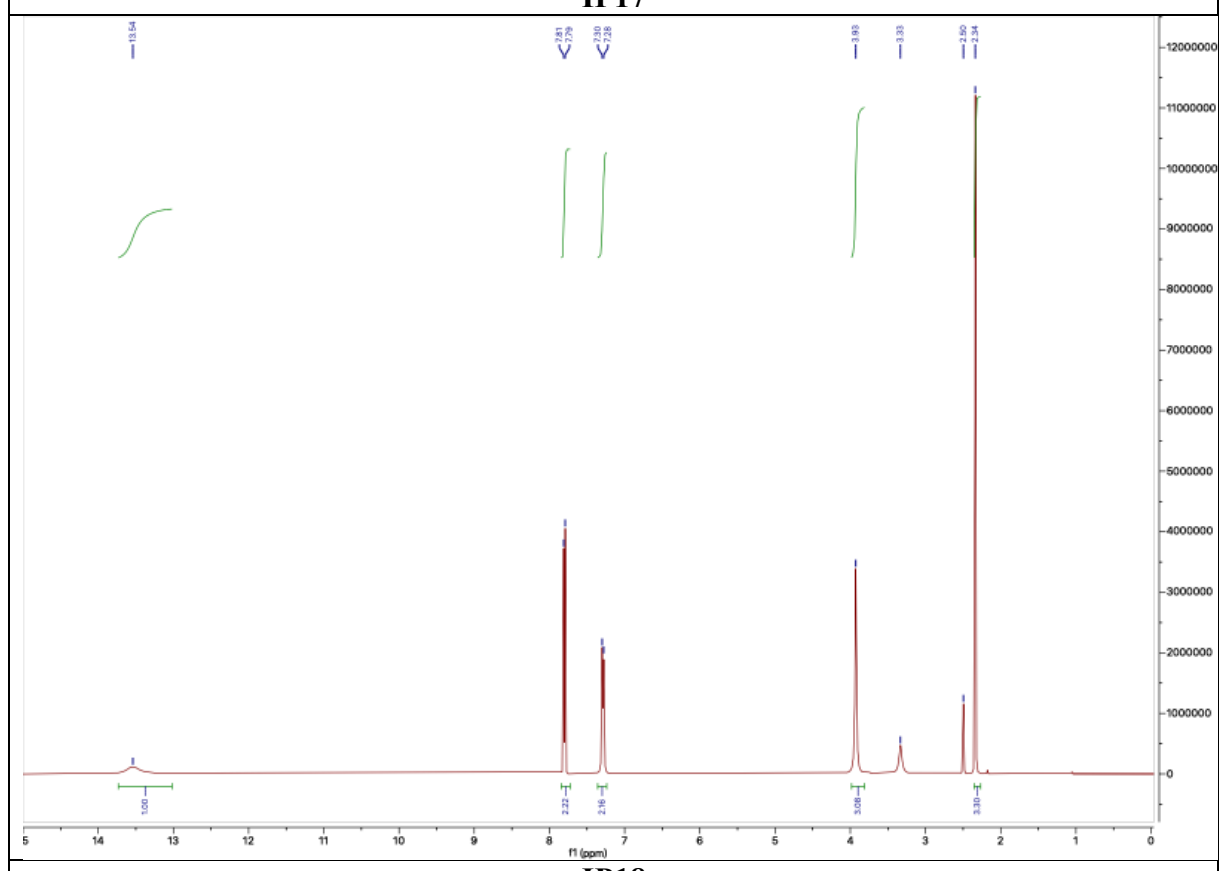
IP14

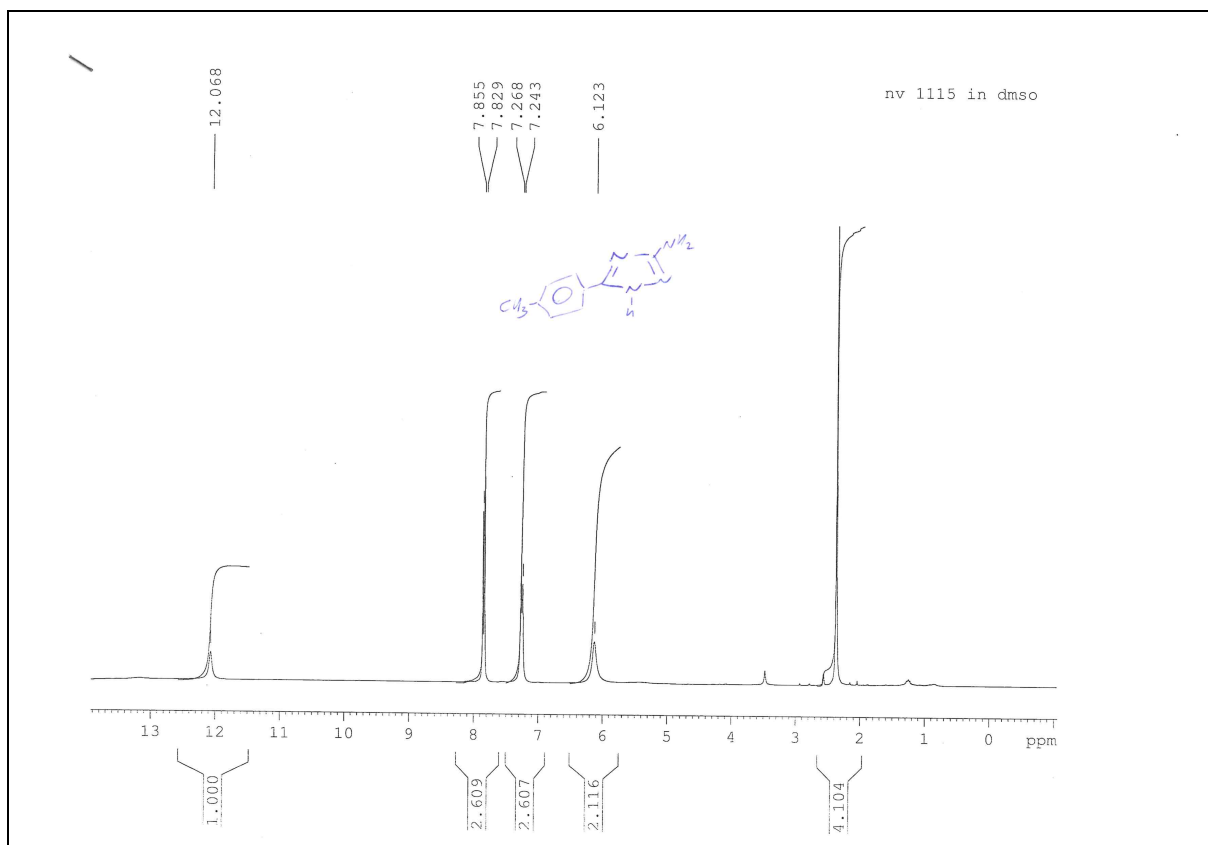


IP15

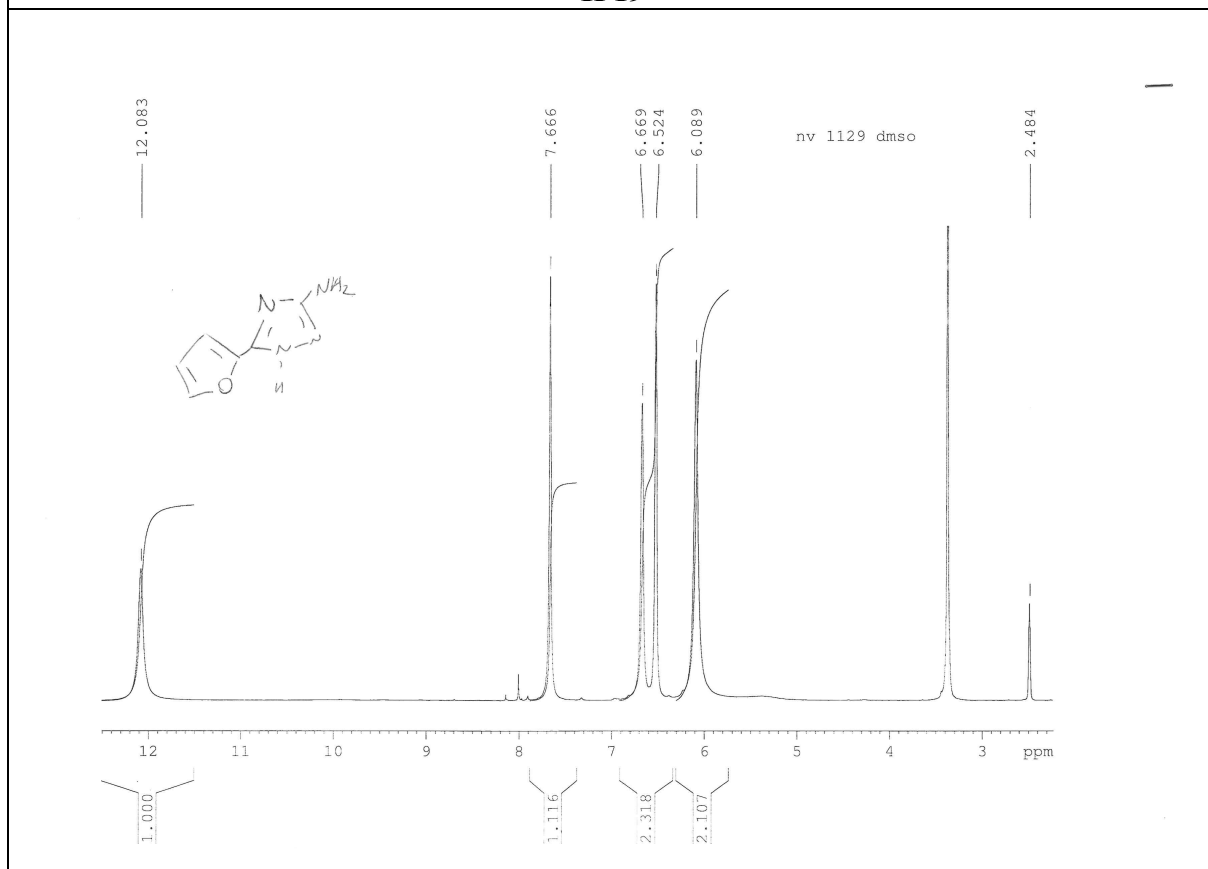


IP17

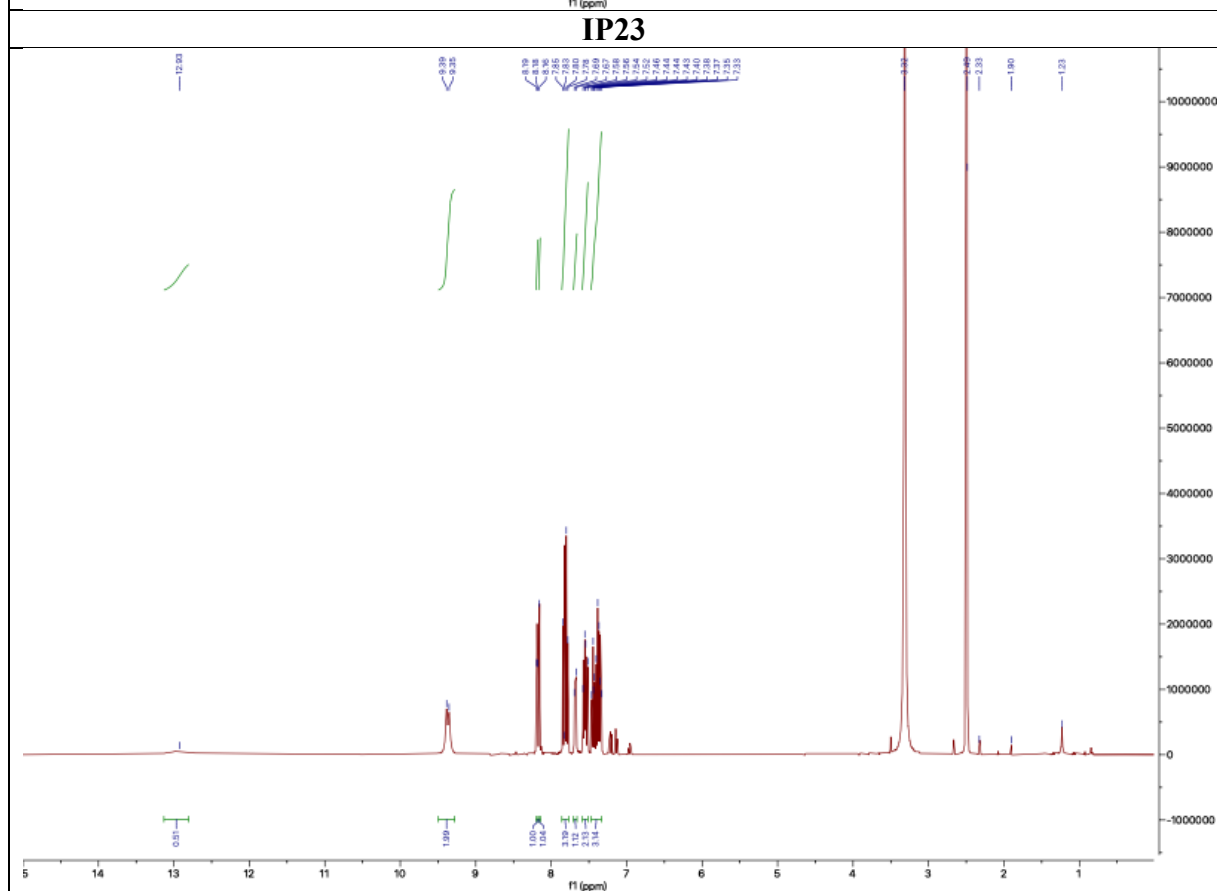
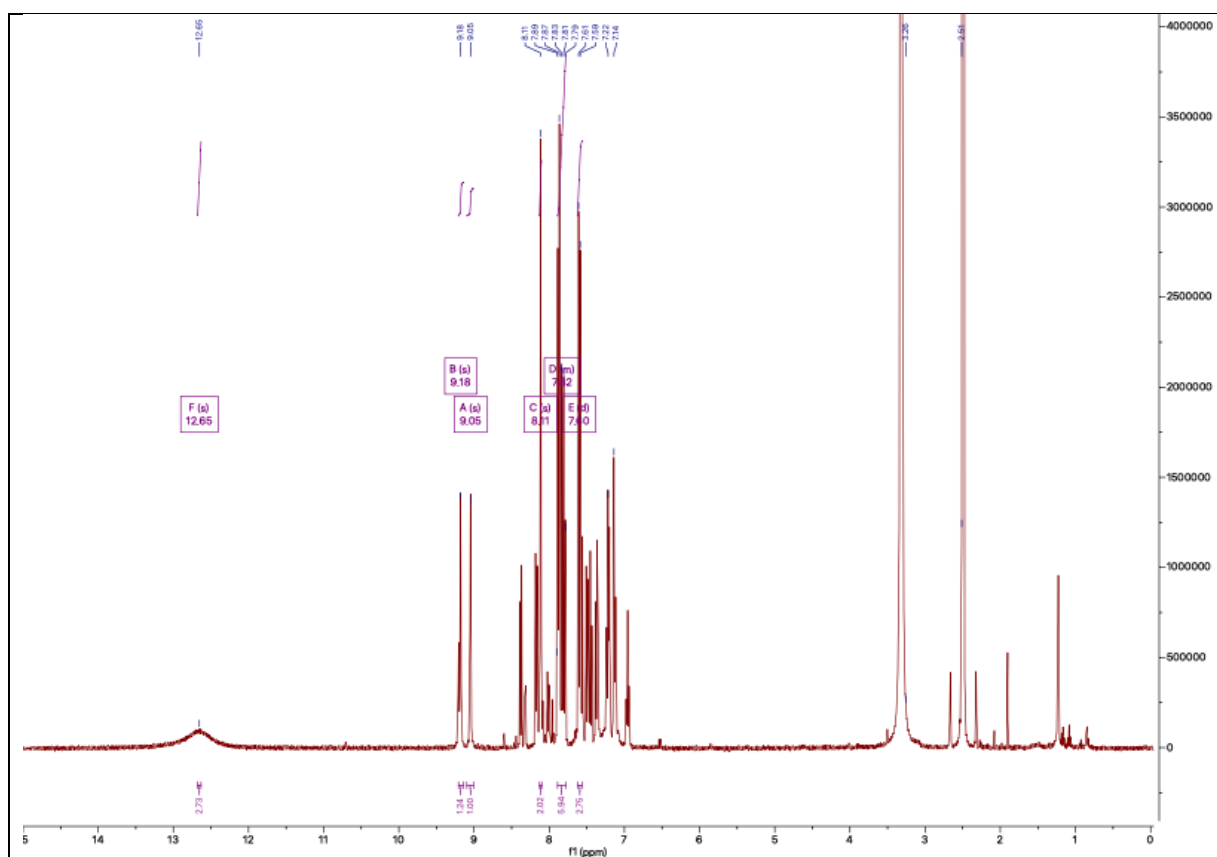


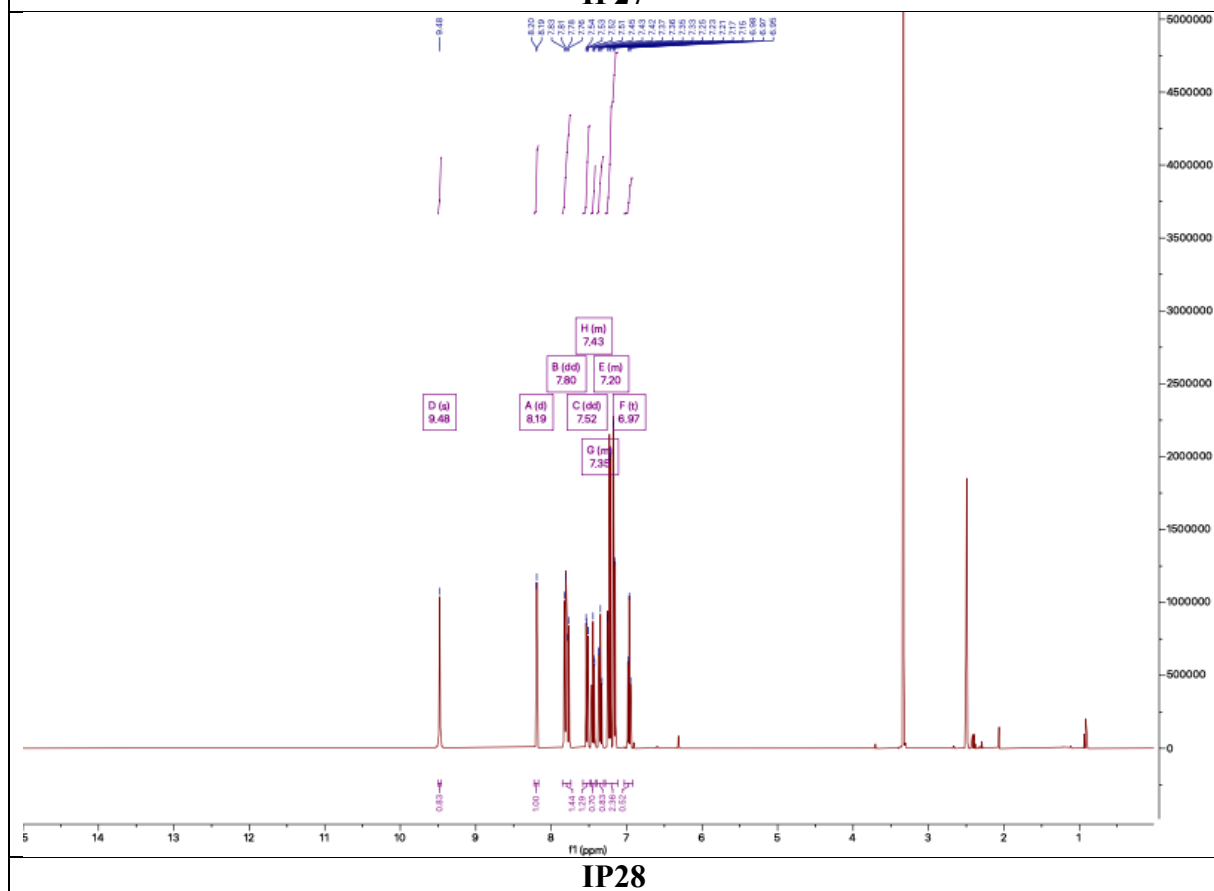
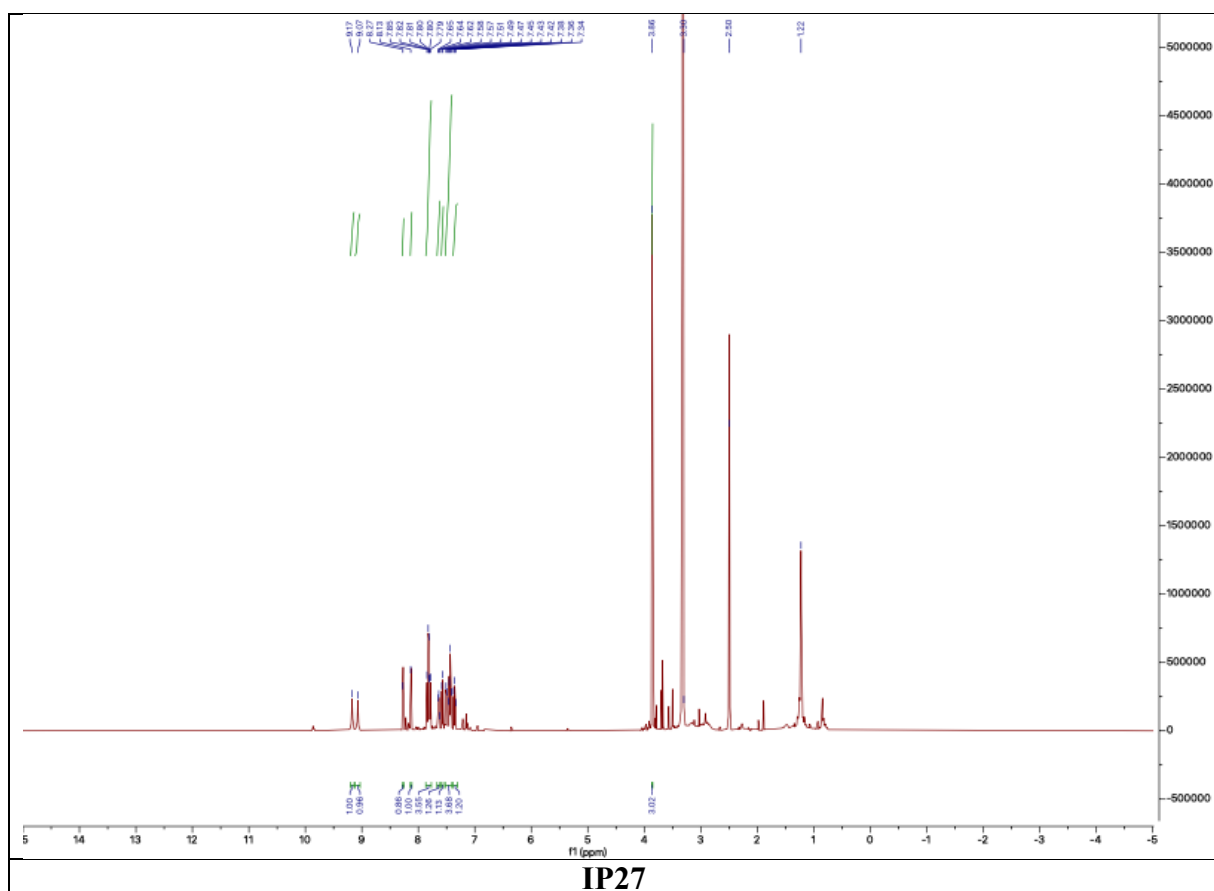


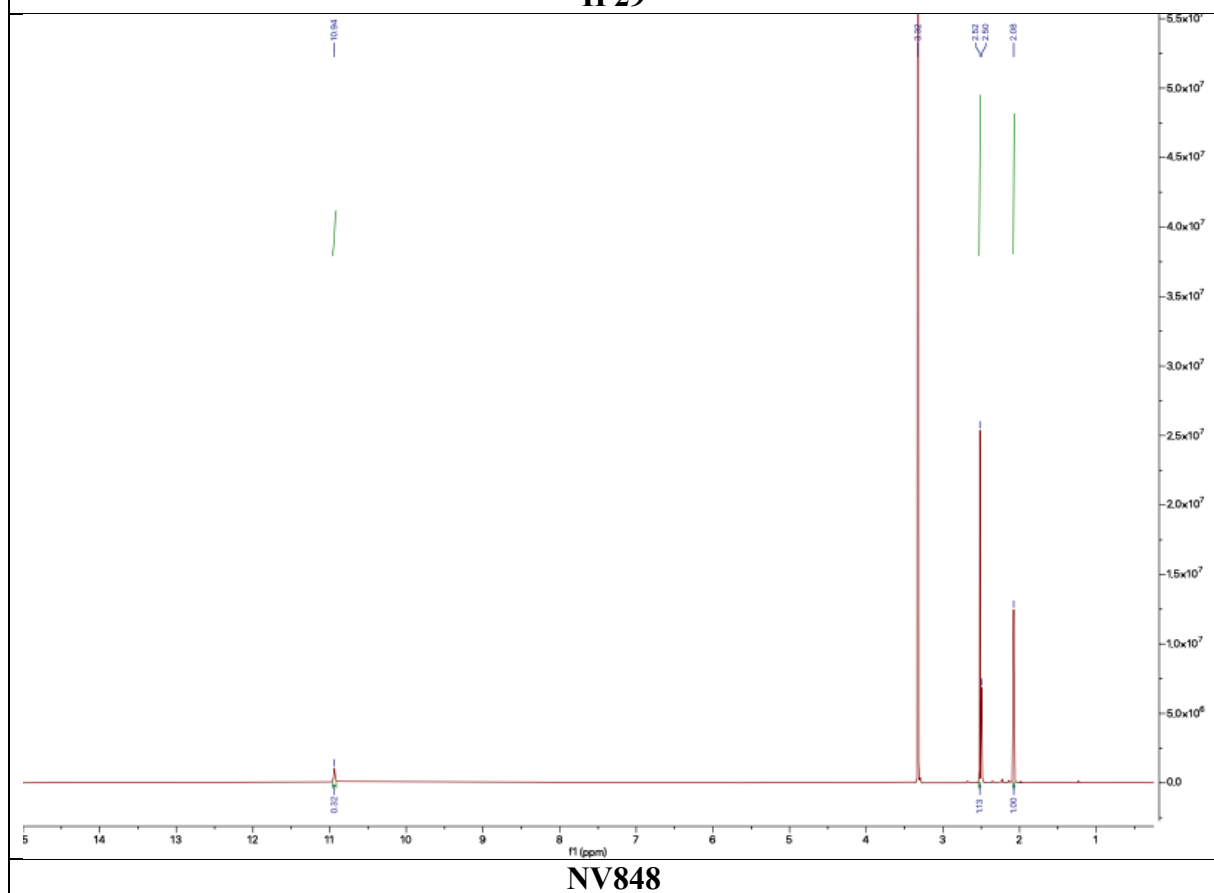
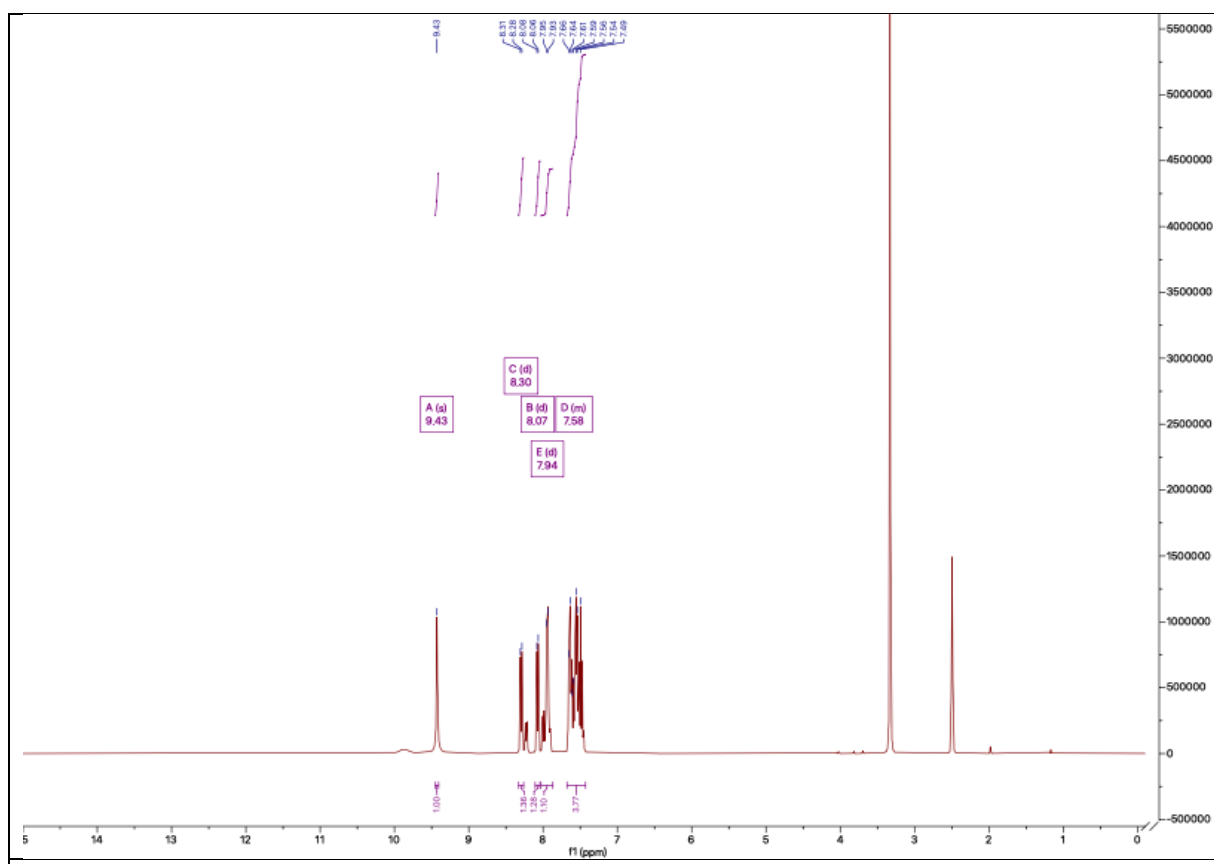
IP19



IP20







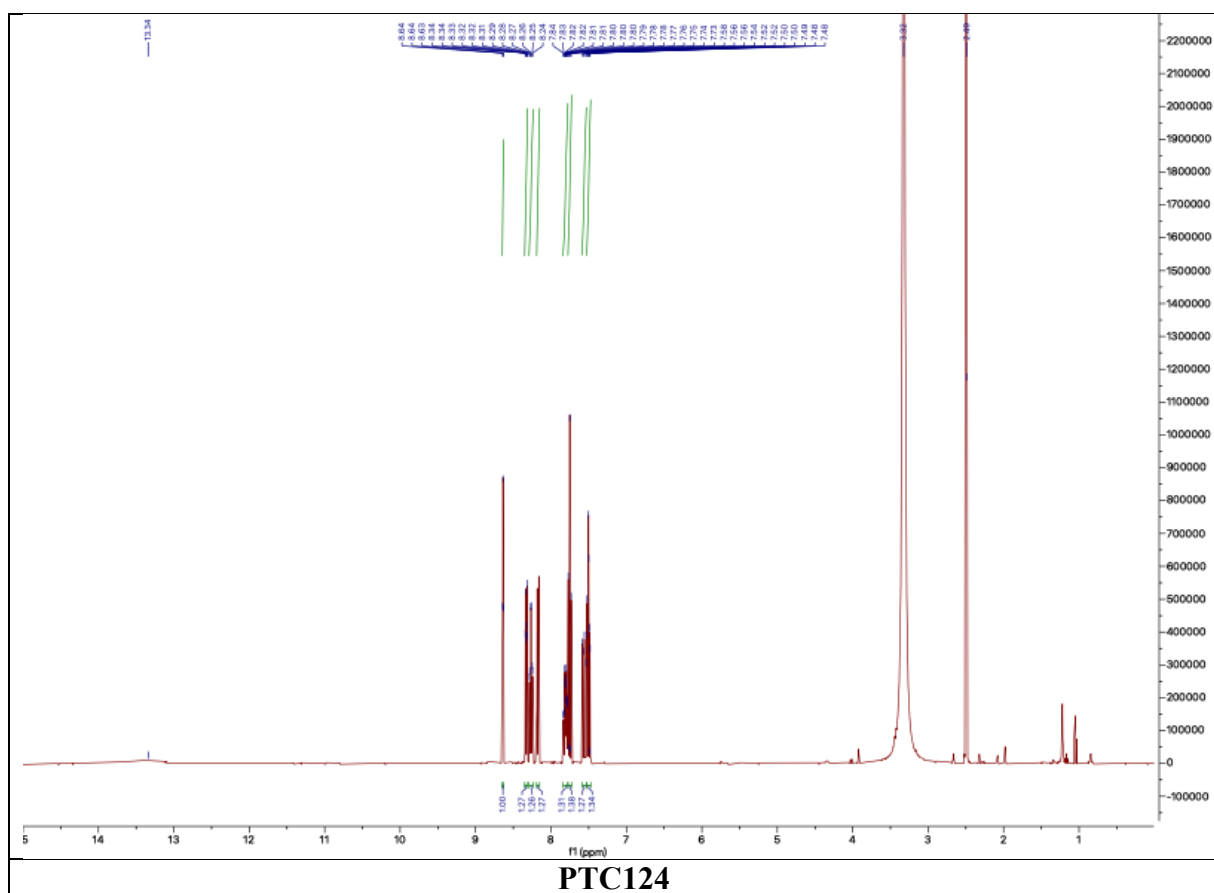
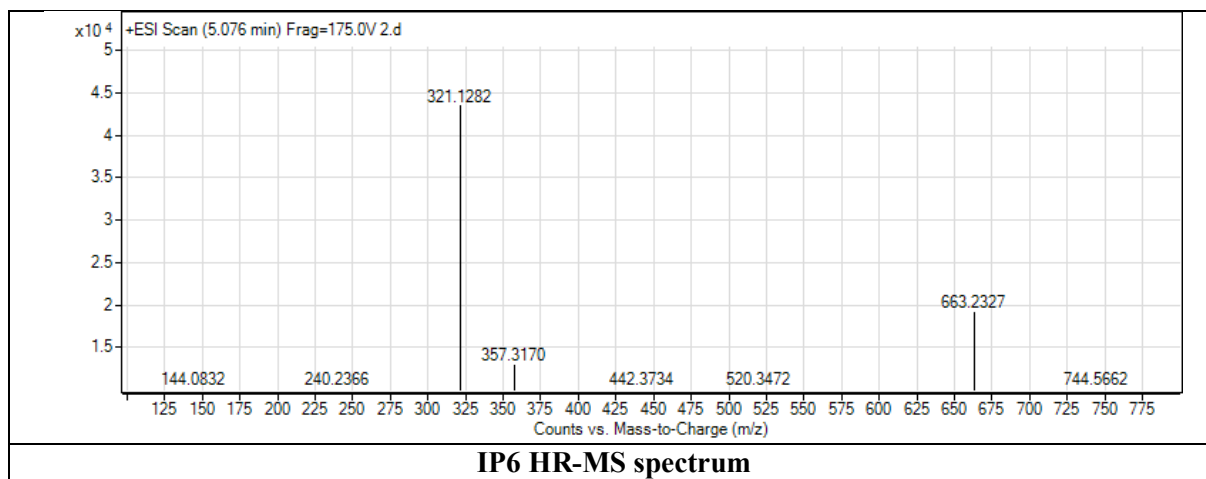
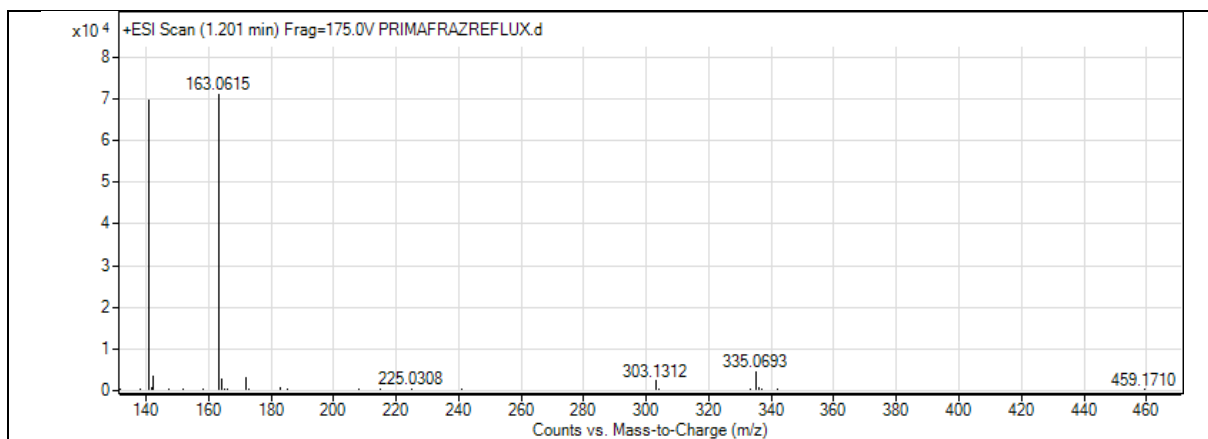
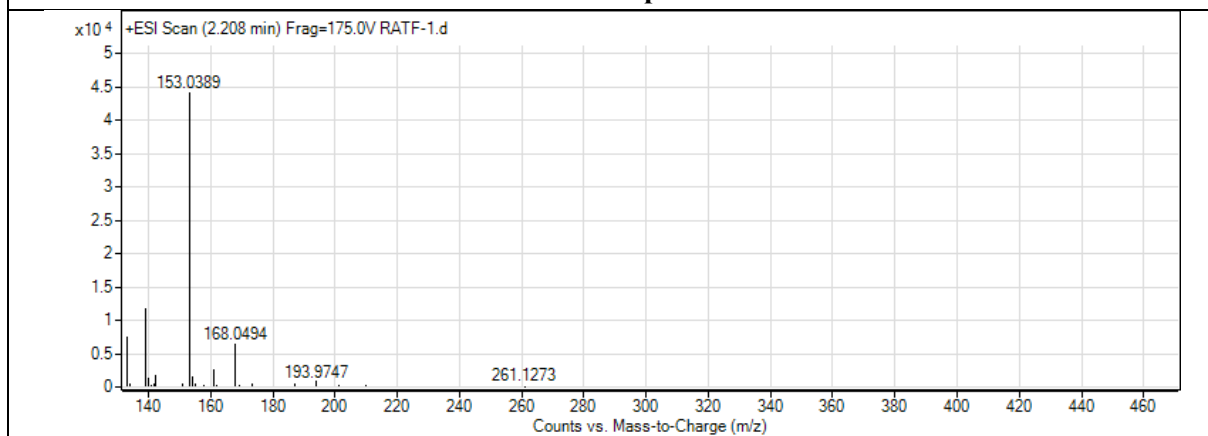


Table A3. HR-MS Data Section

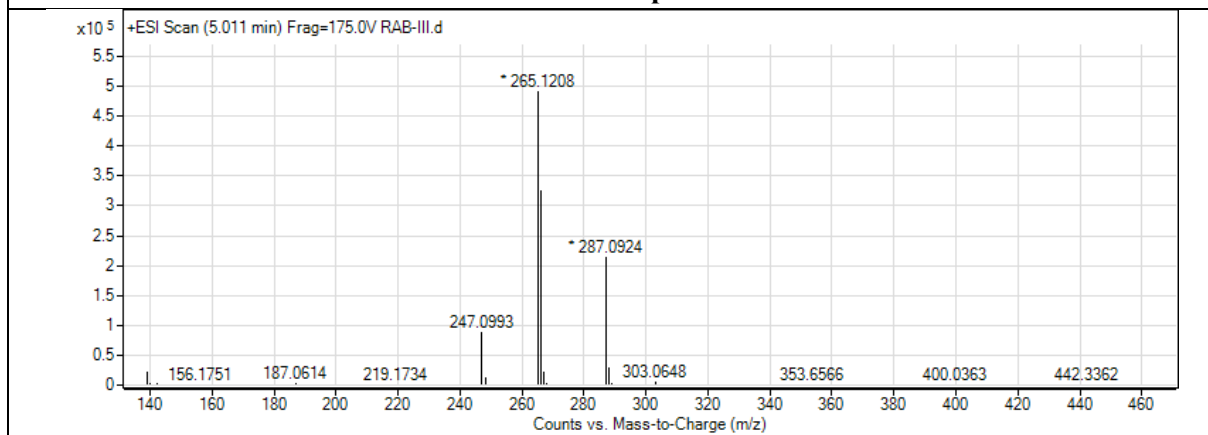




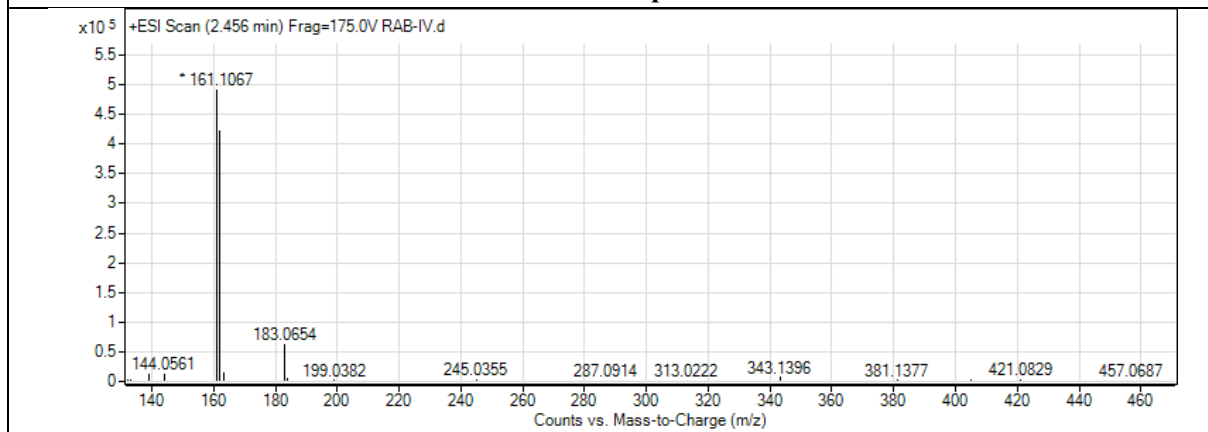
IP8 HR-MS spectrum



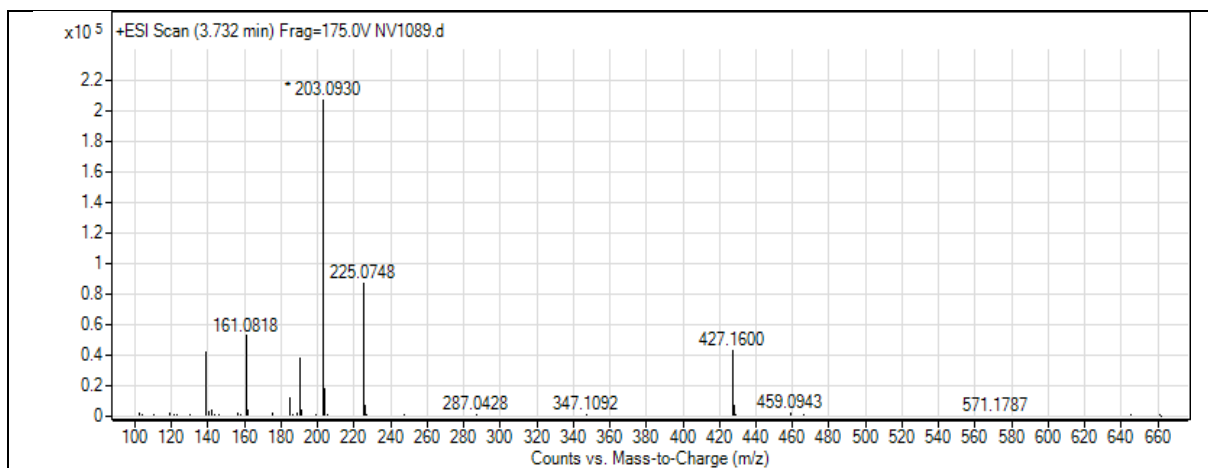
IP10 HR-MS spectrum



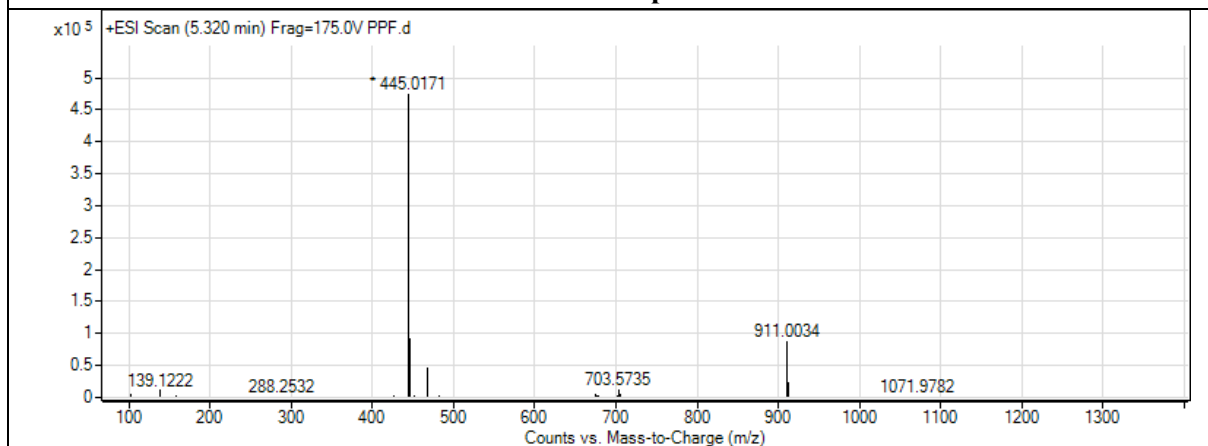
IP12 HR-MS spectrum



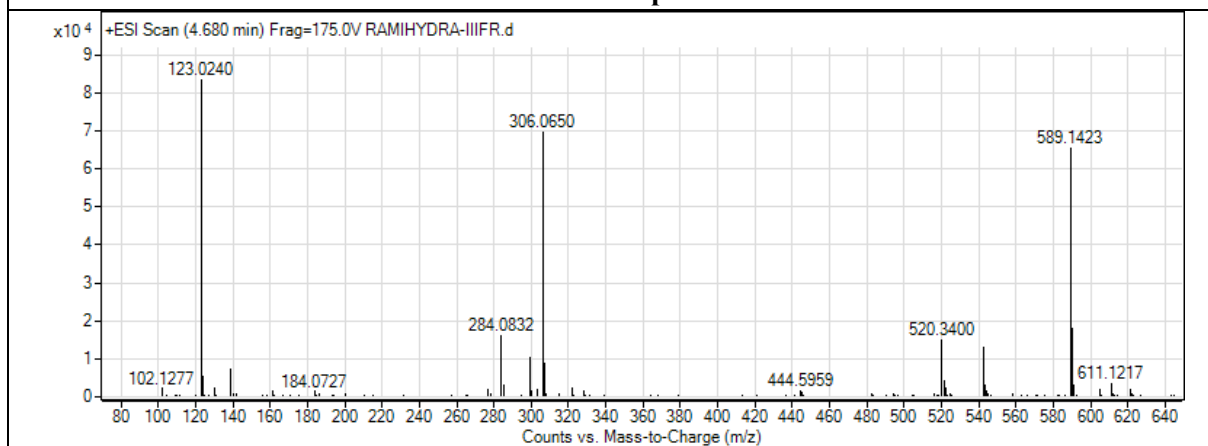
IP13 HR-MS spectrum



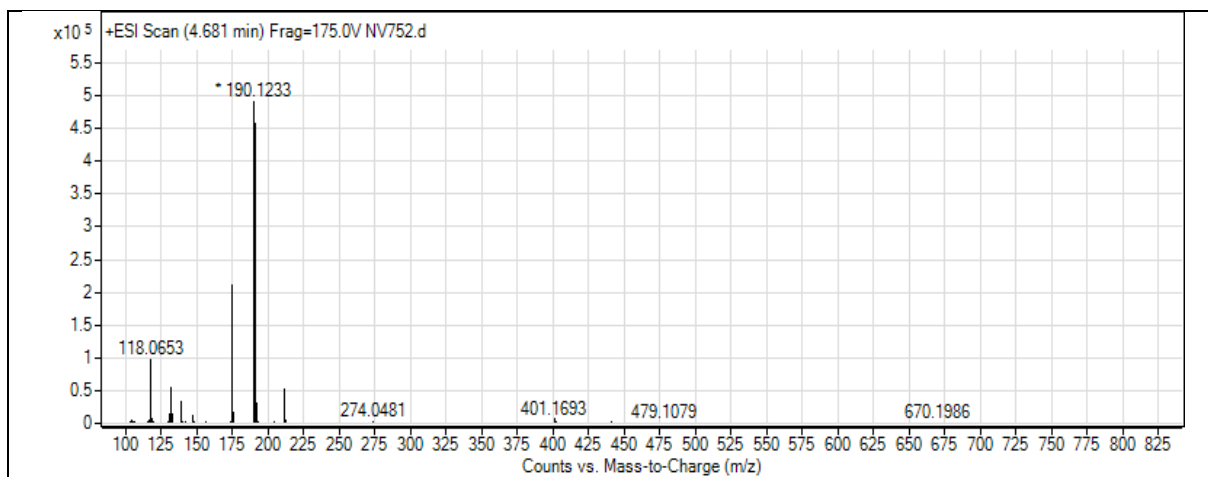
IP14 HR-MS spectrum



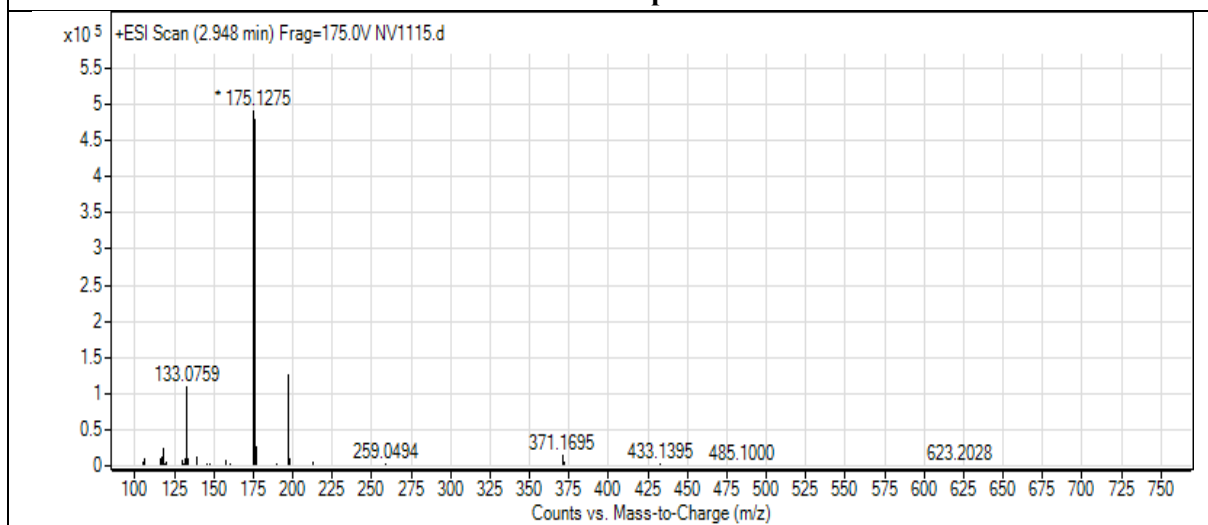
IP15 HR-MS spectrum



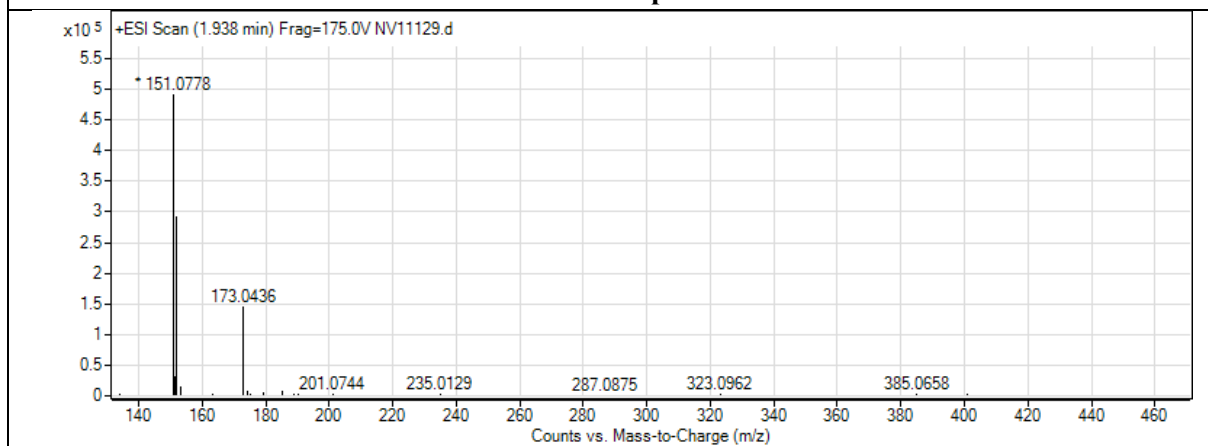
IP17 HR-MS spectrum



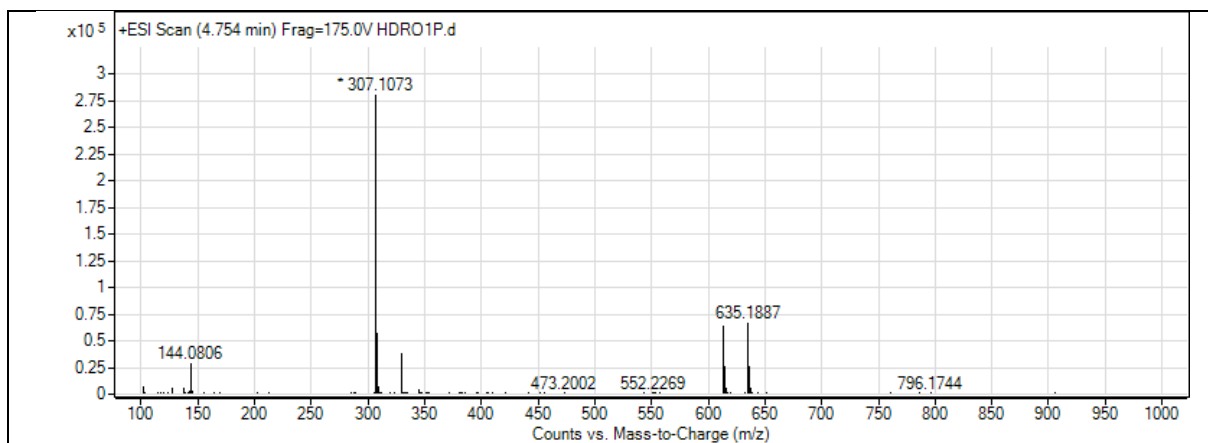
IP18 HR-MS spectrum



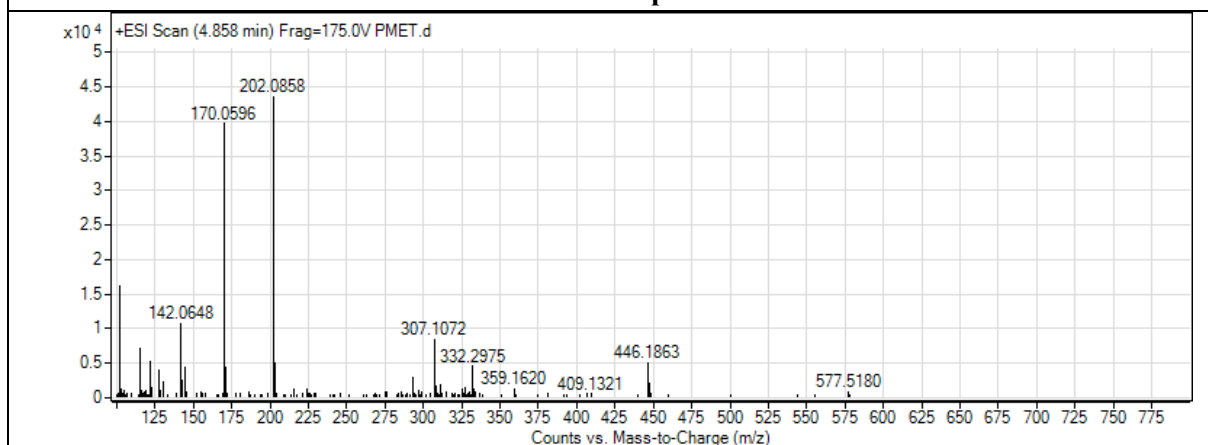
IP19 HR-MS spectrum



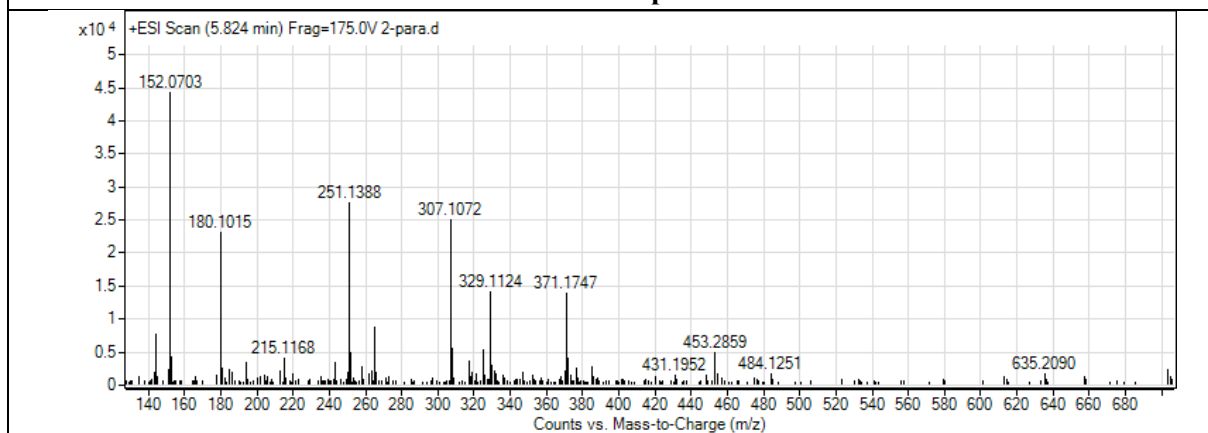
IP20 HR-MS spectrum



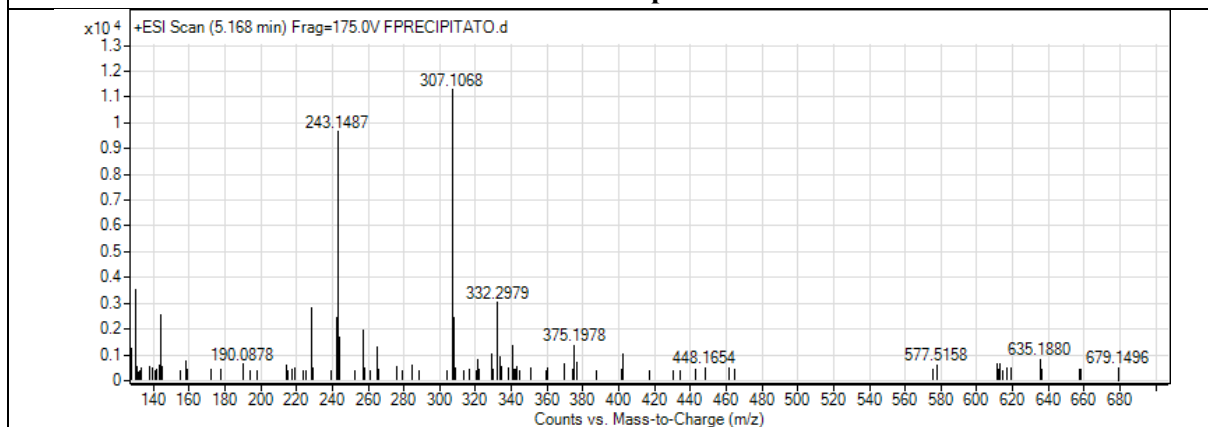
IP21 HR-MS spectrum



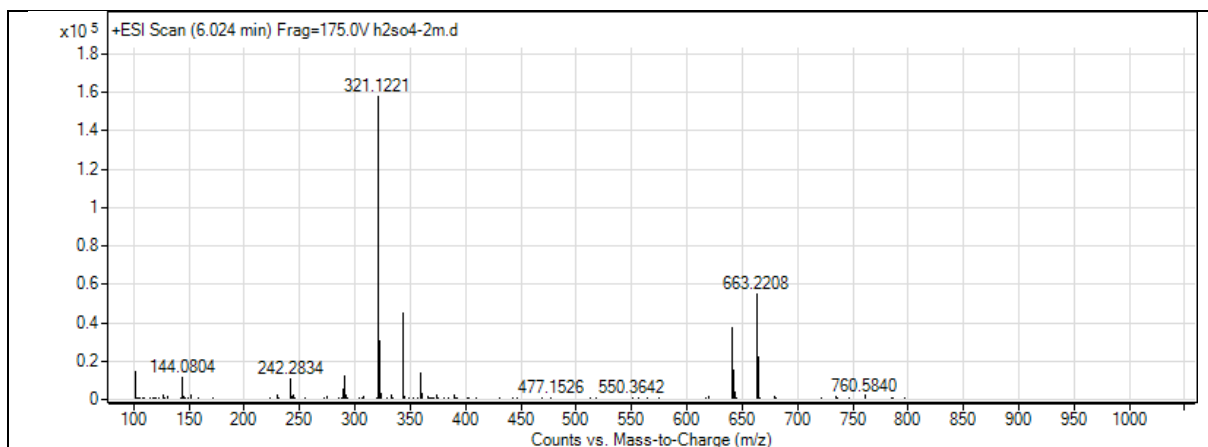
IP22 HR-MS spectrum



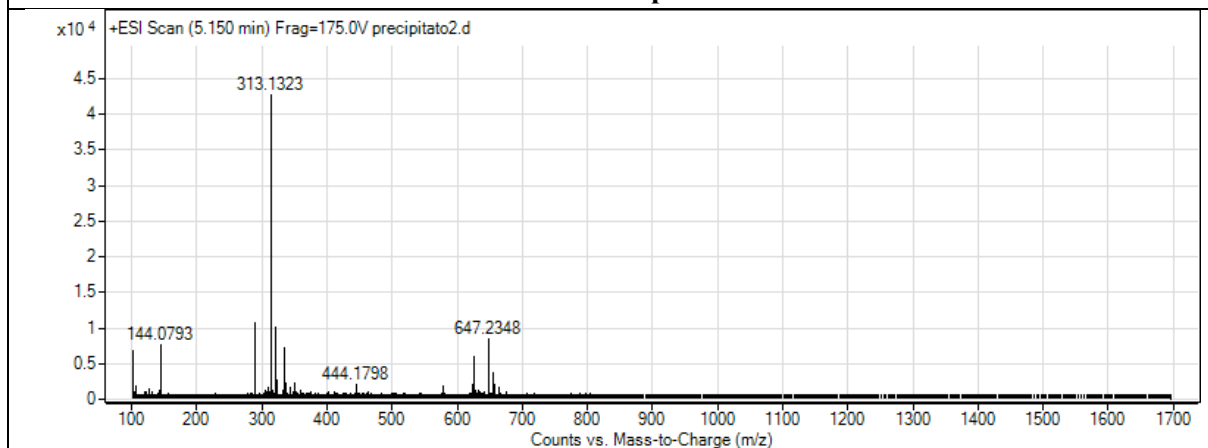
IP23 HR-MS spectrum



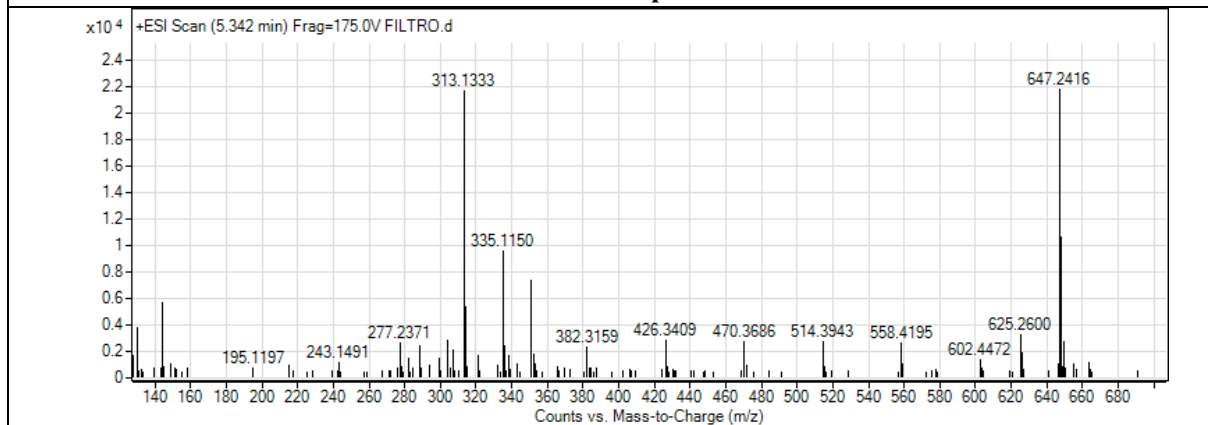
IP24 HR-MS spectrum



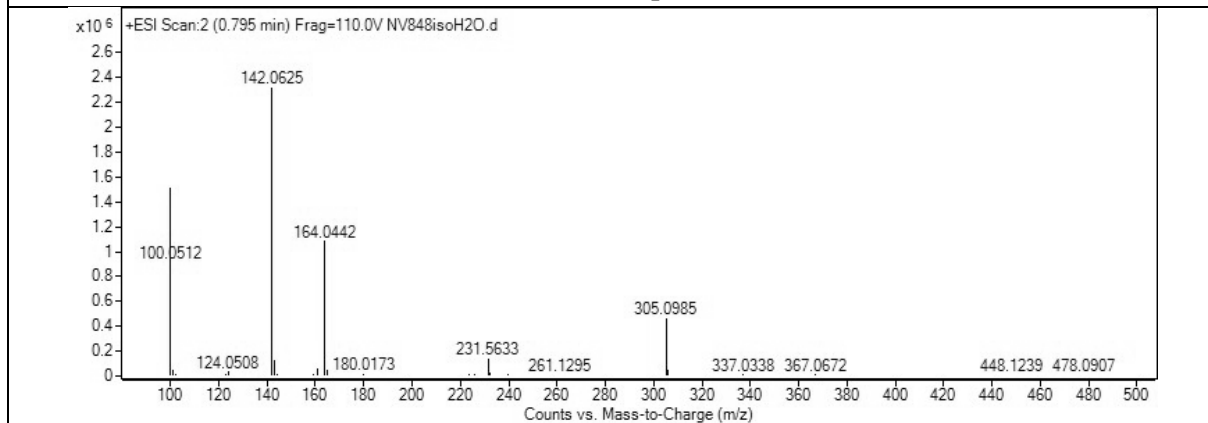
IP27 HR-MS spectrum



IP28 HR-MS spectrum



IP29 HR-MS spectrum



NV848 HR-MS spectrum

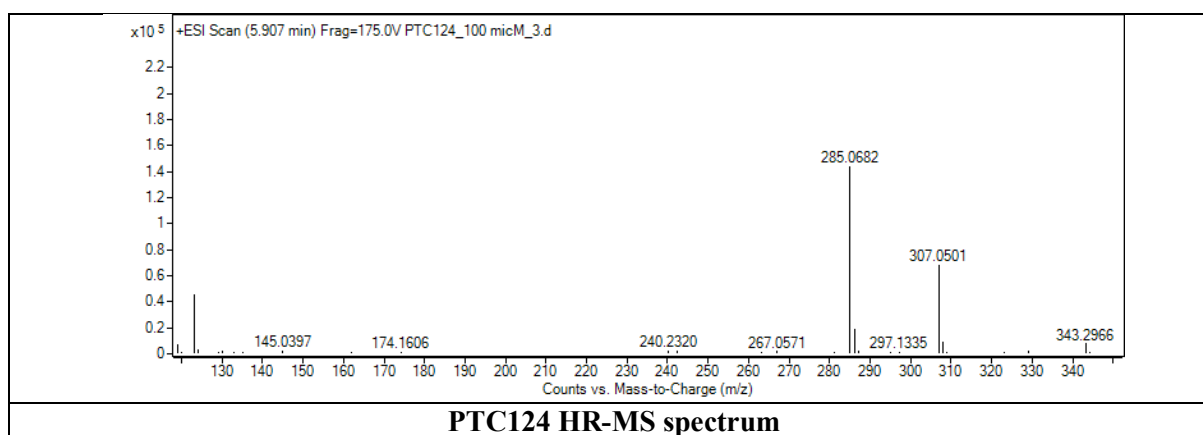


Table A4. Results from Model 1 screening against the Zinc Database.

Name	DATABASE ID	Status	Property
Vistusertib	DB11925	Investigational	serine/threonine protein kinase mTOR inhibitor
Drometrizole trisiloxane	DB11585	Approved	fluroforo ad broad-spectrum utilizzato nelle sunscreen
Novobiocin	DB01051	Approved then withdrawn	topoisomerase and gyrase inhibitor
2-(1,3-thiazol-4-yl)-1H-benzimidazole-5-sulfonamide	DB08083	Experimental	carbonic anhydrase
BMS-986094	DB11966	Investigational	INX-08189 has been used i+E7:E8n trials studying the treatment of Hepatitis C, HCV. Interaction with 40s subunit. Inhibitor RNA polymerase
Cambendazole	DB11380	Human trial, Vet approved	inhibitor Tubulin alpha 1a-chain and Beta 4b-chain
N-1H-indazol-5-yl-2-(6-methylpyridin-2-yl)quinazolin-4-amine	DB08450	Experimental	inhibitor TGF-beta (transforming growth factor beta) receptor 1
2,5-DICHLORO-N-(5-CHLORO-1,3-BENZOXAZOL-2-YL)BENZENESULFONAMIDE	DB07312	Experimental	inhibitor of fructose-1,6-bisphosphatase (FBPase-1)
Icariin	DB12052	Investigational	inhibitor of cGMP-specific 3',5'-cyclic phosphodiesterase
Imino[2-(2-oxo-1,2-dihydro-3-pyridinyl)-1H-benzimidazol-5-yl]methanaminium	DB04442	Experimental	inhibitor Trypsine-1(not confirmed)
Hemi-Babim	DB01767	Experimental	inhibitor prototrombine, inhibitor Trypsine-1 (not confirmed), inhibit mPRO and papaine like protease 8 (Sars Cov-2)
CRA_10656	DB04470	Experimental	inhibitor Trypsine-1
Bis(5-Amidino-Benzimidazolyl)Methane	DB01705	Experimental	tripatase inhibito, inhibitor Trypsine 1, botulinium neurotoxin type B target
AZD-8055	DB12774	Investigational	inhibitor sereine/treonine kinasi mTOR
Caflanone	DB17263	Investigational	sars CoV proliferation limits
Decernotinib	DB12566	Investigational	inhibitor Tyrosine-protein kinase JAK3
Bludigo (carminio indaco)	DB11577	Approved	agonist of alpha adrenergic receptor, inhibitor guanylyl cylcase.
Veliflapon	DB16346	Investigational	inhibitor Arachidonate 5-lipoxygenase-activating protein
Cirtuvivint	DB17269	Investigational	first-in-class CDC-like kinase (CLK) inhibitor

Albaconazole	DB12073	Investigational	inhibitor cythocromes P450
Balaglitazone	DB12781	Investigational	modulator Peroxisome proliferator-activated receptor gamma
Clorobiocin	DB03966	Experimental	topoisomerase and gyrase inhibitor
Ceralasertib	DB14917	Investigational	serine/threonine protein kinase inhibitor ATR
Cenerimod	DB12705	Investigational	high selective modulator f+B26:E37or s1p1
N,4-dimethyl-3-[(1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino]benzamide	DB08242	Experimental	interact with Mitogen-activated protein kinase 14
Cloprostenol	DB11507	Vet approved	agonist of prostagladin and tromboxane receptor
Filgotinib	DB14845	Approved, Investigational	inhibitor janus kinase 1
Imazaquin	DB04582	Experimental	inhibitor acetohydroxy synthase
Indigo	DB16862	Experimental, Investigational	bind with aryl hydrocarbon receptor
Paquinimod	DB13118	Investigational	inhibitor S100A9
2-(6-methylpyridin-2-yl)-N-pyridin-4-ylquinazolin-4-amine	DB07267	Experimental	inhibitor ALK5 (Tgf-beta type I receptor) KINASE
TAK-593	DB13093	Investigational	modulator of Vascular endothelial growth factor receptor 2 and Platelet-derived growth factor receptor beta
(6,7-Difluoro-Quinazolin-4-Yl)-(1-Methyl-2,2-Diphenyl-Ethyl)-Amine	DB03678	Experimental	inhibitor of scytalone dehydratase
Zopolrestat	DB08772	Experimental	modulator aldose reductase
Tanshinone I	DB16886	Investigational	regulator of apoptotic pathway
MK-0873	DB13029	Investigational	inhibitor cAMP-specific 3',5'-cyclic phosphodiesterase
AC-430	DB12535	Investigational	inhibitor janus kinase 2
1-(2-cyclopropylethyl)-3-(1,1-dioxo-2H-1,2,4-benzothiadiazin-3-yl)-6-fluoro-4-hydroxy-2(1H)-quinolinone	DB08278	Experimental	inhibitor of HVC RNA polimerasi
3-(4-CHLOROPHENYL)-5-(METHYLTHIO)-4H-1,2,4-TRIAZOLE	DB08062	Experimental	inhibitor prototrombine
Pagoclone	DB04903	Investigational	modulator of Gamma-aminobutyric acid receptor subunit
Abacavir	DB01048	Approved	inhibitor of Reverse transcriptase/RNaseH, inhibitor of Gag-Pol polyprotein
Thiabendazole	DB00730	Approved	inhibitor of Fumarate reductase flavoprotein subunit
Laquinimod	DB06685	Investigational	modulator of: Nuclear factor NF-kappa-B p100 subunit, Transcription factor p65, Proto-oncogene c-Rel, C-X-C motif chemokine 2
Roquinimex	DB11366	Investigational	modulator of interleukin-2, inhibitor of angiotensin, decrease TNF alpha level
Xipamide	DB13803	Approved, Investigational	decrease sodium reabsorption in kidney
Gavorestat	DB16707	Investigational	inhibitor of aldose reductase
Brequinar Analog	DB03480	Experimental	act on Dihydroorotate dehydrogenase (quinone), mitochondrial
Brequinar	DB03523	Experimental	modulator of Dihydroorotate dehydrogenase (quinone), mitochondrial
Tasquinimod	DB05861	Investigational	modulator of Protein S100-A9
Rivoglitazone	DB09200	Experimental, Investigational	agonist of Peroxisome proliferator-activated receptor gamma
JQ1	DB17021	Investigational	inhibitor of Bromodomain-containing protein 4
Efatutazone	DB11894	Investigational	agonist of Peroxisome proliferator-activated receptor gamma

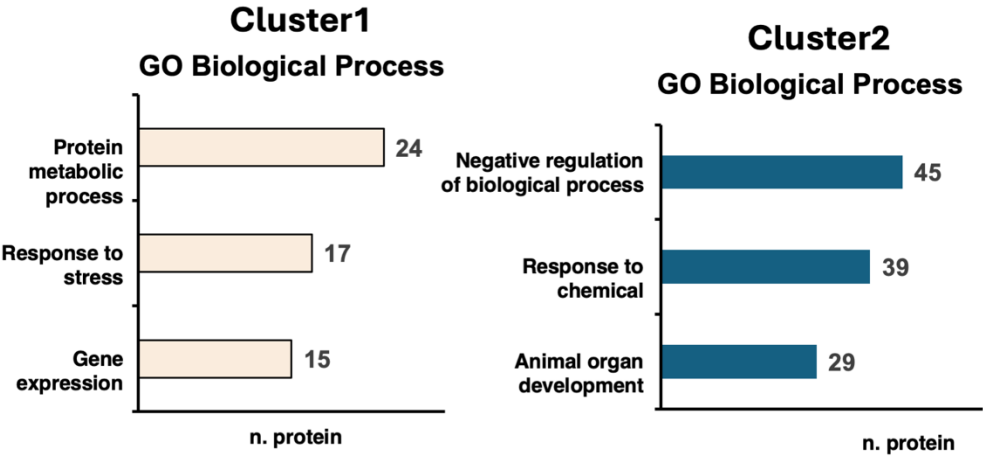
APPENDIX B

Table 1B. Mice genotype and treatment.

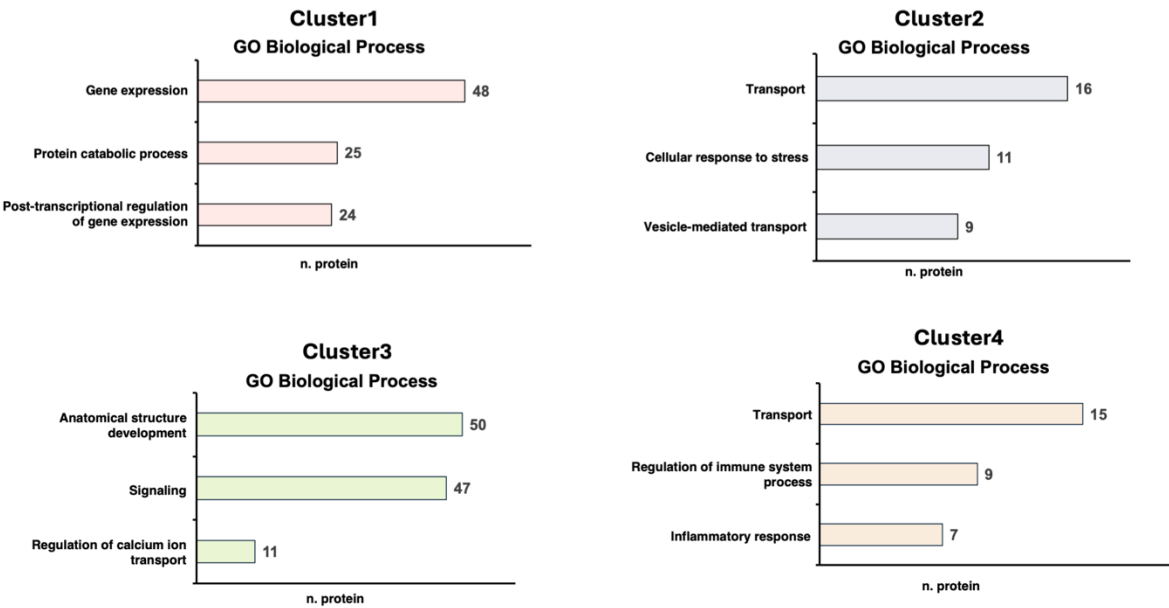
ID MICE	Genotype	Treatment	Data sacrifice
T16	G542X/G542X	H2O	13/10/22
T41	G542X/G542X	H2O	02/02/23
T58	G542X/G542X	H2O	24/02/23
T194	G542X/G542X	H2O	15/11/23
T238	G542X/G542X	H2O	11/07/24
T19	WT	H2O	13/10/22
T40	WT	H2O	02/02/23
T51	WT	H2O	24/02/23
T169	G542X/G542X	PTC124	03/10/23
T200	G542X/G542X	PTC124	15/11/23
T223	G542X/G542X	PTC124	06/02/24
T225	WT	PTC124	20/02/24
T226	WT	PTC124	20/02/24
T228	WT	PTC124	20/02/24
T229	WT	PTC124	20/02/24
T230	WT	PTC124	20/02/24
T3	G542X/G542X	NV848	13/10/22
T5	G542X/G542X	NV848	13/10/22
T6	G542X/G542X	NV848	13/10/22
T11	G542X/G542X	NV848	13/10/22
T17	G542X/G542X	NV848	13/10/22
T2	WT	NV848	13/10/22
T4	WT	NV848	13/10/22
T8	WT	NV848	13/10/22
T13	WT	NV848	13/10/22
T15	WT	NV848	13/10/22

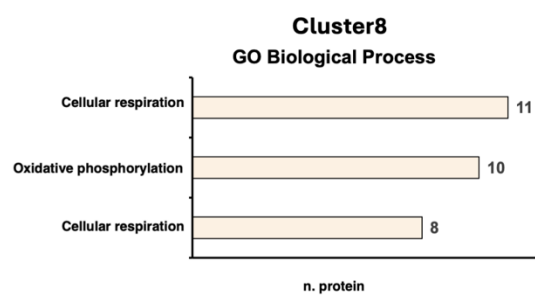
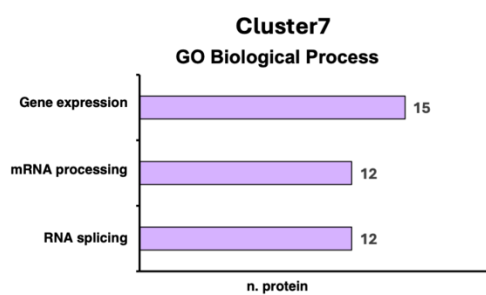
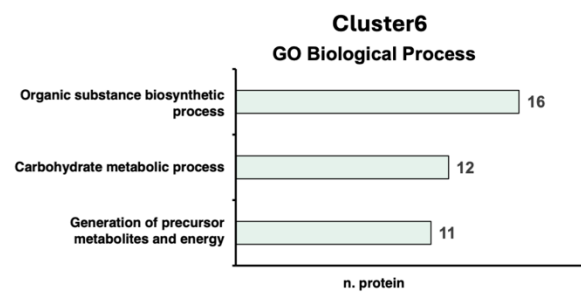
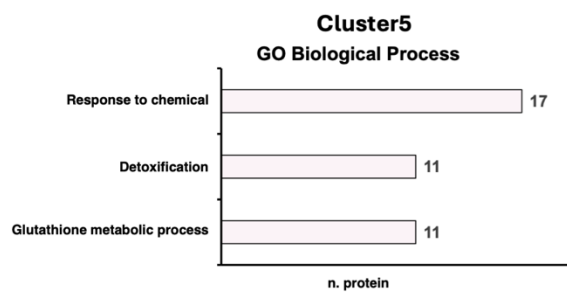
Gene Ontology (GO) enrichment analysis results for identified clusters.

Gene Ontology (GO) enrichment analysis results for identified clusters in WTPTC124 DEPs PPI

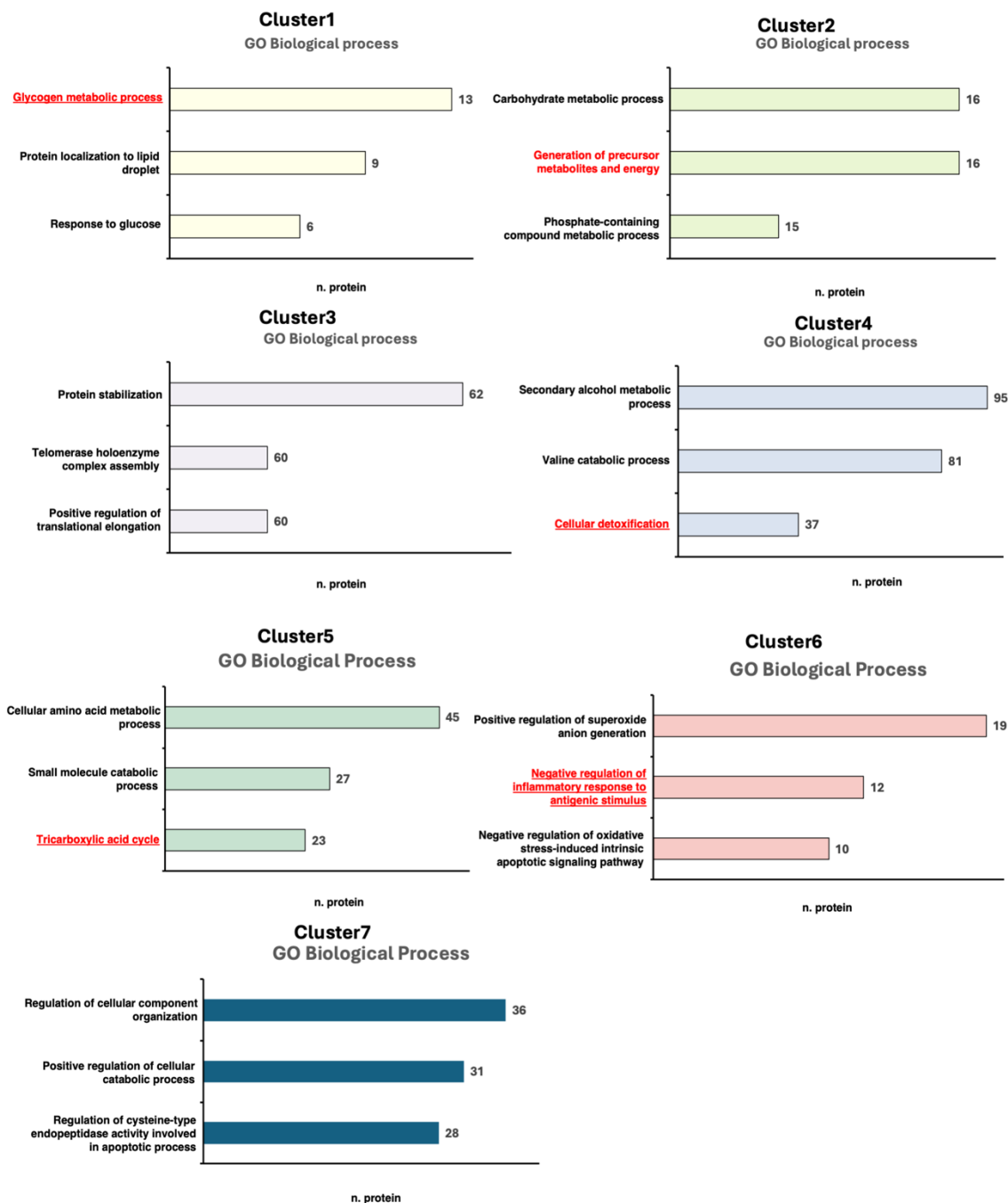


Gene Ontology (GO) enrichment analysis results for identified clusters in Pancreas CFTR-related DEPs PPI

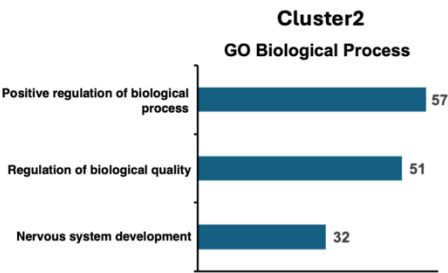
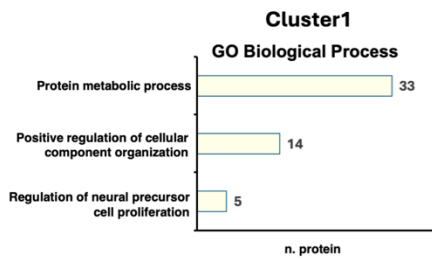




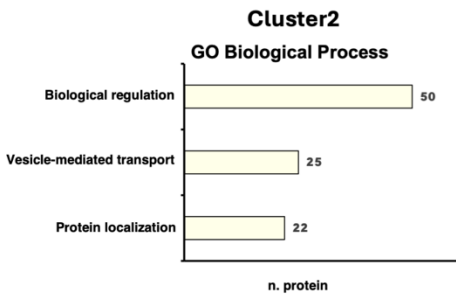
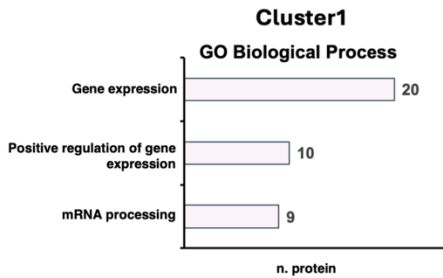
**Gene Ontology (GO) enrichment analysis results for identified clusters in Liver
CFTR-related DEPs PPI**



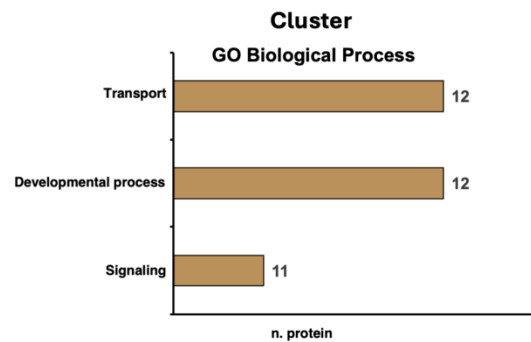
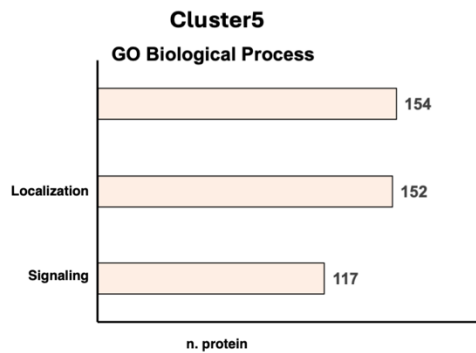
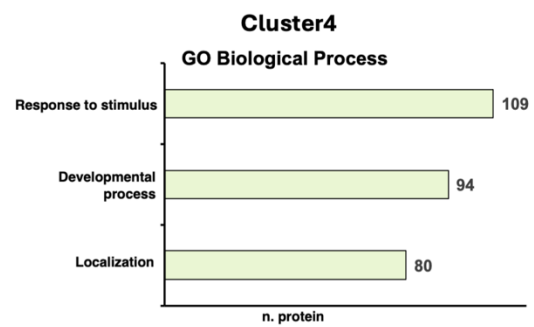
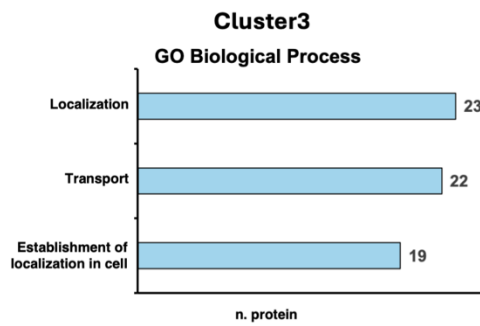
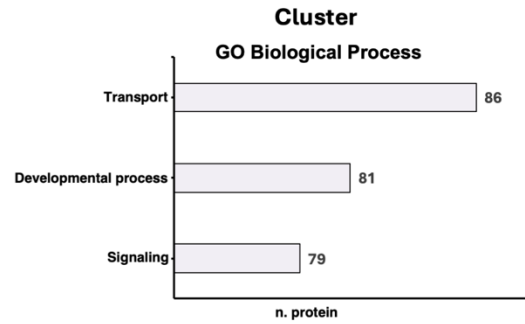
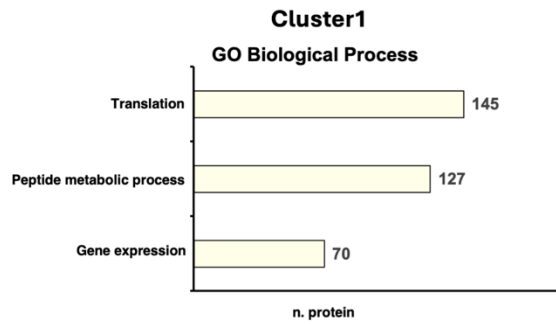
Gene Ontology (GO) enrichment analysis results for identified clusters in Brain WTNV848 DEPs PPI



Gene Ontology (GO) enrichment analysis results for identified clusters in Brain WTPTC124 DEPs PPI



**Gene Ontology (GO) enrichment analysis results for identified clusters in Brain
CFTR-related DEPs PPI**



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AKNOWLEDGMENT

I would like to express my deepest gratitude to Prof. Ivana Pibiri for being a steadfast point of reference and a constant source of inspiration throughout my doctoral journey. Her unwavering trust in my abilities and ideas, together with her invaluable support in my personal and professional growth, and her elegant and kind guidance in my research path, have been truly inestimable.

I am sincerely grateful to Prof. Lentini for guiding me in the exploration of molecular biology and genetics, supporting my growth with competence and professionalism, and always showing enthusiasm and a proactive spirit towards the proposals made.

My heartfelt thanks go to Dr. Ignazio Fiduccia and Dr. Carla Rizzo for all the support provided, for helping me overcome moments of difficulty, and for their constant companionship and reliable presence throughout my journey.

I warmly thank the FCT NOVA University of Lisbon, in particular Prof. José Luis Capelo Martínez and Prof. Hugo M. Santos, for hosting me during my research stay abroad, for embracing with scientific openness the research proposal on *in vivo* models with constant enthusiasm, for allowing me to discover and appreciate the field of proteomics, and for their invaluable contribution to my scientific training.

I also wish to acknowledge with gratitude Prof. Mariagrazia Zizzo and Dr. Ilenia Cruciata for their valuable support in providing me with *in vivo* mice model tissue, which has been instrumental to my research.

I am truly grateful to the BIOSCOPE GROUP, especially Dr. André Figueiredo, Dr. Inês M. Freitas, and Dr. Luís B. Carvalho, for welcoming me and integrating me as one of their own during my experience in Portugal.

Finally, I thank Dr. Emanuele Vitale and Dr. Michele Menditto for their support in the biological assays.

This work has been carried out with the financial support of the Italian National Recovery and Resilience Plan (PNRR), which I gratefully acknowledge for funding the grant that made this research possible.