Biological characterization of CFTR corrector ARN23765 in live cells

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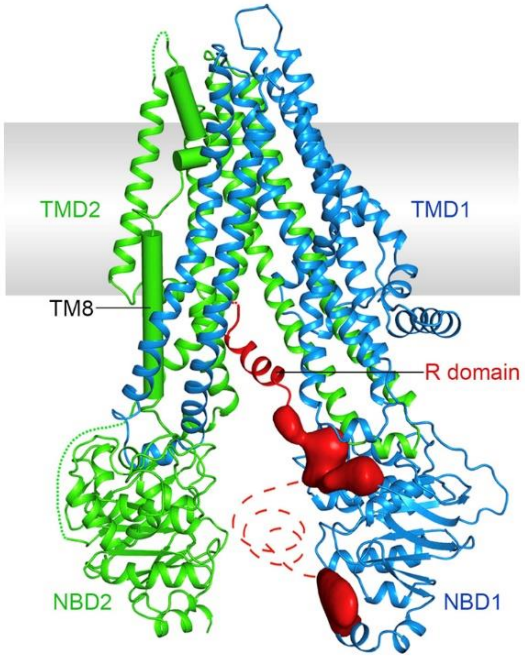
Cystic Fibrosis (CF)

CF is caused by mutations in the **Cystic Fibrosis Transmembrane conductance Regulator** (**CFTR**) gene that lead to loss-of-function or loss-of-expression of the CFTR protein.

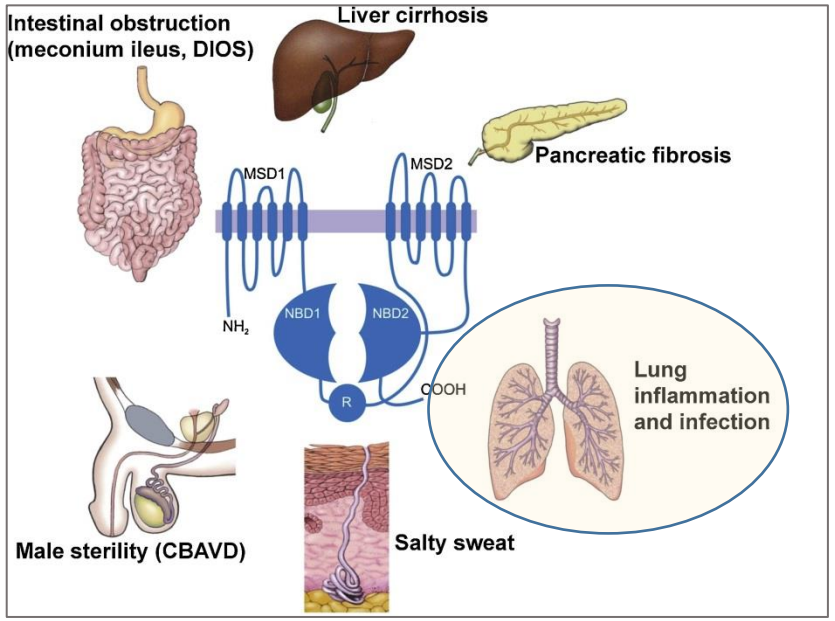


Molecular structure of human CFTR determined in the dephosphorylated, ATP-free form

Liu et al., *Cell* **2017**, *169*, 85-89

CFTR is an epithelial ion channel involved in anions transport (Cl^- and HCO_3^-) in multiple organs.

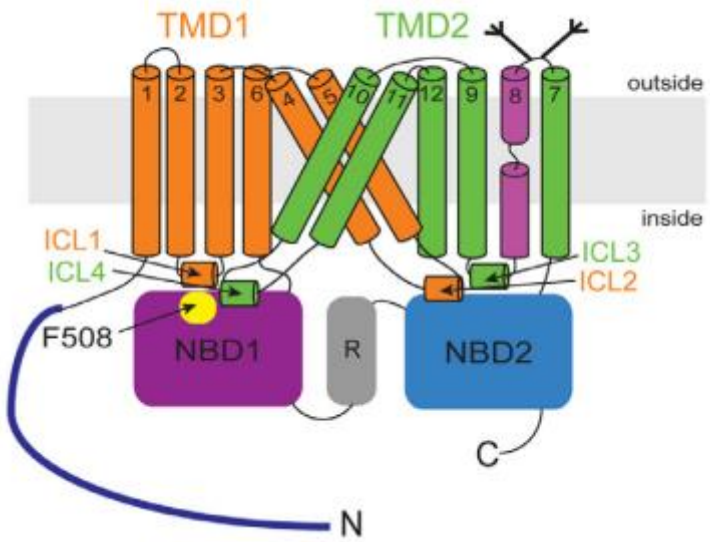
Effect on organ function of CFTR mutations causing severe disease.



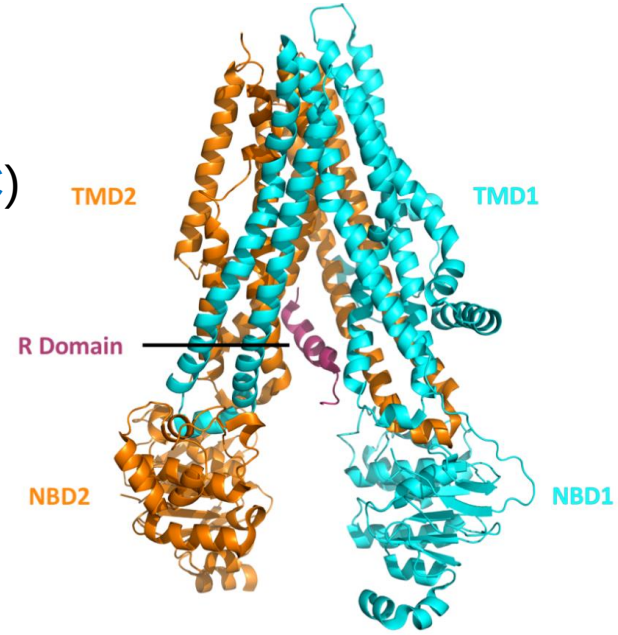
Adapted from: Ikpa *et al., Int. J. Biochem. Cell Biol*. **2014**, *52*, 192-200

CFTR structure

CFTR is an ion channel that belongs to the ATP-binding cassette (ABC) transporter family of proteins



Schematic representation of CFTR with its characteristic ABC transporter architecture



Overall structure of human CFTR in the dephosphorylated, ATP-Free conformation

CFTR is a membrane glycoprotein with 1480 amino acids, and consists of:

- two transmembrane domains (**TMD1** and **TMD2**, each consisting of 6 transmembrane α-helices) that form an ion permeation pathway

- two cytosolic nucleotide-binding domains (**NBD1** and **NBD2**) that bind and hydrolyse ATP

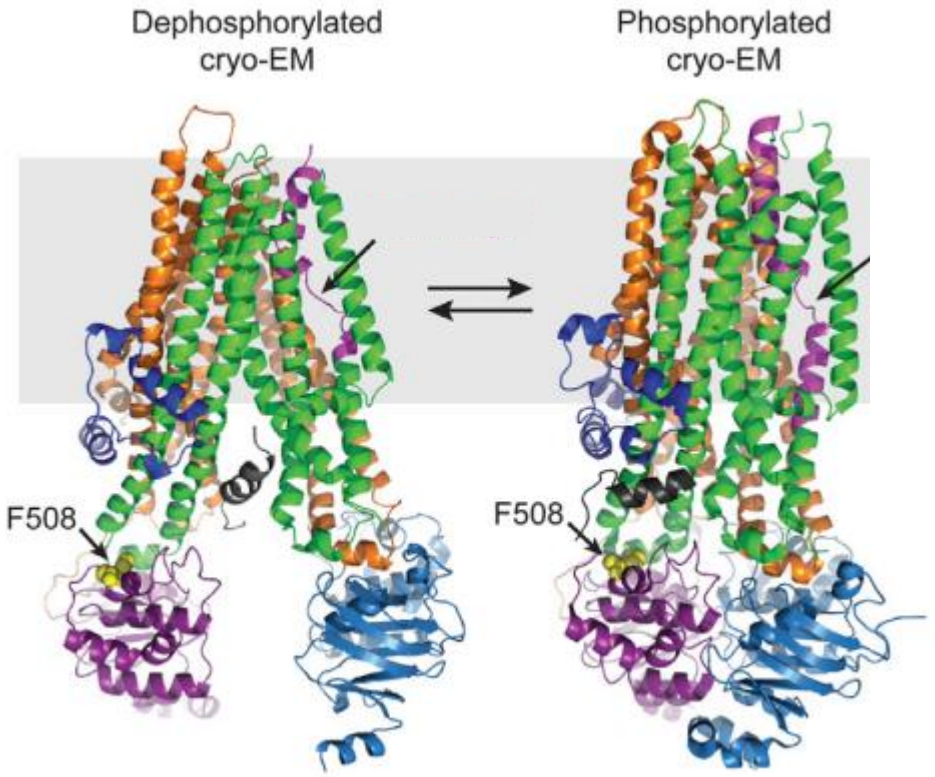
- a unique cytosolic regulatory (**R**) domain that includes several phosphorylation sites by protein kinase A (PKA)

Kleizen *et al., J. Cyst. Fibrosis* **2020**, *19*, S19-S24

CFTR function

In the absence of phosphorylation and ATP, CFTR forms a ***pore-closed*** conformation in which the *NBDs are separated*, and the R domain sterically precludes NBD dimerization

In the phosphorylated and ATP-bound CFTR conformation, the *NBDs form a closed dimer* with two ATP molecules bound at their interface



Orthogonal views of the cryo-EM structures of human CFTR (resolution (2.7Å)

The *ion channel opens* when R-domain is phosphorylated by PKA and ATP is bound at the NBDs.
Phosphorylation displaces the disordered R domain allowing NBD dimerization and ***pore-opening***

Subsequent ATP hydrolysis destabilizes the NBD dimer and favors return to the anion closed conformation

Kleizen et al., *J. Cyst. Fibrosis* **2020**, *19*, S19-S24

Fiedorczuk et al., *Cell* **2022**, *185*, 158-168

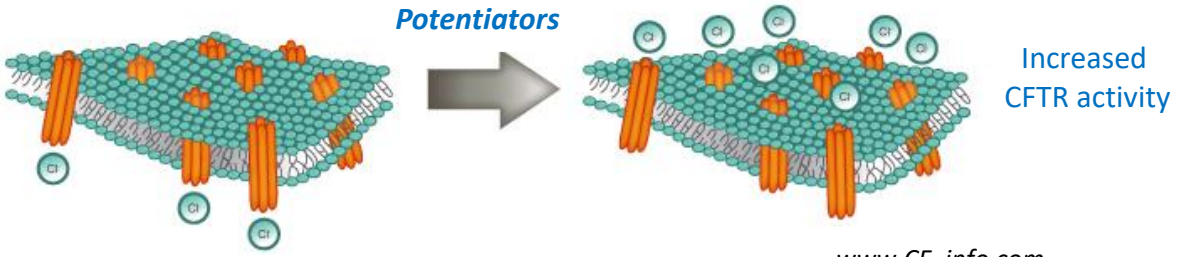
Levring et al., *Nature* **2023**, *616*, 606-614

CFTR modulators

CFTR modulators are small molecules that target specific defects caused by mutations in the *CFTR* gene (e.g., *read-through agents, potentiators, correctors, stabilizers and amplifiers*)

Potentiators

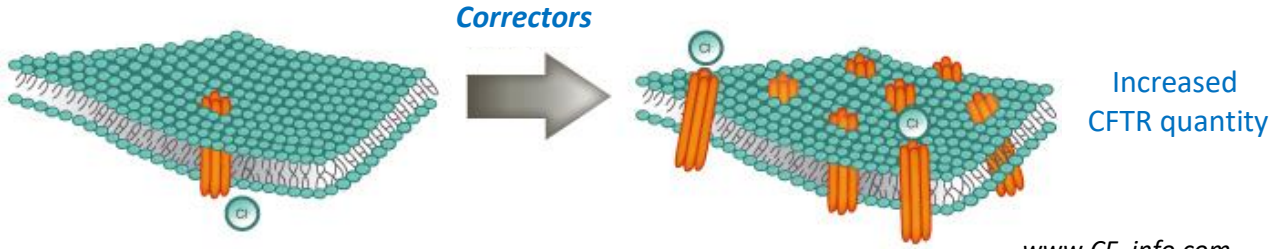
increase the flow of chloride through CFTR channels at the cell surface (modulate CFTR function)



www.CF_info.com

Correctors

increase the processing and trafficking of CFTR proteins to the cell surface (modulate the quantity of CFTR)

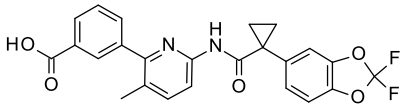


www.CF_info.com

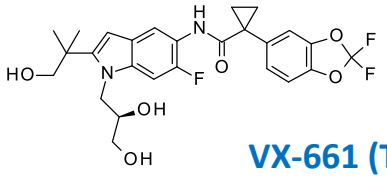
Correctors classification

(based on postulated mechanism)[1]

Type I correctors (**VX-809**, **VX-661**) primarily stabilize the NBD1-ICL4 and NBD1-ICL1 interface



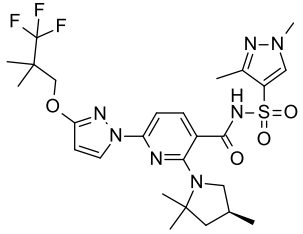
VX-809 (Lumacaftor)



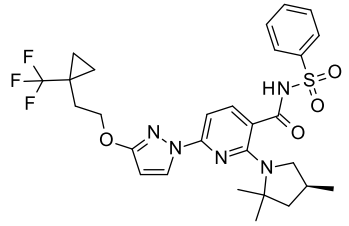
VX-661 (Tezacaftor)

Type II correctors target NBD2 and/or its interface

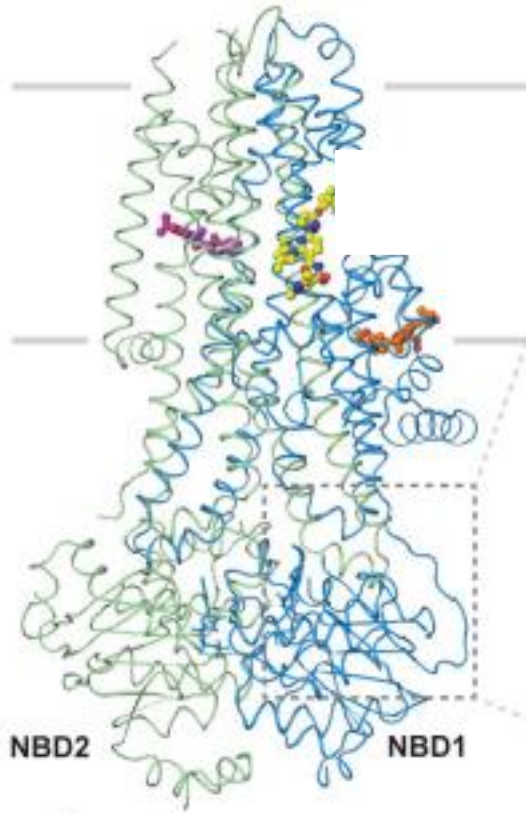
Type III correctors (**VX-445**, **VX-659**) stabilizes ΔF508-NBD1



VX-445 (Elexacaftor)



VX-659



Cryo-EM structure of *Trikafta*-corrected ΔF508-CFTR. Orthogonal views of Δ508/E1371Q CFTR in complex with ivacaftor, elexacaftor, and tezacaftor.[2]

1. Okiyoneda *et al., Nat. Chem. Biol*. **2013**, *9*, 444-454
2. Fiedorczuk *et al., Science* **2022**, 378, 284-290

Search for new modulators of mutant CFTR

The *Task Force for Cystic Fibrosis (TFCF)* Project

A collaborative drug discovery project aimed at the identification of new drugs for the treatment of CF

Istituto Giannina Gaslini (IGG)

Luis J. V. Galietta **(now at TIGEM)**

Nicoletta Pedemonte

Istituto Italiano di Tecnologia (IIT)

Tiziano Bandiera

Project funded by FFC

Fondazione Ricerca Fibrosi Cistica - Onlus

italian cystic fibrosis research foundation

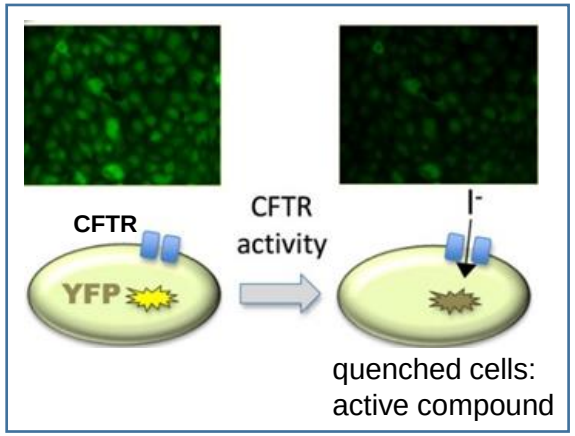
http://www.fibrosicisticaricerca.it/

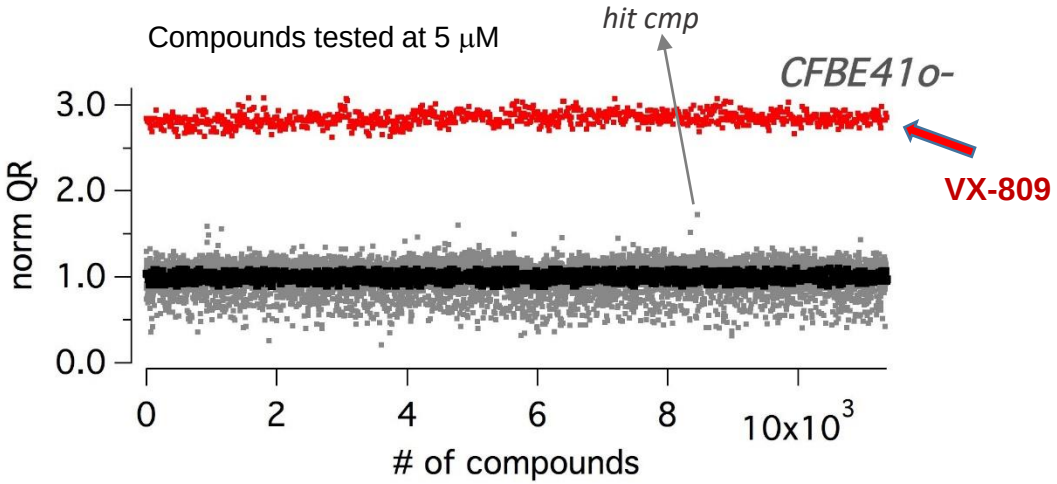
Phenotypic HTS of IIT compound collection

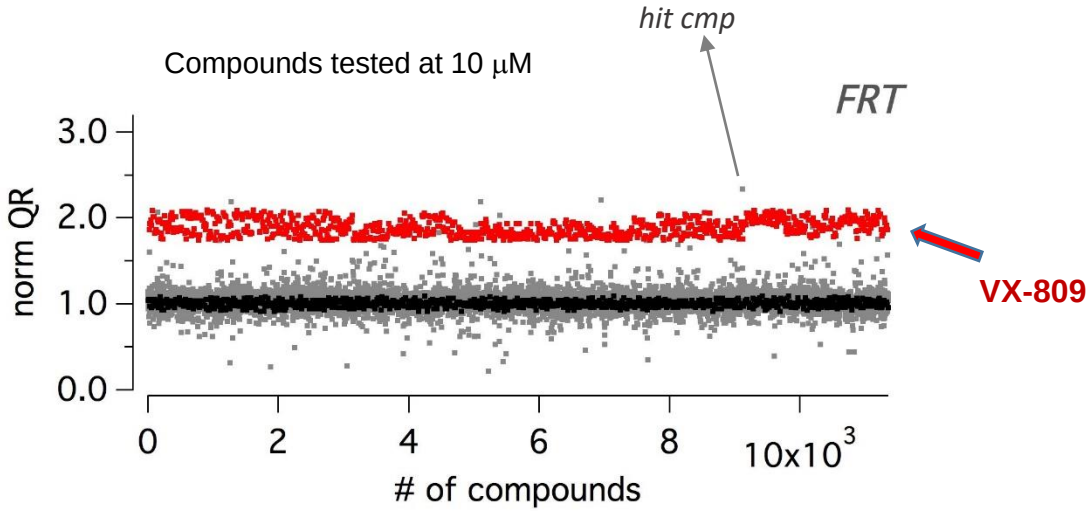
A collection of 11,334 maximally diverse compounds was screened on two cell lines:

- ***CFBE41o-*** and
- ***FRT***

Both cell lines stably express F508del-CFTR and the halide-sensitive yellow fluorescent protein (HS-YFP)[1].



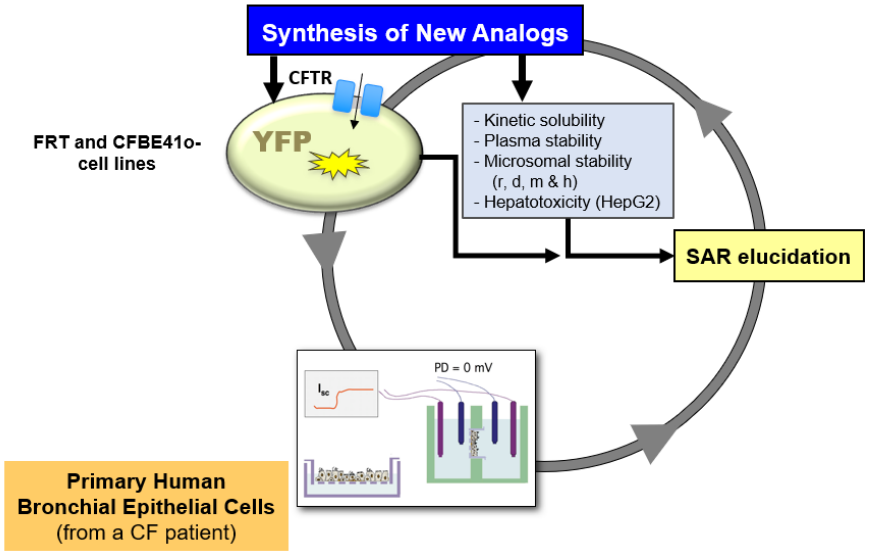


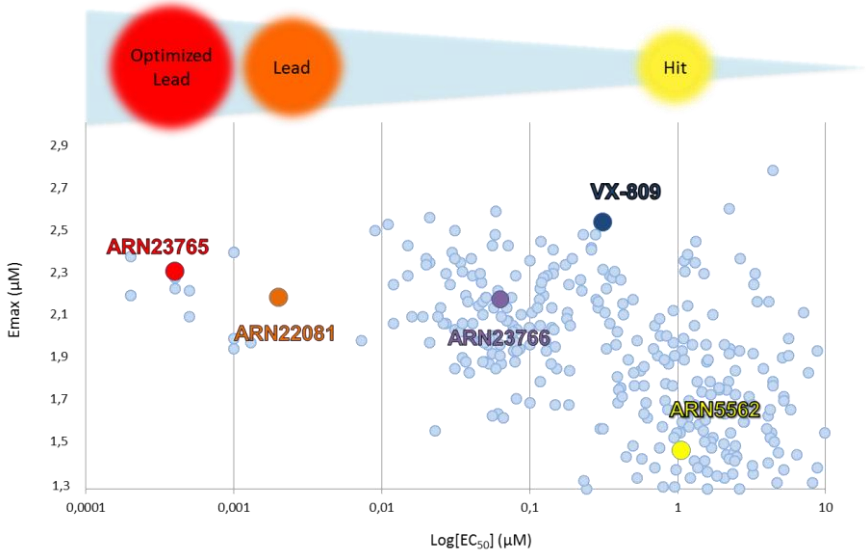


CFBE41o-: Cystic Fibrosis Bronchial Epithelial cells; **FRT**: Fisher Rat Thyroid cells

1. Galietta *et al., J. Biol. Chem*. **2001** *276*, 19723-19728

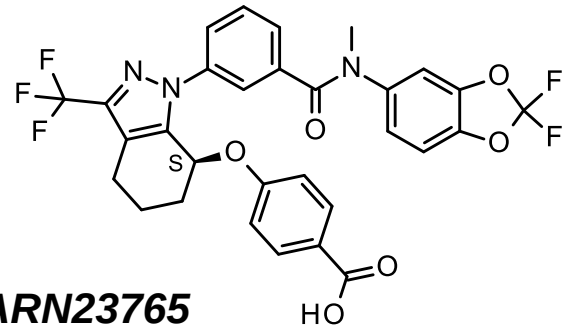
Discovery of ARN23765





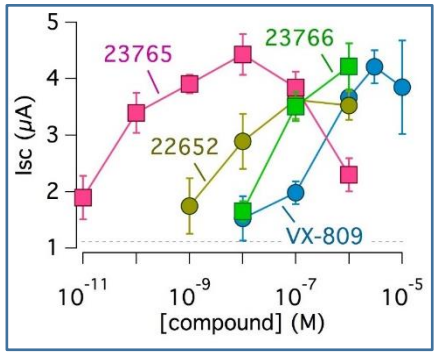
ca. 400 compounds synthesized and tested

E_{max} *vs.* EC_{50} on F508del-CFTR FRT cells (HS-YFP assay)



ARN23765

F508del/F508del HBE cells

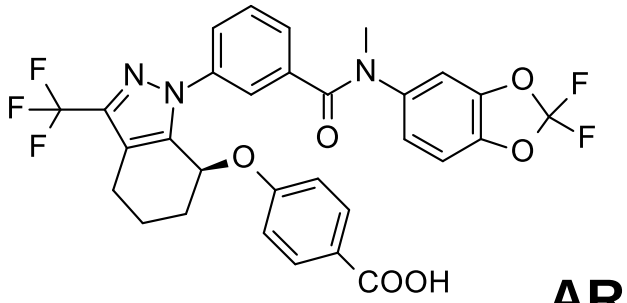


ARN23765 EC_{50} : **0.038** nM

VX-809 EC_{50} : ~**200** nM

Pedemonte, Bertozzi *et al., Sci. Adv.* **2020**, 6, eaay9669

ARN23765: Target/mechanism of action???

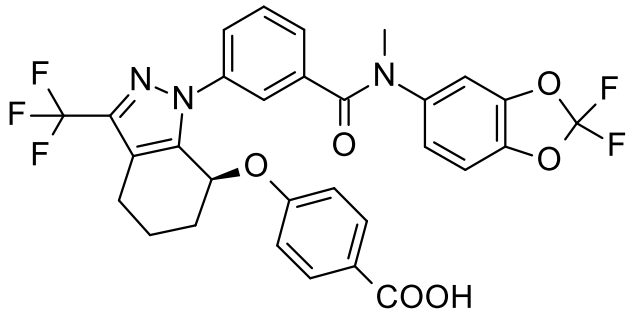


ARN23765 was identified after a ***phenotypic screening**** campaign in two cell lines (FRT and CFBE41o-) overexpressing F508del-CFTR

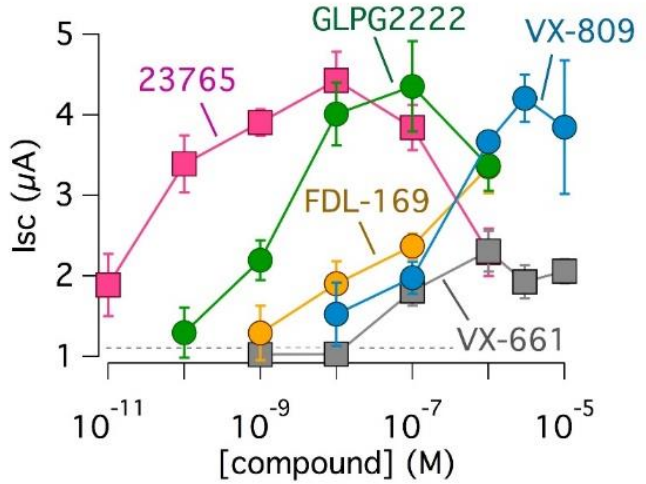
Phenotypic screening is a type of screening used in drug discovery *to identify substances/hits* (e.g., small molecules, peptides) with desirable efficacy that *alter the phenotype of a cell* in a specific manner, but often with ***unknown modes of action***.

Follow-up ***target identification (and validation)*** often through the use of chemoproteomics are essential to successful drug discovery research to identify the mechanisms through which a phenotypic hit works.

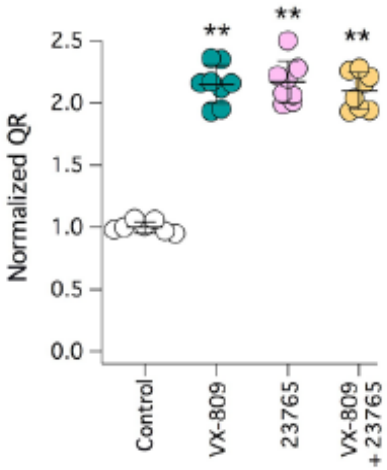
ARN23765: Target/mechanism of action ID



ARN23765 shows sub-nanomolar activity in rescuing F508del-CFTR in primary HBE cells from a F508del/F508del CF patient

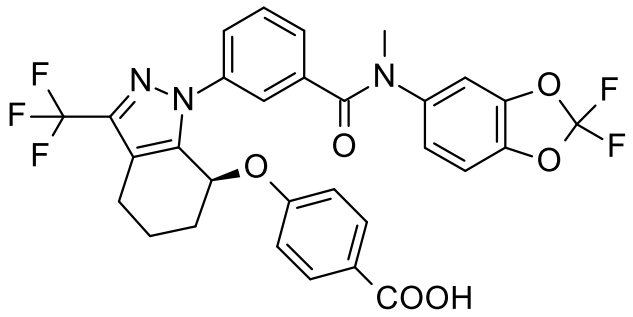


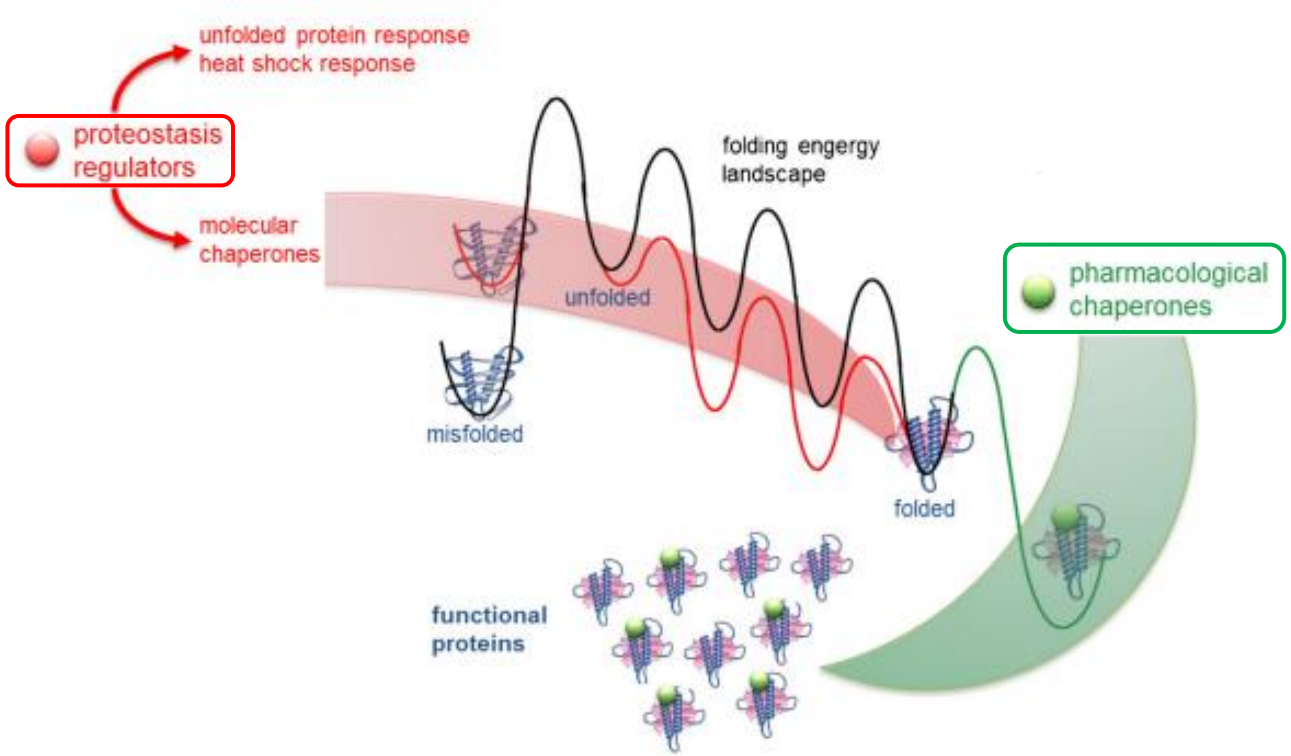
No additive effect elicited in combination with VX-809 (type I corrector) in CFBE41o- cells



[HBE: Human bronchial epithelial cells]

ARN23765: Target/mechanism of action ID





Mode of action of proteostasis regulators and pharmacological chaperones

Gersting et al., *J. Inherit. Metab. Dis.* **2014**, *37*, 505-523

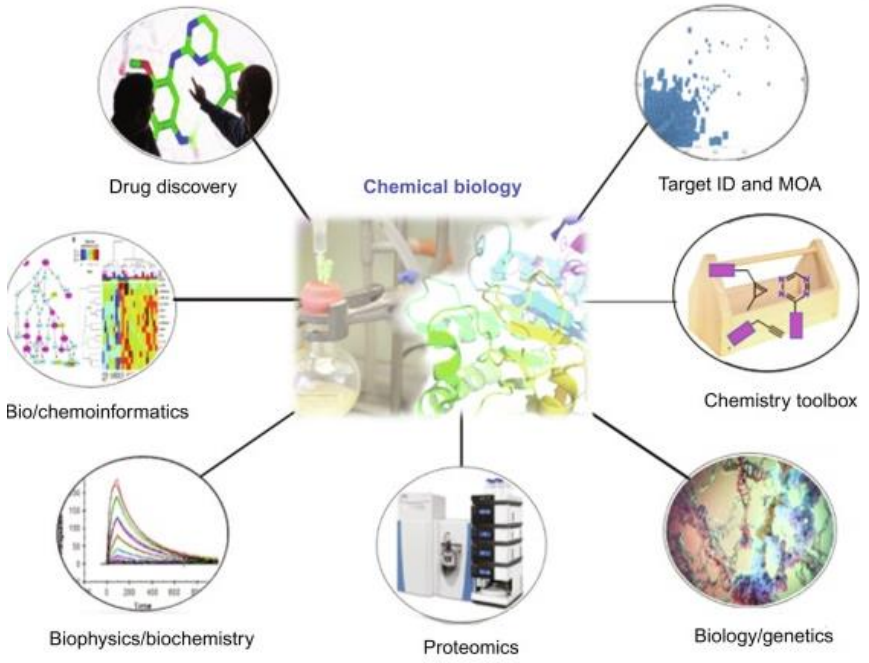
ARN23765 biological target(s) and mechanism/site of action were not known:

- ***CFTR molecular chaperone*** (promoting folding/trafficking of mutant CFTR via direct binding)?
- ***Proteostasis regulator*** (restoring mutant CFTR delivery to the plasma membrane)?

Chemical Biology

Chemical Biology is a scientific discipline between the fields of ***chemistry*** and ***biology***.

It involves the application of *chemical techniques*, *analysis*, and *(small)molecules* produced through *synthetic chemistry*, to the study and manipulation of biological systems

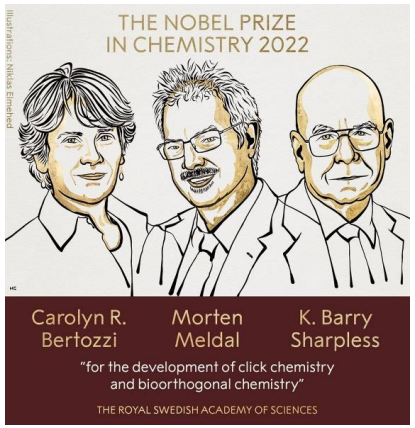


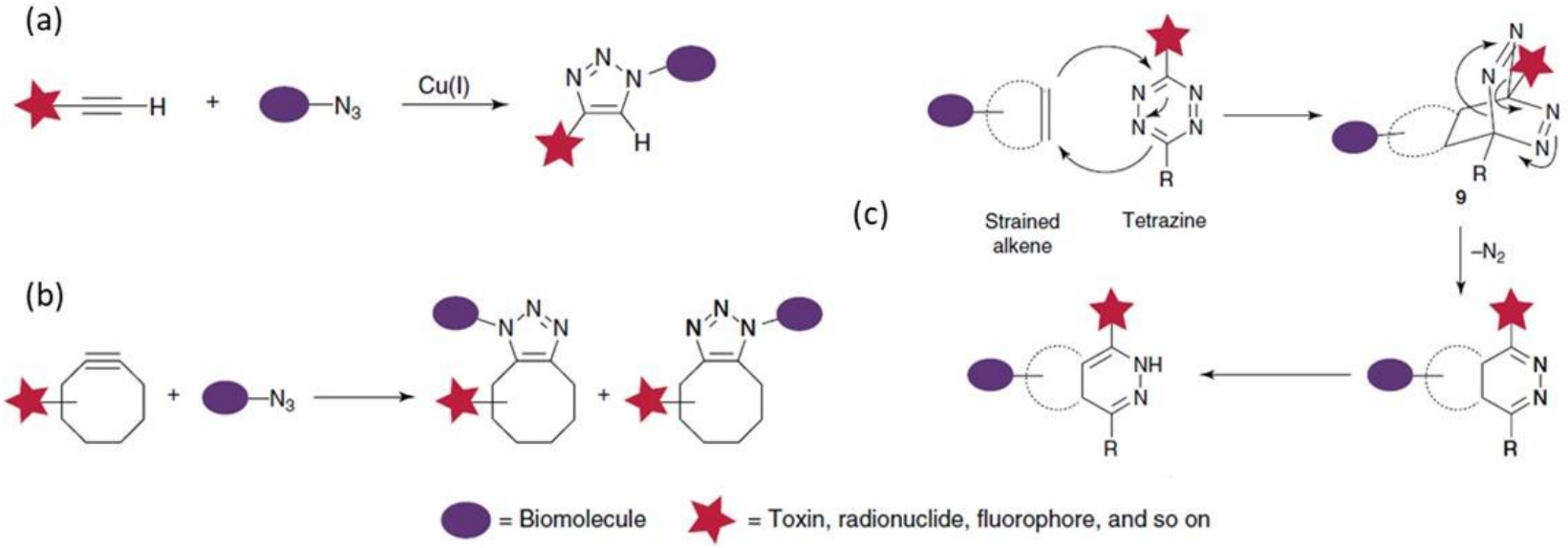
Biochemistry studies the chemistry of biomolecules and regulation of biochemical pathways within and between cells

Castaldi *et al., Annual Rep. Med. Chem.* **2017**, 50, 335-370

Chemical Biology & Bioorthogonal Chemistry

Bioorthogonal chemistry refers to any chemical reaction that can occur inside of living systems (e.g., cells, tissues, organs) without interfering with native biochemical processes



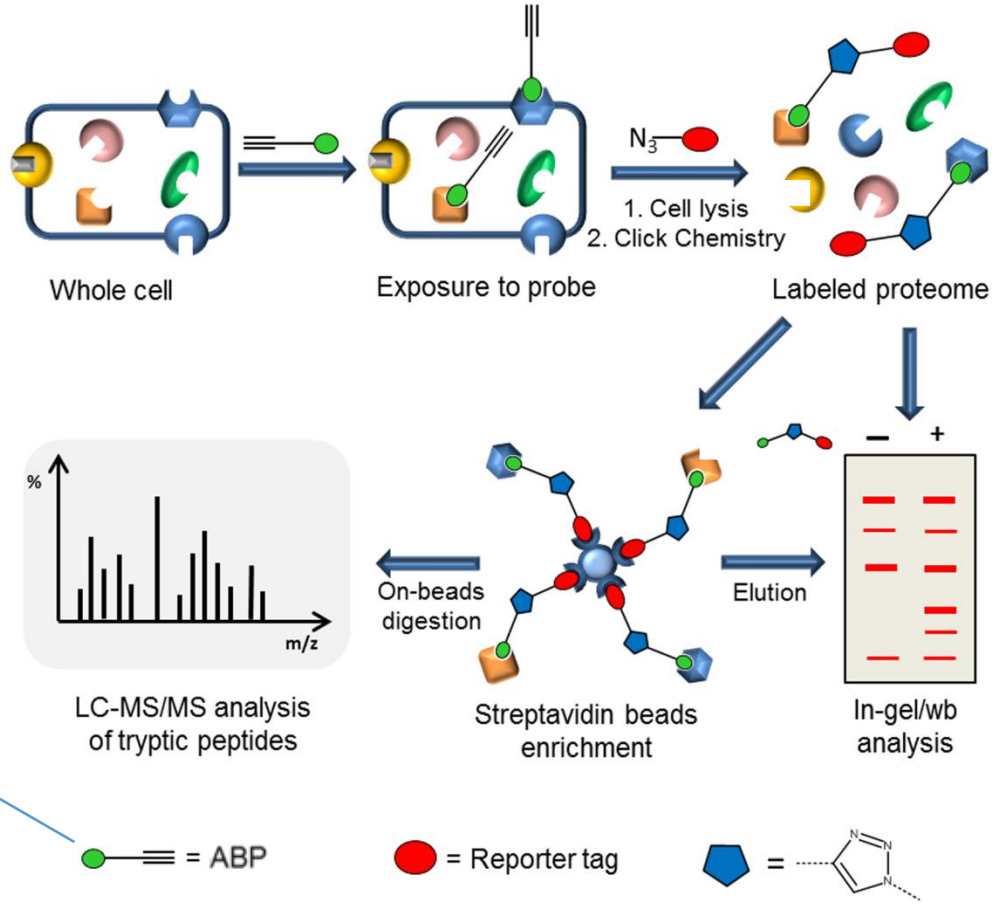


Bioorthogonal reactions can occur between complementary chemical groups not present in living biological systems (also *in vivo)* and have been exploited in for diagnostic and therapeutic applications in humans.

Sletten, Bertozzi. *Angew. Chem. Int. Ed.* **2009**, 48, 6974-6998

Activity-based Protein Profiling (*ABPP*) for target ID and validation

ABPP is a powerful chemical biology strategy for profiling of functional states of proteins in native biological systems.



ABP: *activity-based probes* are active-site-directed chemical probes to enable visualization of the active form of proteins.

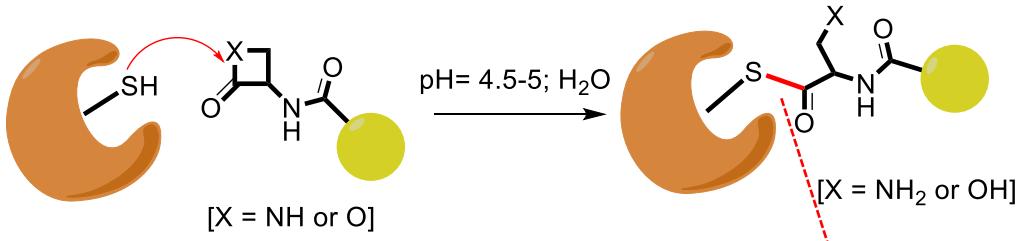
*[Adapted from: Romeo et al. ACS Chem. Biol. **2015**, 10, 2057-2064]*

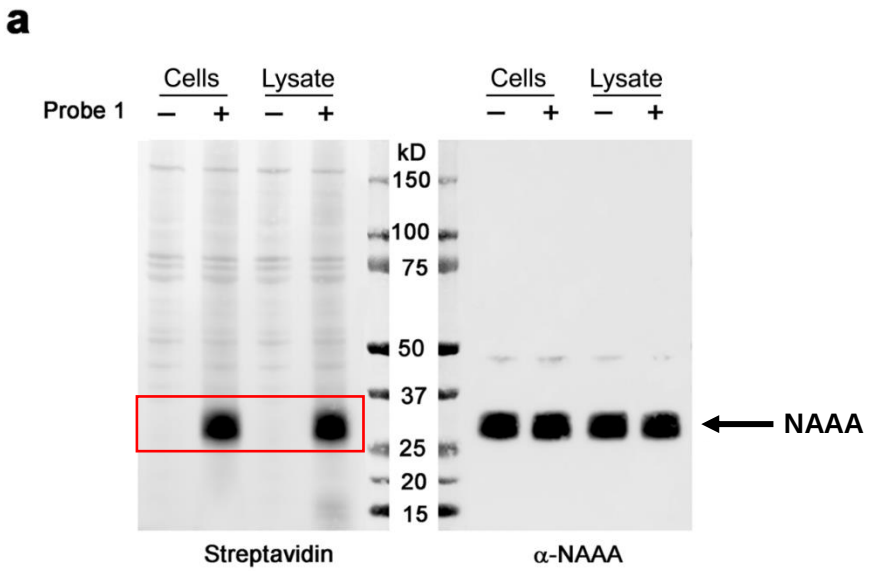
Wang *et al*., *Front Pharmacol*. **2018**, 9, 353

Cravatt *et al*., *Annu Rev Biochem*. **2008**, 77, 383-414

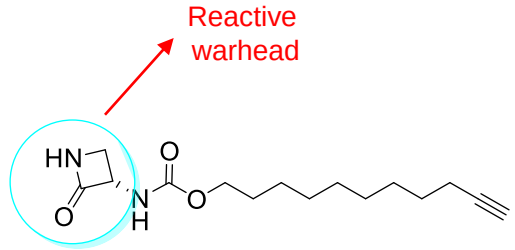
ABPP for target ID and validation: our experience

N-Acylethanolamine-hydrolyzing Acid Amidase (NAAA) is lysosomal *cysteine hydrolase* responsible for the deactivation of fatty acid ethanolamides (FAEs), primarily palmitoylethanolamide (PEA)





a) WB analysis of h-NAAA-overexpressing HEK293 cells or lysate incubated with **Probe 1** (+) or DMSO (−). WB membranes were probed with a streptavidin−HRP conjugate or an anti-NAAA antibody (α-NAAA).



Probe 1
h-NAAA IC_{50} = 0.006 μM
r-NAAA IC_{50} = 0.013 μM

Romeo *et al., ACS Chem. Biol.* **2015**, 10, 2057-2064

ABPP for target ID and validation: our experience

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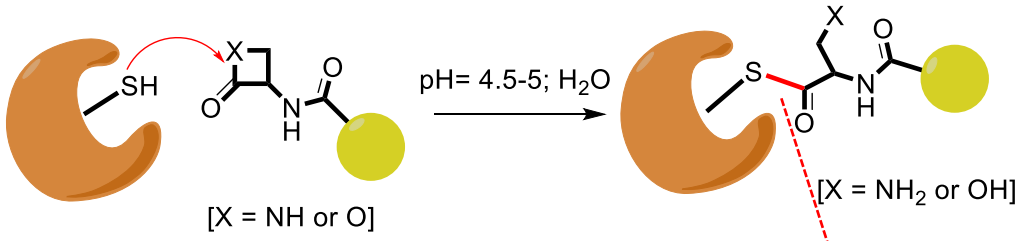

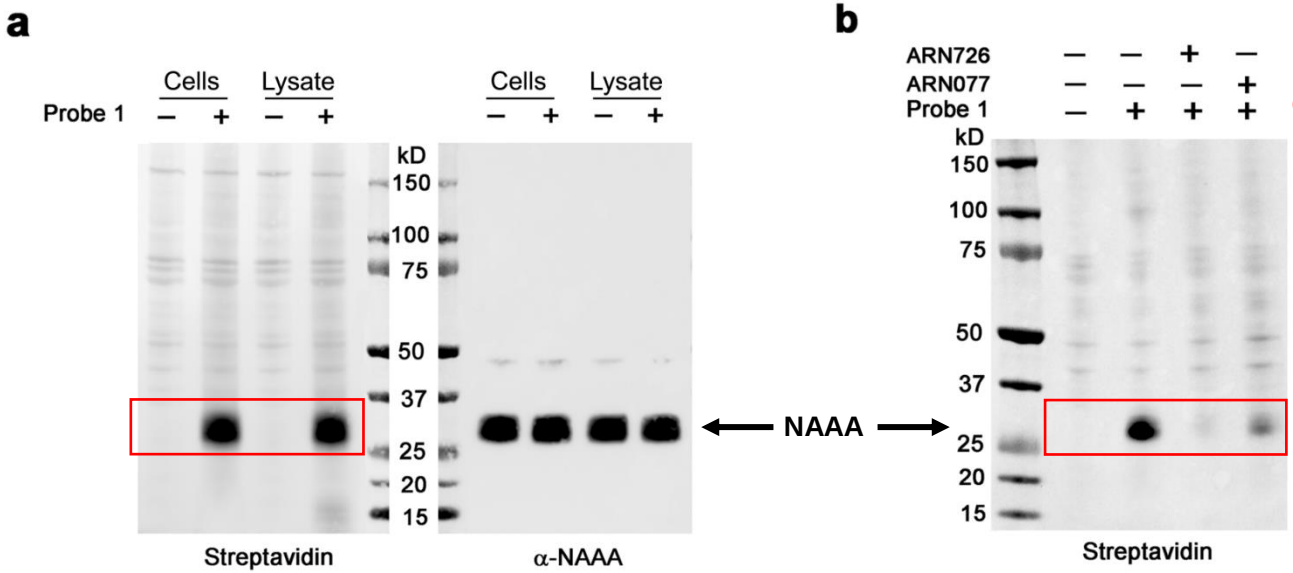

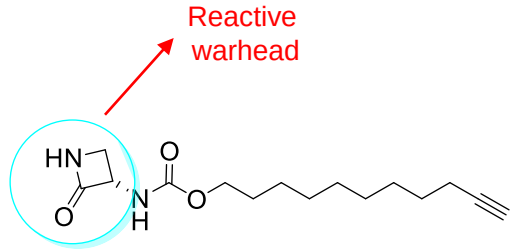

Probe 1
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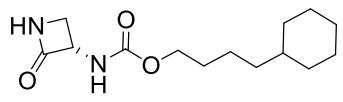
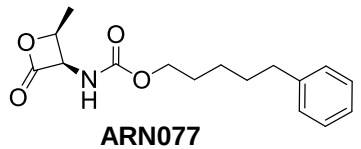
r-NAAA IC_{50} = 0.013 μM



ARN726
h-NAAA IC_{50} = 0.027 μM



ARN077
h-NAAA IC_{50} = 0.007 μM

a) WB analysis of h-NAAA-overexpressing HEK293 cells or lysate incubated with **Probe 1** (+) or DMSO (−). WB membranes were probed with a streptavidin−HRP conjugate or an anti-NAAA antibody (α-NAAA)
b) h-NAAA-HEK intact cells preincubated (10x) with **ARN726** or **ARN077** before addition of **Probe 1**.

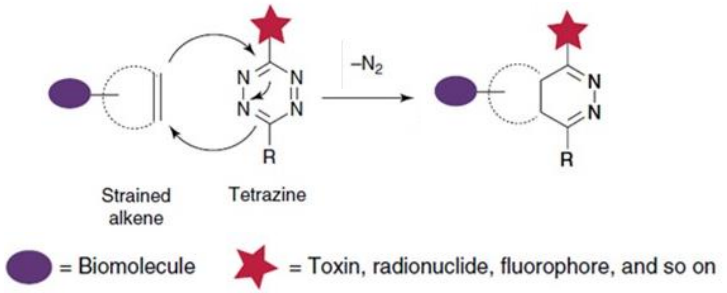
Romeo *et al., ACS Chem. Biol.* **2015**, 10, 2057-2064

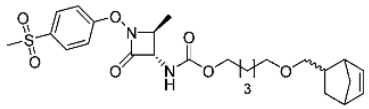
ABPP for target ID and validation: our experience

N-Acylethanolamine-hydrolyzing Acid Amidase (NAAA) is lysosomal *cysteine hydrolase* responsible for the deactivation of fatty acid ethanolamides (FAEs), primarily palmitoylethanolamide (PEA)

In-cell bioimaging

(NAAA-overexpressing HEK-293 cells)





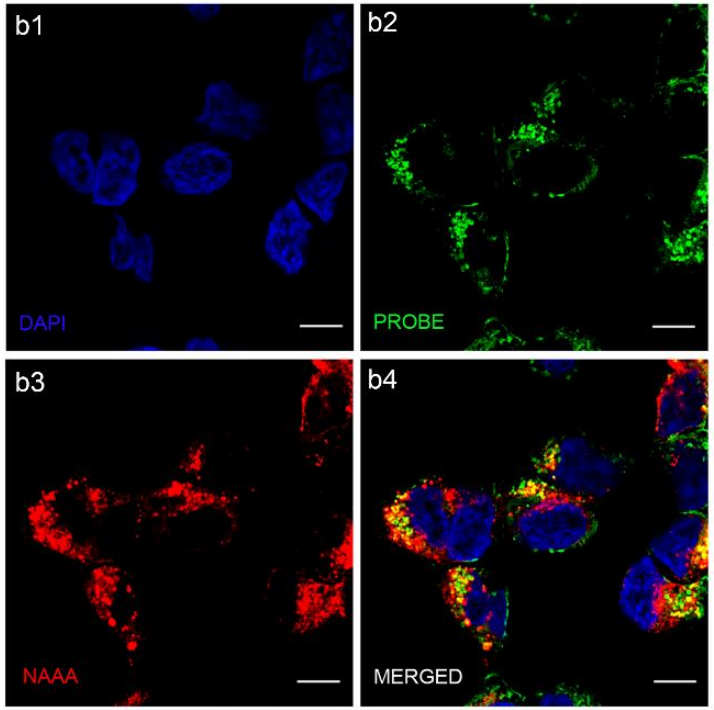
NAAA probe

ABPP for target ID and validation: our experience

N-Acylethanolamine-hydrolyzing Acid Amidase (NAAA) is lysosomal *cysteine hydrolase* responsible for the deactivation of fatty acid ethanolamides (FAEs), primarily palmitoylethanolamide (PEA)

In-cell bioimaging

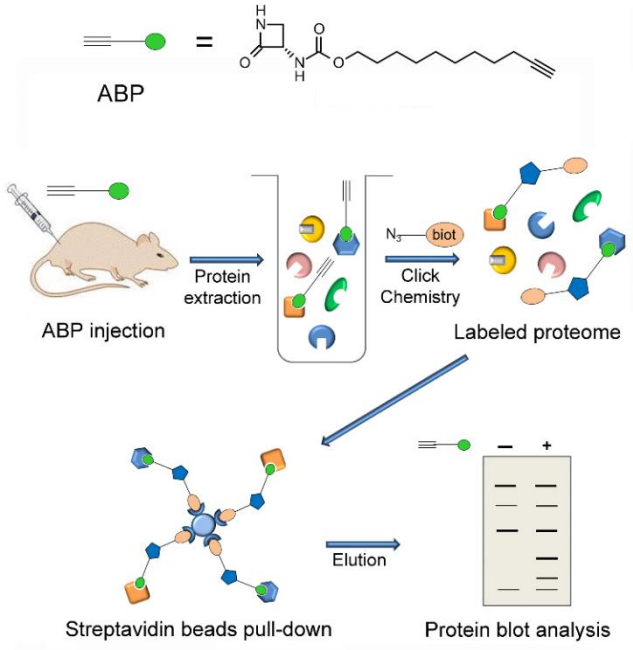
(NAAA-overexpressing HEK-293 cells)

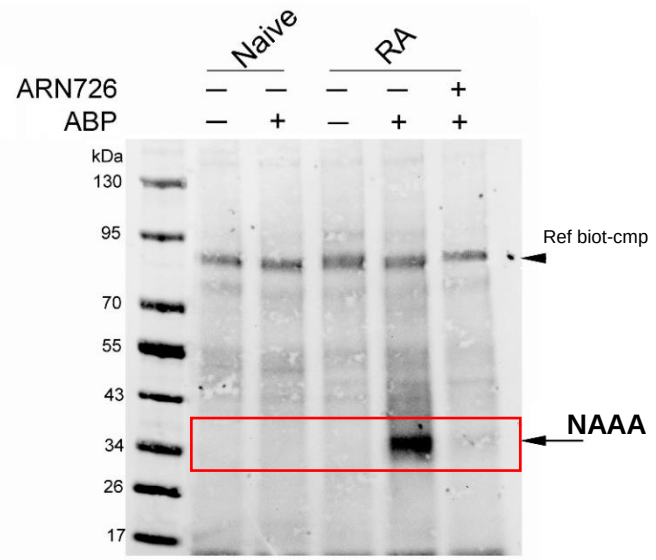


Petracca et al., *Chem Comm* **2017**, 53, 11810-11813

In-vivo

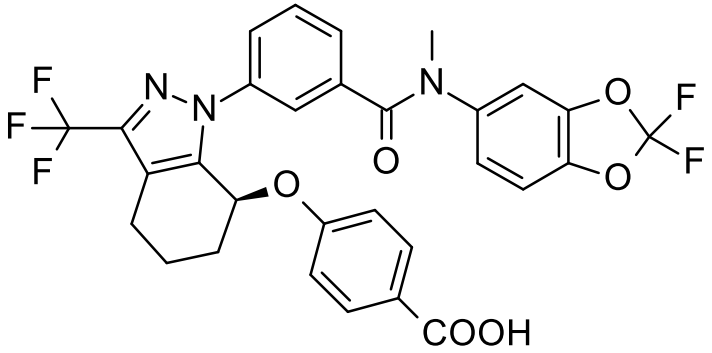
(Rheumatoid Arthritis mouse model)



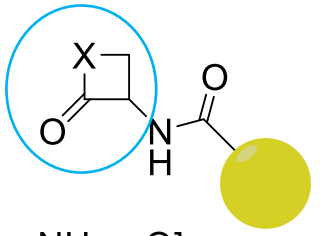


Bonezzi et al., *J. Pharmacol. Exp. Ther.* **2016,** 356, 656-663

ARN23765: Target/mechanism of action ID

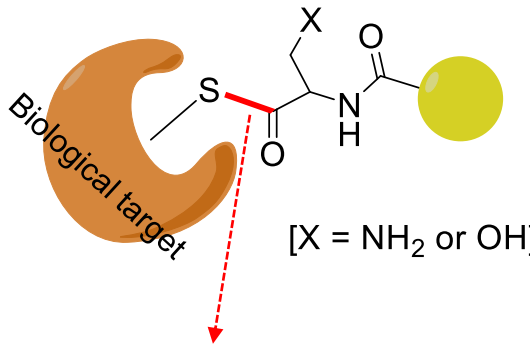


Reactive, Electrophilic warhead



[X = NH or O]

bioconjugation

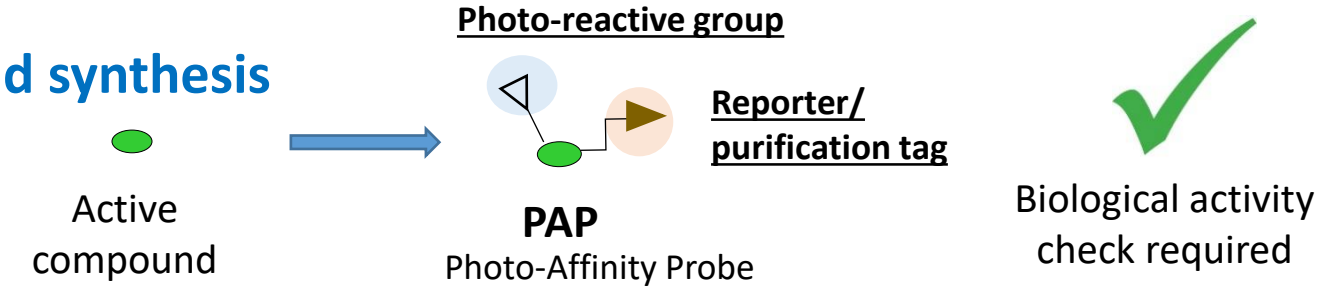


[X = NH_2 or OH]

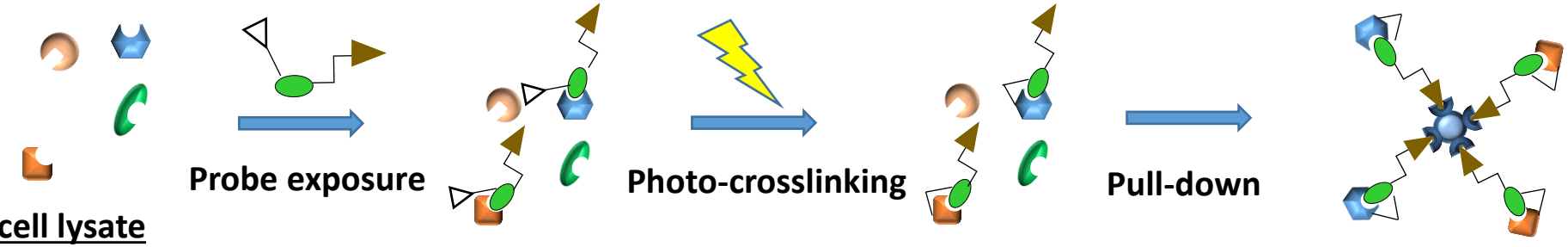
New covalent bond

Photo-affinity labelling (*PAL*) for target ID

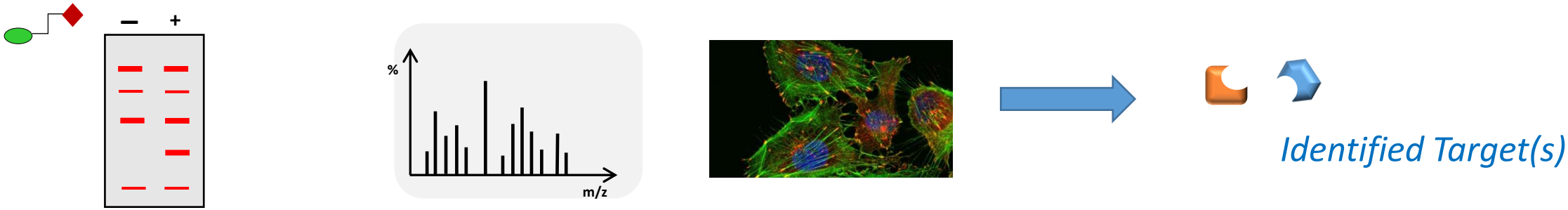
1) PAP design and synthesis



2) Target capturing



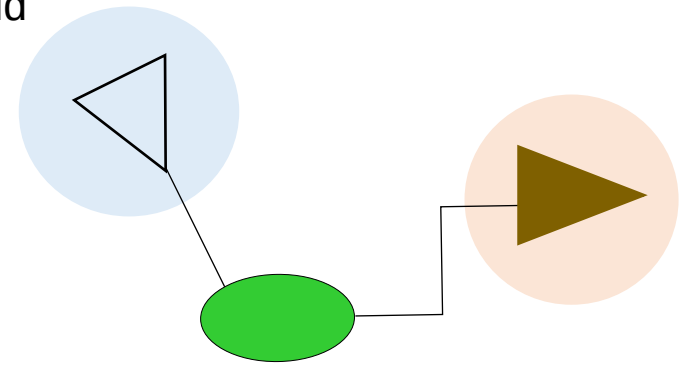
3) Target analysis and ID



Ge *et al.*, *RCS Adv.* **2018**, 8, 29428
Hill *et al.*, *J. Med. Chem.* **2018**, 61, 6945-6963
Dubinsky *et al.*, *Bioorg. Med. Chem.* **2012**, 20, 554-570

Photo-affinity probes (PAPs)

Photo-reactive group
(cross-links the parent scaffold to biomolecule)

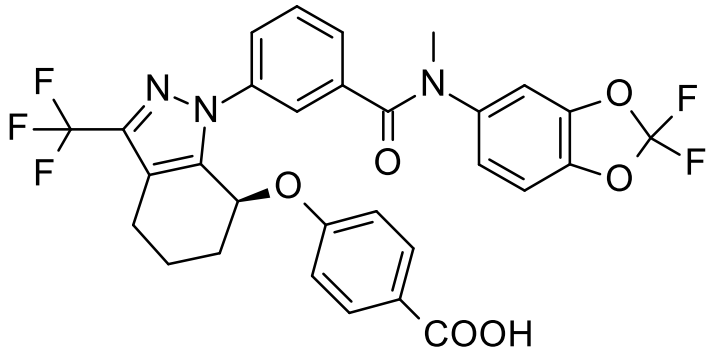


Reporter/purification tag (chemical handle)
(enhances adducts purification, isolation and detection)

Bioactive motif
(provides selectivity and affinity for target)

- high degree of ***similarity*** to the parent compound
- ***comparable activity*** and affinity levels (SAR understanding is critical)
- little ***steric interference***
- ***stability*** in the dark at a range of pHs
- ***activation*** at wavelengths that minimize damage to biological molecules

ARN23765: Target/mechanism of action ID



Identification of **ARN23765** target protein(s) and mechanism/site of action
in live cells

(wt- and F508del-CFTR CFBE41o-)

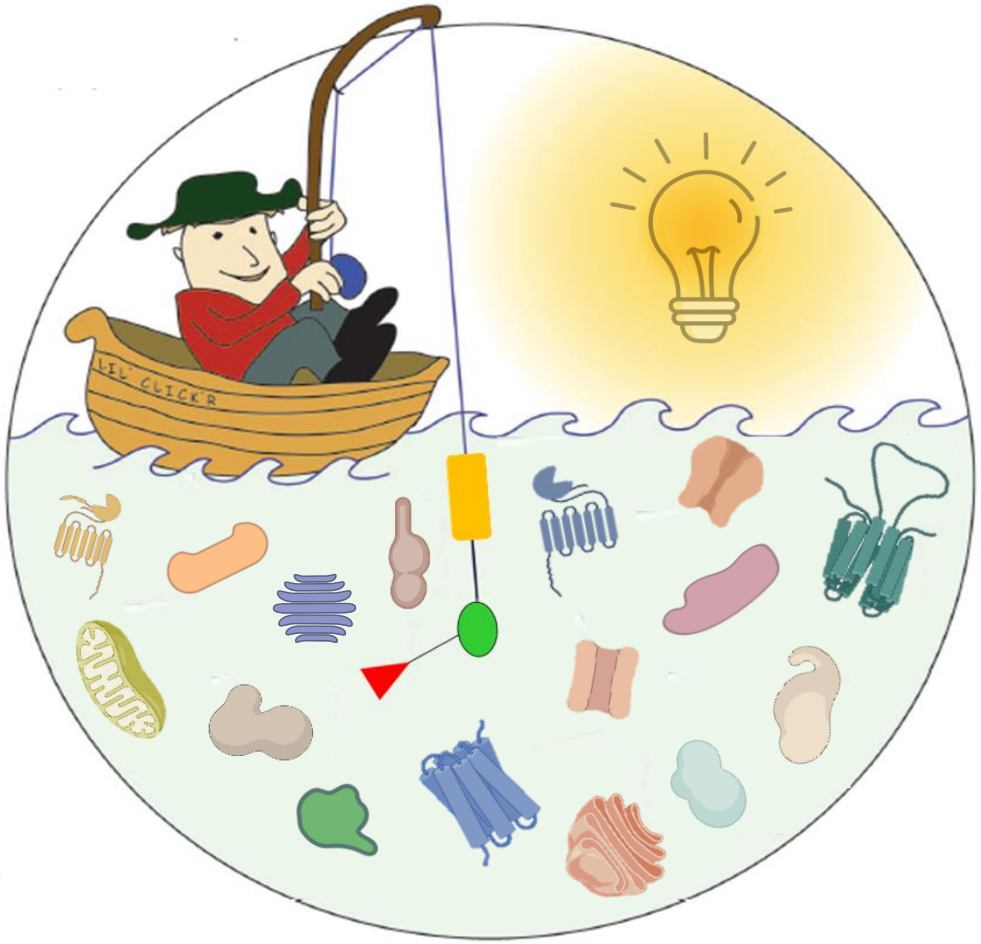
Discovery of other proteins related and/or unrelated (*i.e.*, off-targets)
to the CFTR interactome will represent an important finding

Fondazione per la Ricerca sulla Fibrosi Cistica - ETS
fibrosicisticaricerca.it

FFC #4/2020

FFC#2/2022

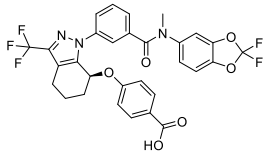
ARN23765: Target/mechanism of action ID



[Adapted from: Sletten, Bertozzi. *Angew. Chem. Int. Ed. Engl.* **2009**, *48*, 6974-6798]

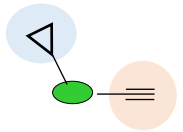
PAL studies with alkyne-PAPs

1) PAP design and synthesis



Active compound

Photo-reactive group



Click handle

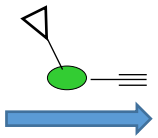
PAP

Photo-Affinity Probe

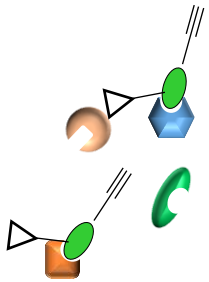
Biological activity check required

2) Target capturing

Cells / cell lysate



Probe exposure



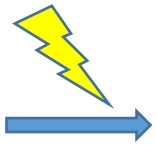
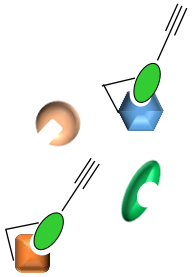
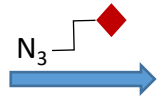


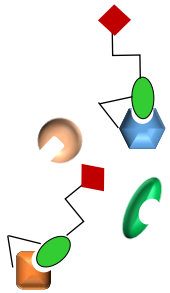
Photo-crosslinking



Biotin-Azide

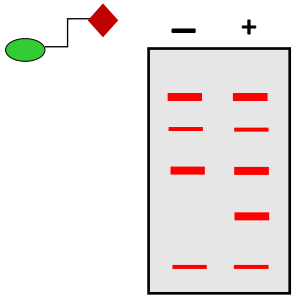


Click chemistry

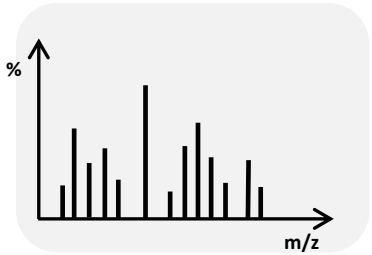


Labeled proteome

3) Target analysis and ID



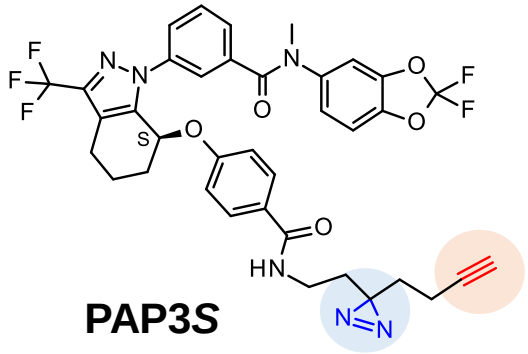
In-gel fluorescence scanning/ Protein blot analysis



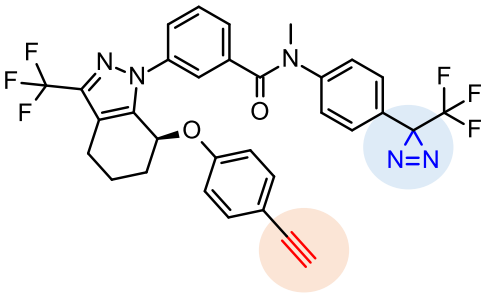
LC-MS analysis

Identified Target(s)

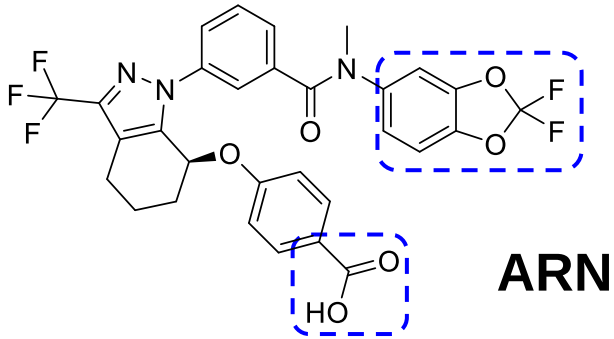
ARN23765-derived alkyne-PAPs



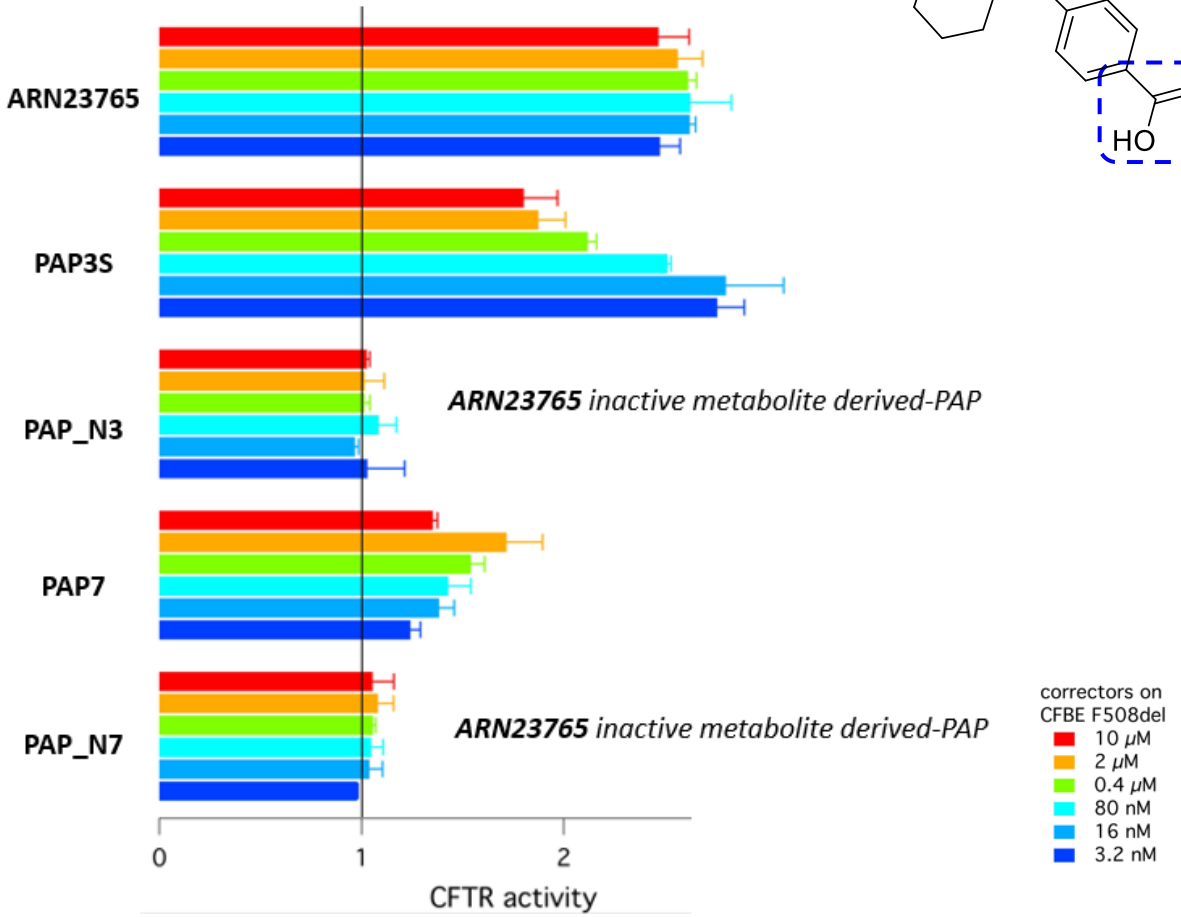
PAP3S



PAP7

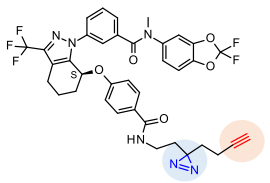


ARN23765

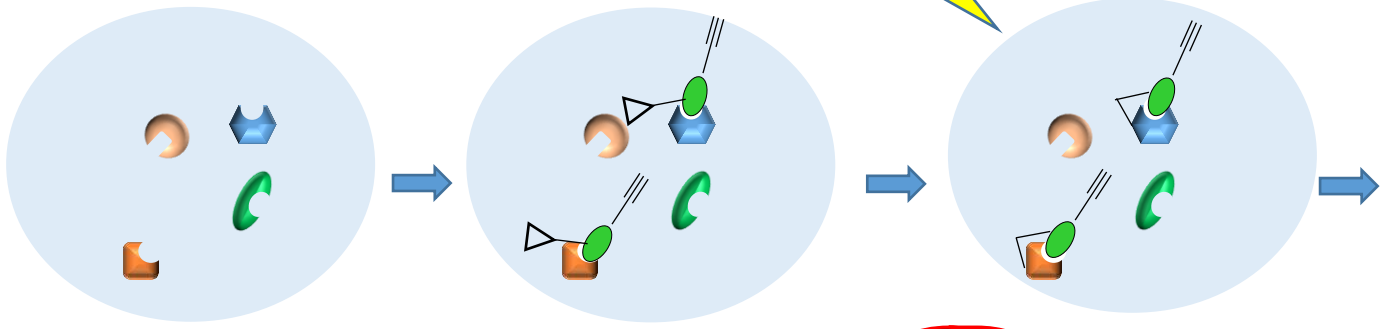


HS-YFP assay in F508del-CFTR CFBE41o- cells

In situ PAL with alkyne-PAPs



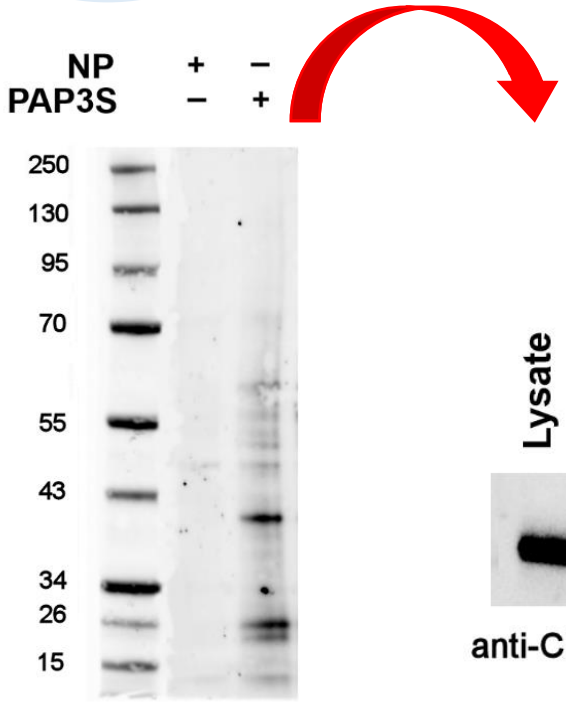
= PAP

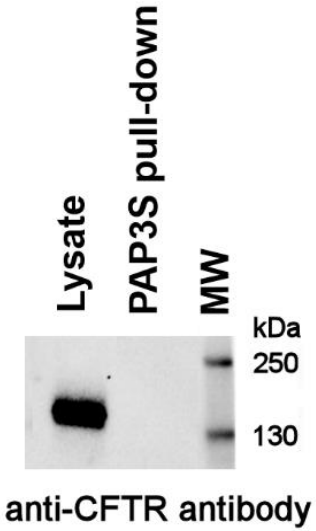


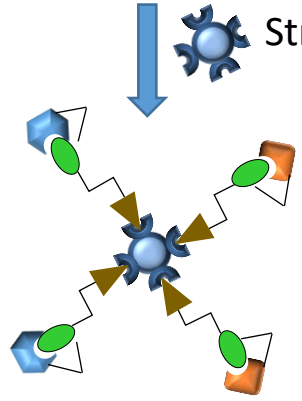
Live cells
wt-CFTR CFBE41o-

Biotin-streptavidin pull-down

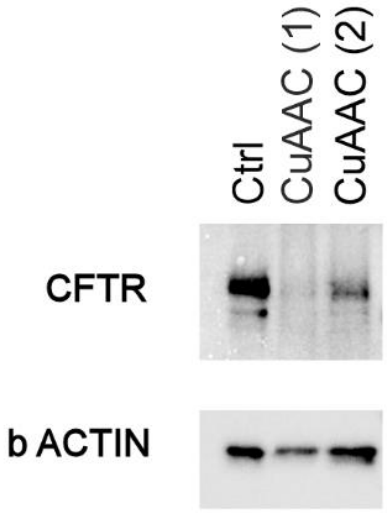
anti-CFTR mAb



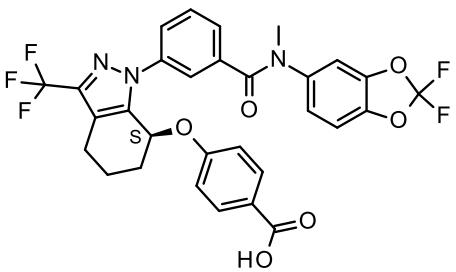




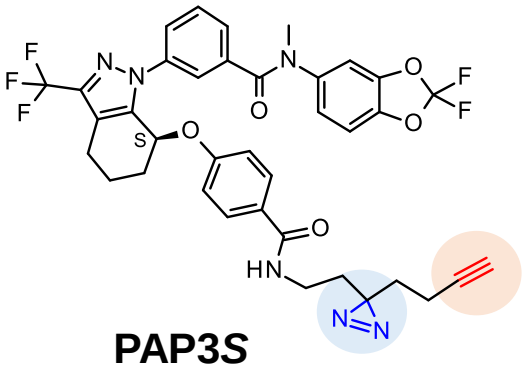
CFTR stability issues



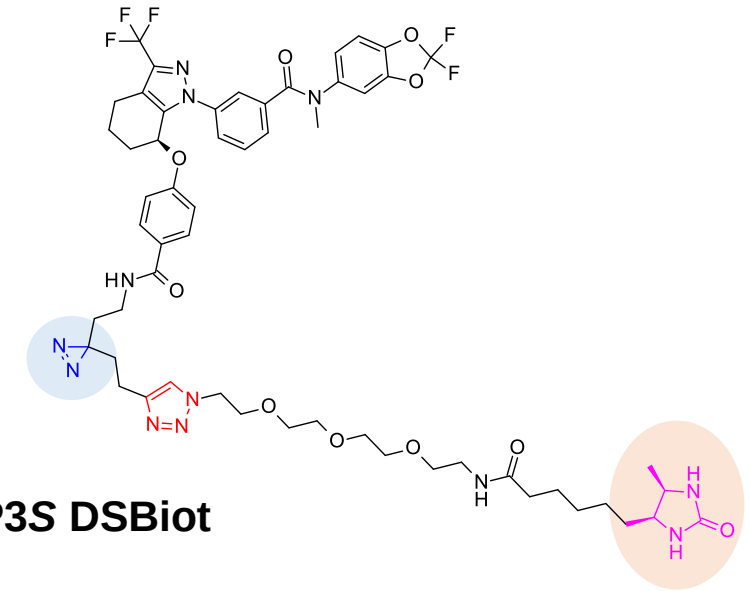
Biotinylated-*PAPs* structure



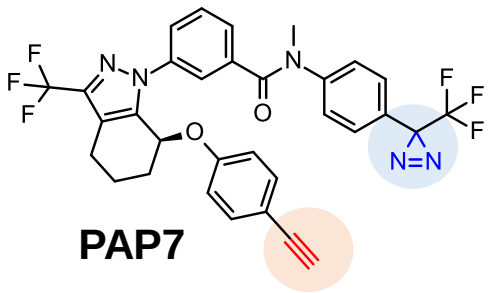
ARN23765



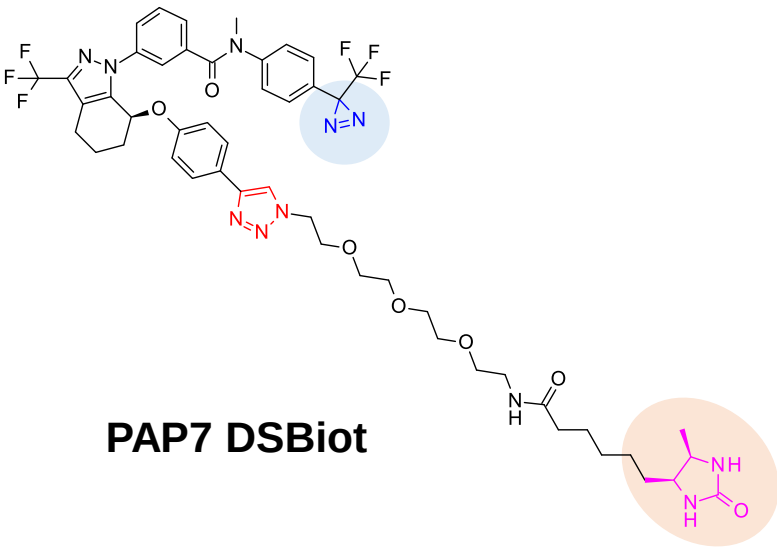
PAP3S



PAP3S DSBiot

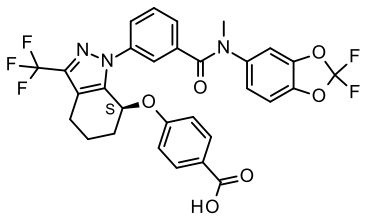


PAP7

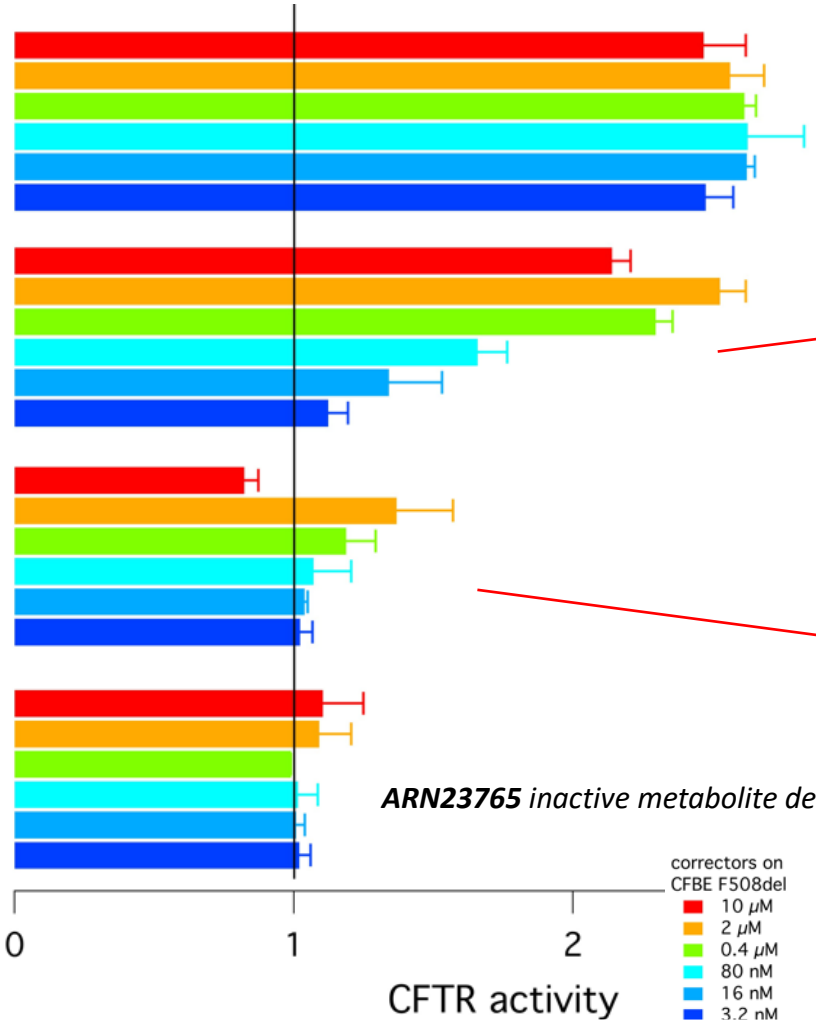


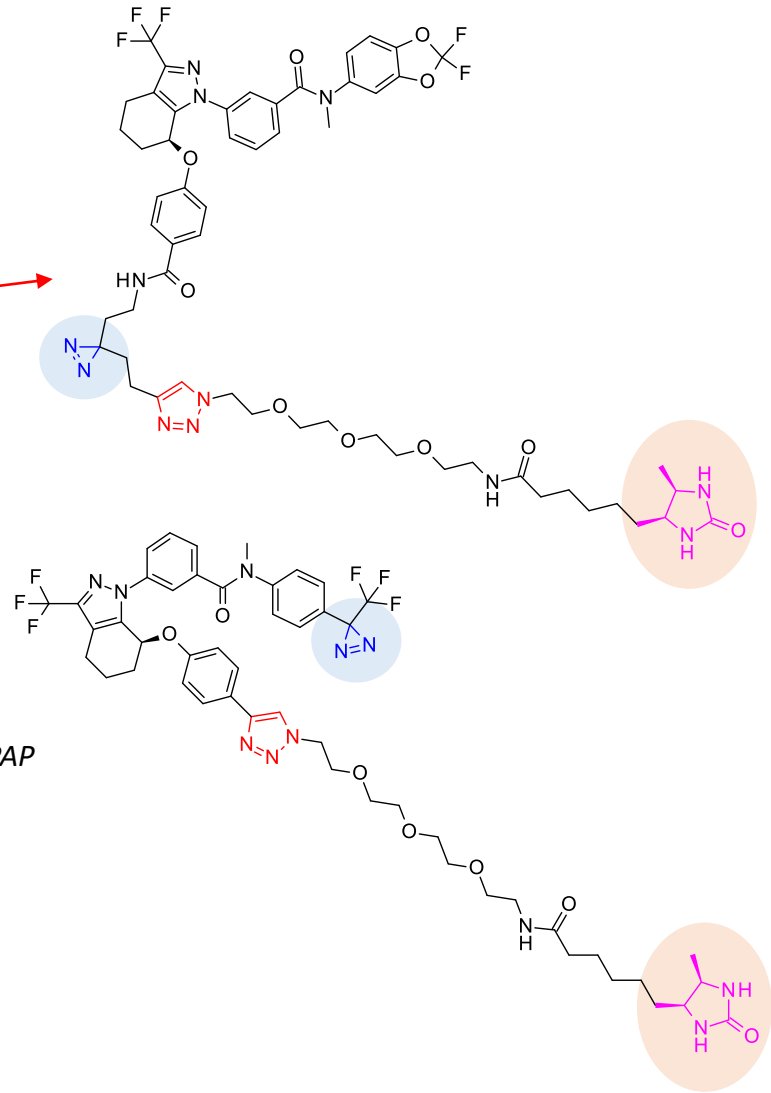
PAP7 DSBiot

Biotinylated-*PAPs* structure and activity



ARN23765

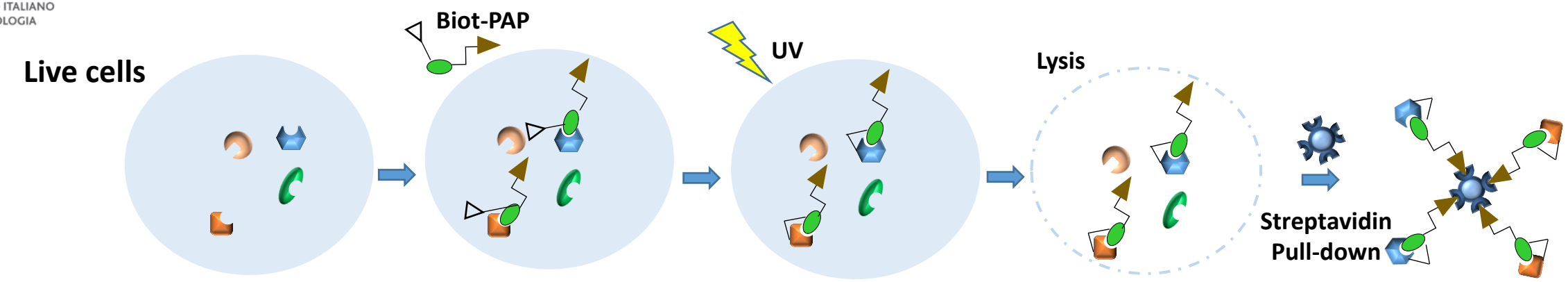




***ARN23765** inactive metabolite derived-PAP*

HS-YFP assay in F508del-CFTR CFBE41o- cells

In cell *PAL* studies with biotinylated-*PAPs*

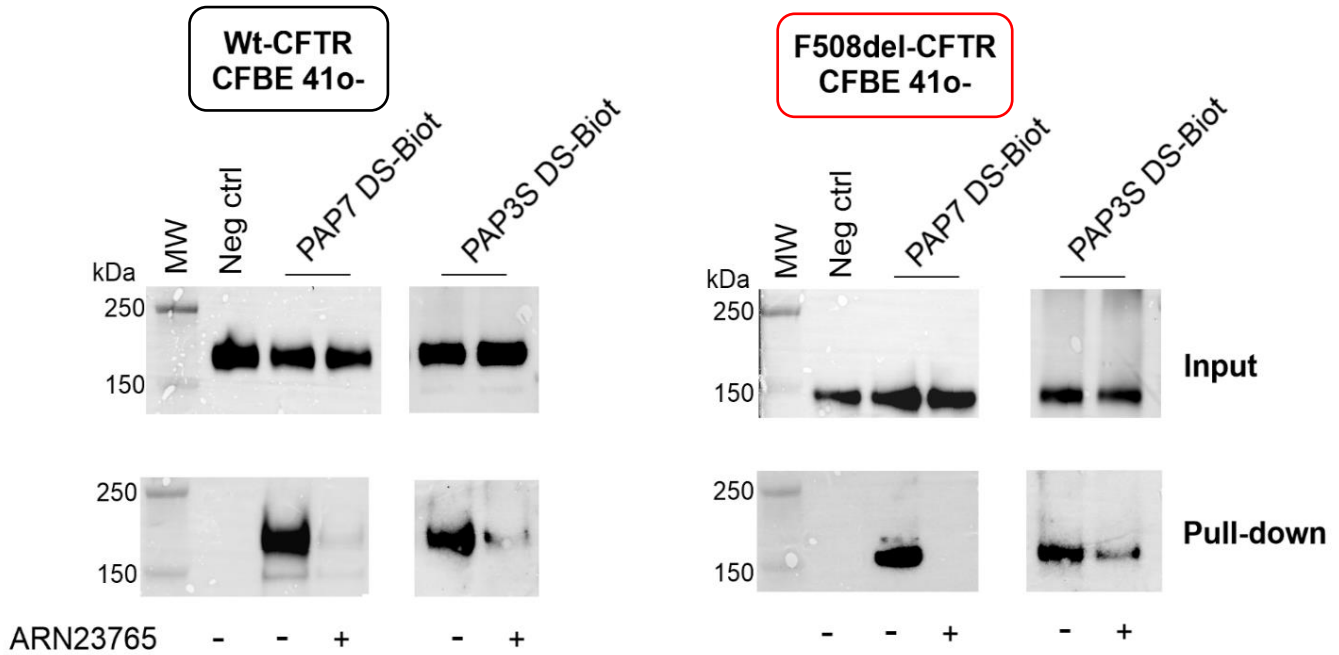


wt-CFTR CFBE41o-

F508del-CFTR CFBE41o-

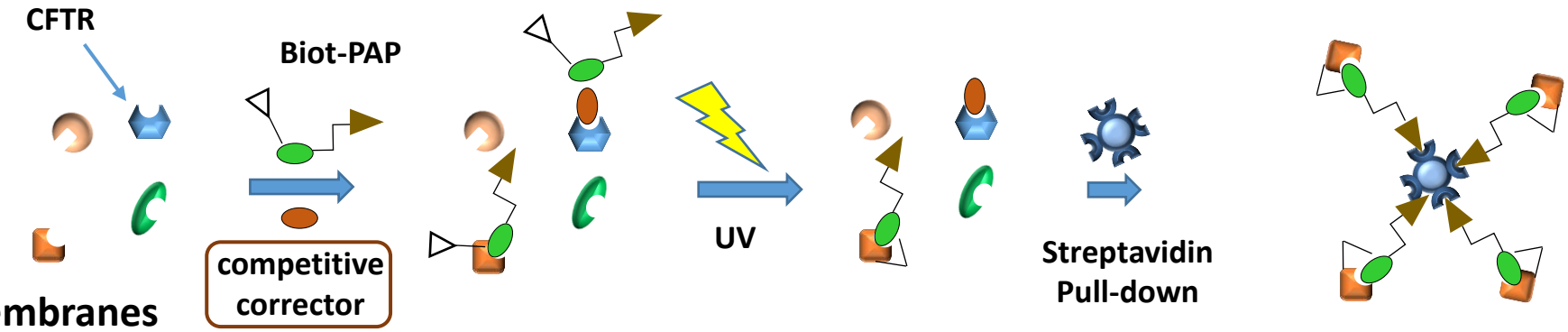
Biotin-streptavidin pull-down

anti-CFTR mAb

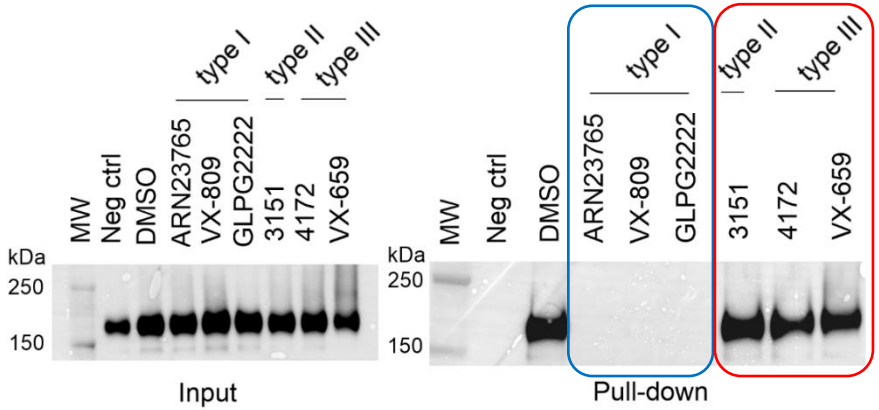


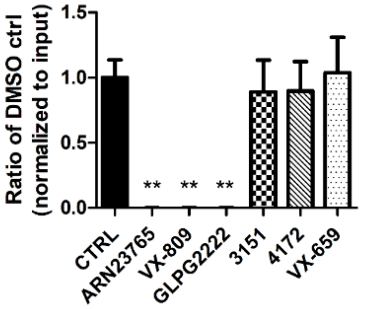
Wt- or F508del-CFTR CFBE41o- cells were incubated for 2h with ***PAP7 DS-biot*** (2.5μM) or ***PAP3S DS-biot*** (5.0μM). PAPs were added alone or in combination with a 10-fold excess of ***ARN23765***.

Competitive *PAL* studies with known correctors



Anti-CFTR antibody

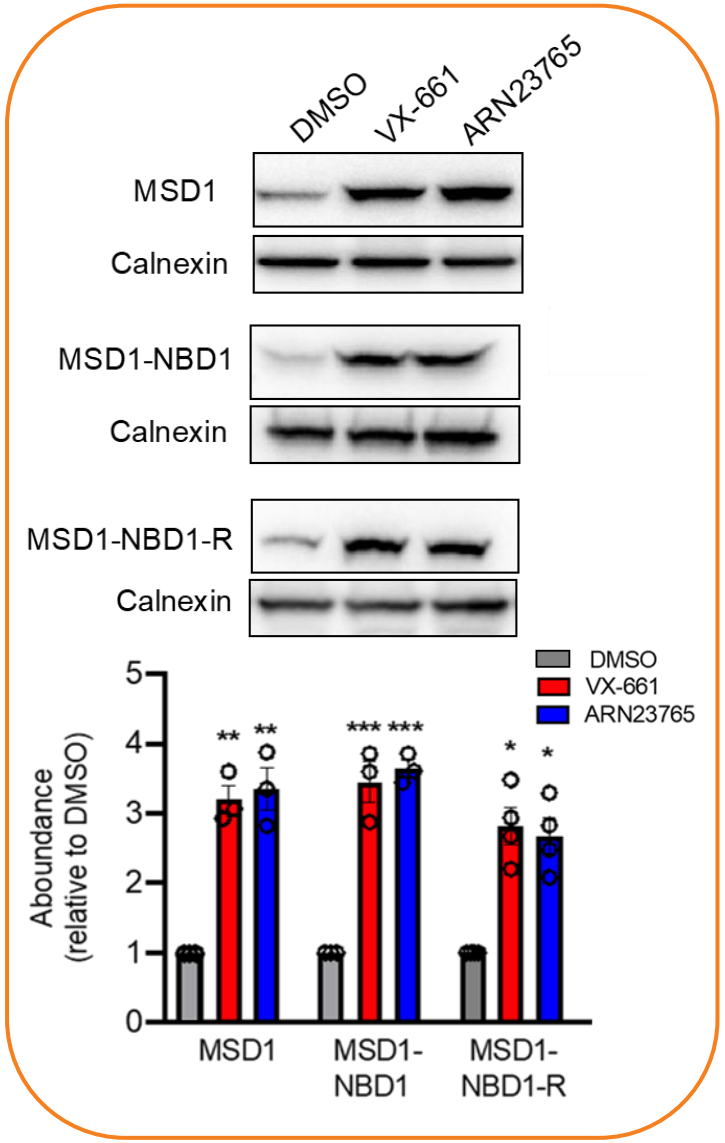


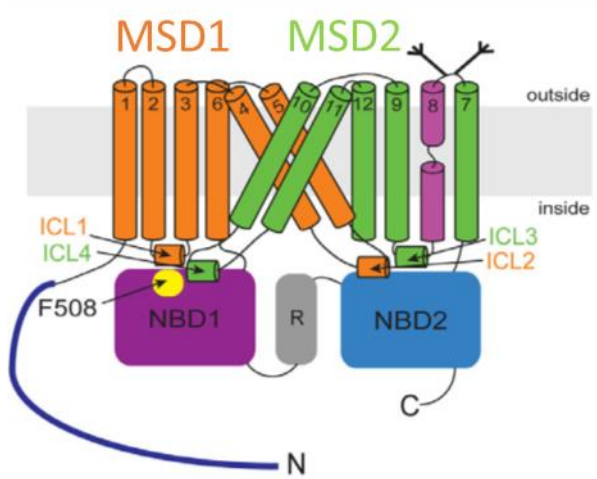


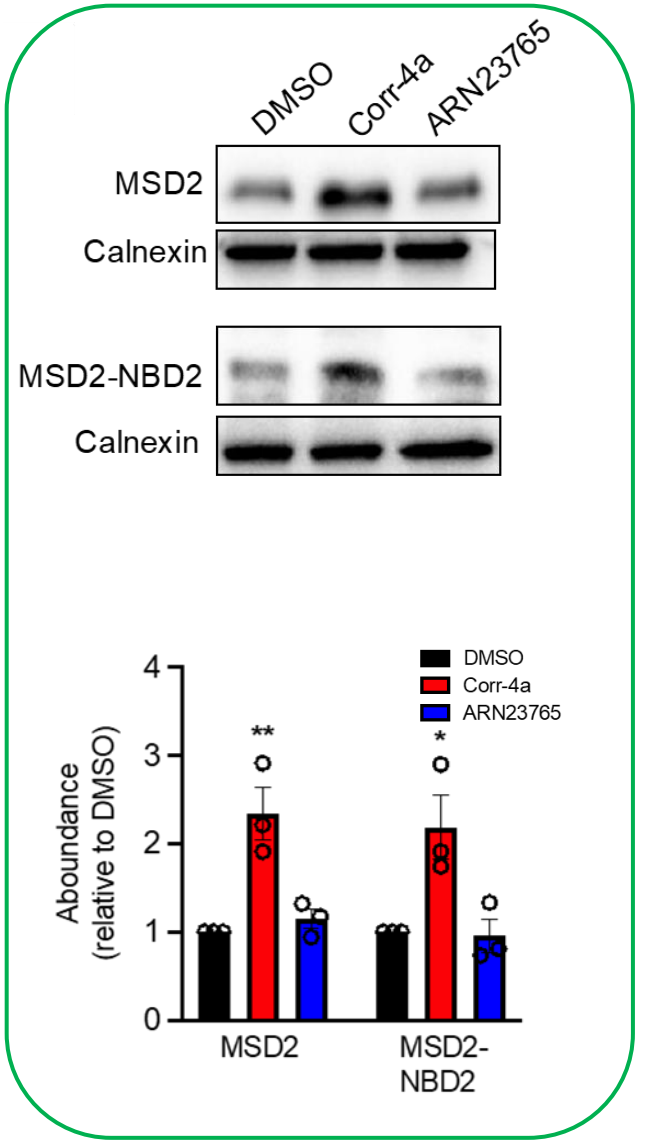
- Type I correctors compete with ARN23765-derived PAP for the binding to CFTR
- Type II and III correctors do not affect ARN23765-derived PAP's binding to CFTR

ARN23765 and type I correctors (may) share a similar binding site on CFTR

ARN23765 mode of action elucidation







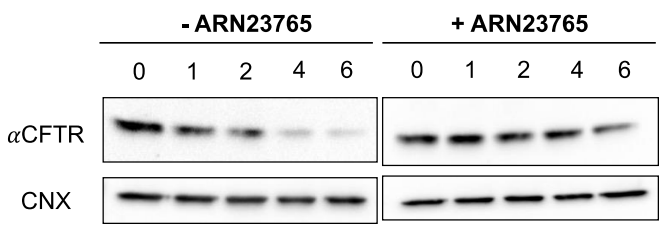
ARN23765 enhances the expression of MSD1-containing fragments

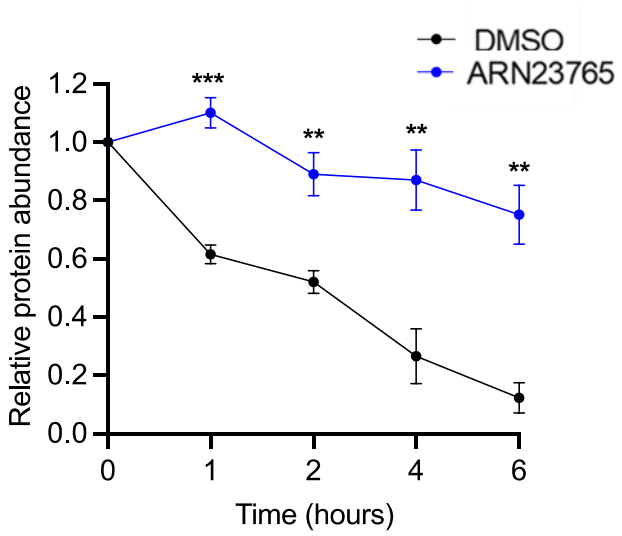
HEK293 cells transfected with CFTR fragments, incubated for 24h with ARN23765 (10nM), VX-661 (3μM) or Corr-4a (10μM) and analyzed by WB

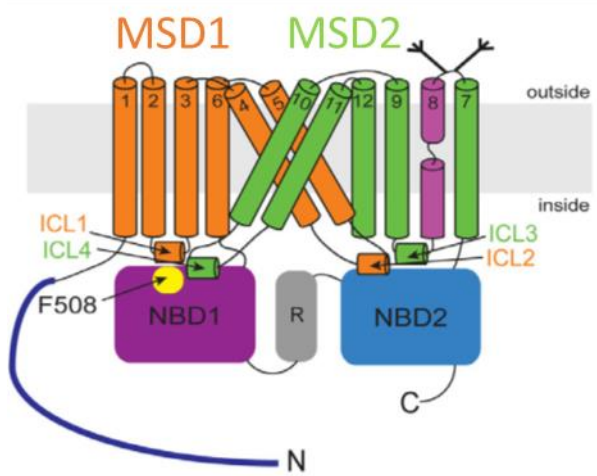
Loo *et al., Biochem. Pharm.* **2017**, 136, 24-31

ARN23765 mode of action elucidation

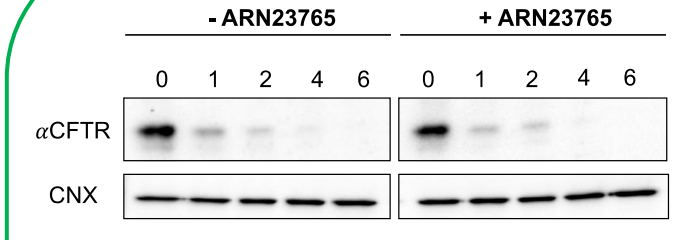
$MSD1_{(1-380)}$

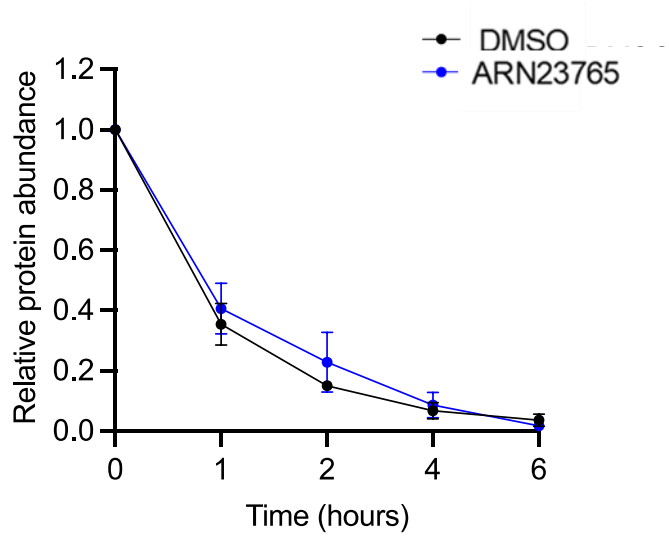






$MSD2_{(837-1196)}$

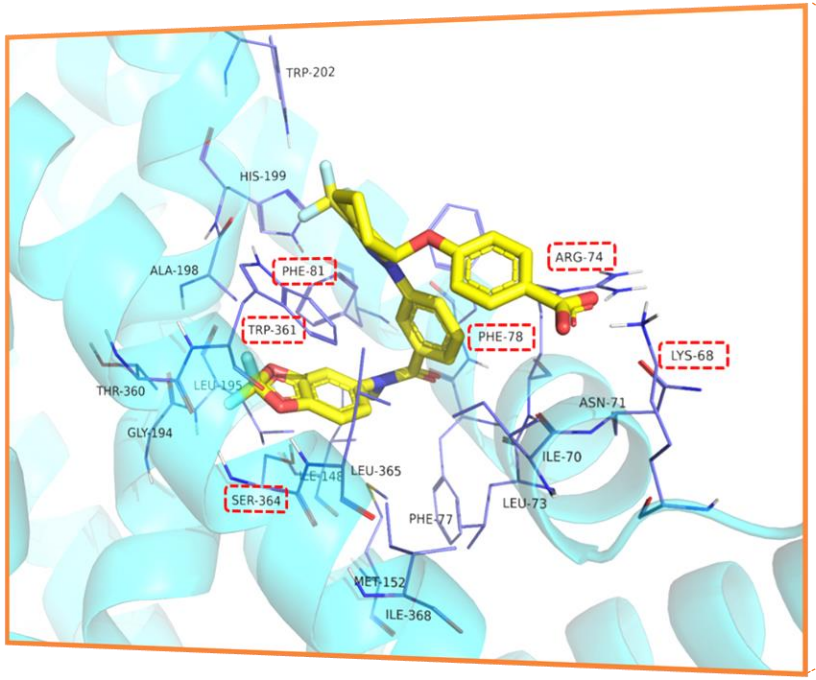


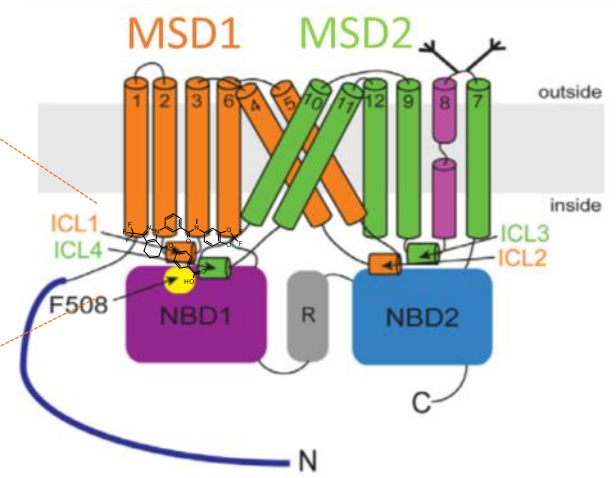


ARN23765 stabilizes MSD1-CFTR domain

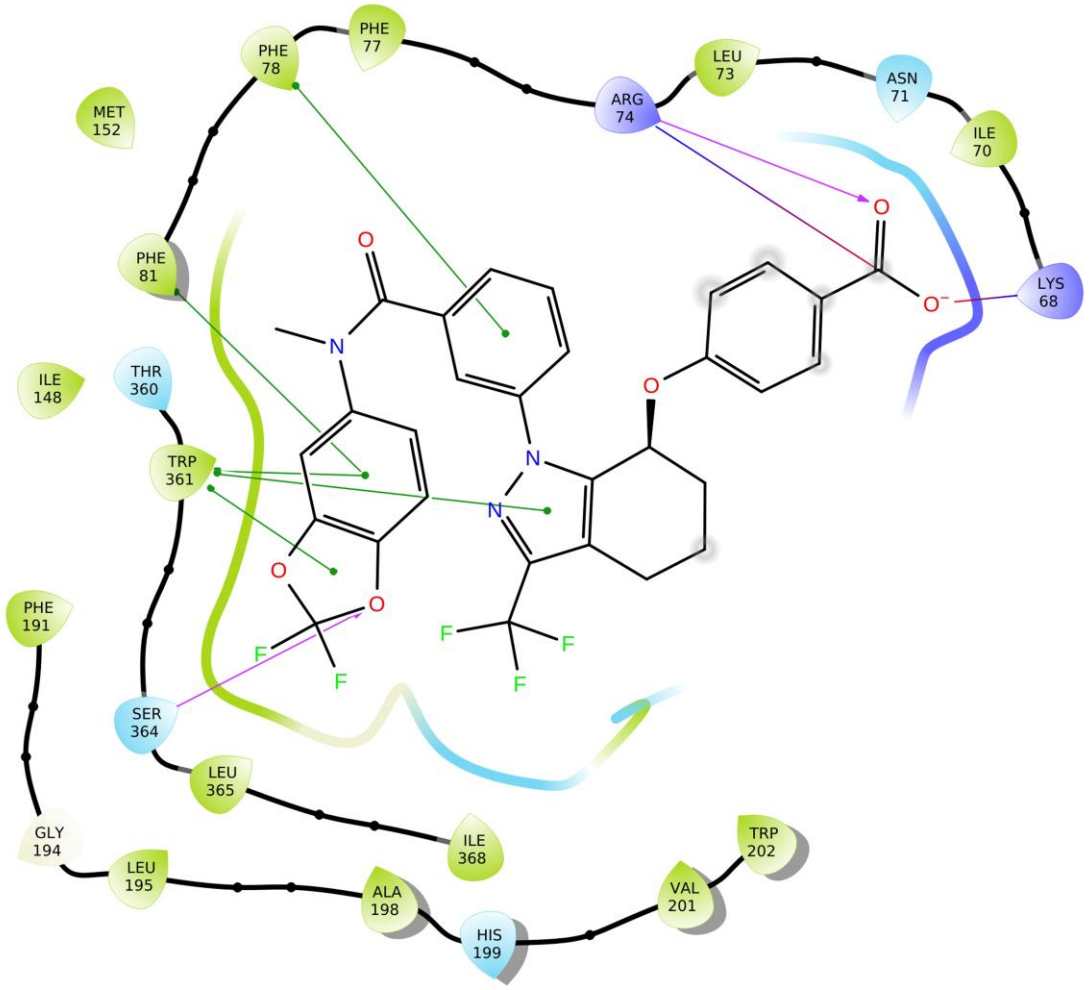
HEK293 cells transfected with $MSD1_{(1-380)}$ or $MSD2_{(837-1196)}$ in the presence or absence of ARN23765 (10 nM); after 24h, protein synthesis inhibited with CXM (200 µM).

ARN23765 docking studies to mutant CFTR





ARN23765 docking studies to mutant CFTR



In-silico mutagenesis scan

			F508del			WT
			8EIO	8EIQ	8EIG	7SVD
Residue	Original	Mutated	d Affinity	d Affinity	d Affinity	d Affinity
A:198	ALA	TYR	40.98	41.80	185.32	25.11
A:361	TRP	ALA	12.92	19.43	17.77	19.45
A:81	PHE	ALA	10.41	11.18	8.03	12.22
A:68	LYS	ILE	8.64	9.36	8.99	9.77
A:74	ARG	ALA	10.85	9.35	5.91	10.50
A:364	SER	PHE	4.85	3.49	3.83	5.69
A:195	LEU	TRP	1.94	-0.04	-0.92	0.49
A:364	SER	ALA	-0.61	-1.18	2.85	-1.52
A:71	ASN	ILE	-1.62	-1.90	2.43	-2.78

Selected mutations:

Ala198Tyr **Trp361Ala**

Phe81Ala **Lys68Ile** **Arg74Ala** **Ser364Phe**

Asn71Ile

ARN23765 site-directed mutagenesis studies

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			F508del			WT
			8EIO	8EIQ	8EIG	7SVD
Residue	Original	Mutated	d Affinity	d Affinity	d Affinity	d Affinity
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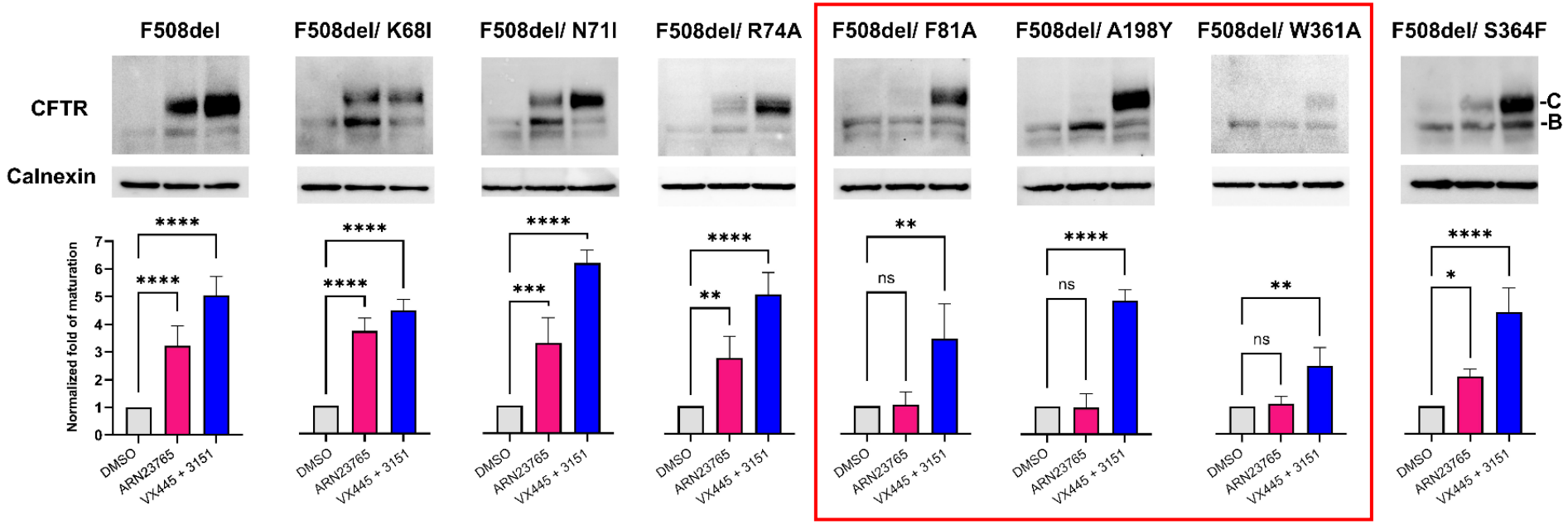
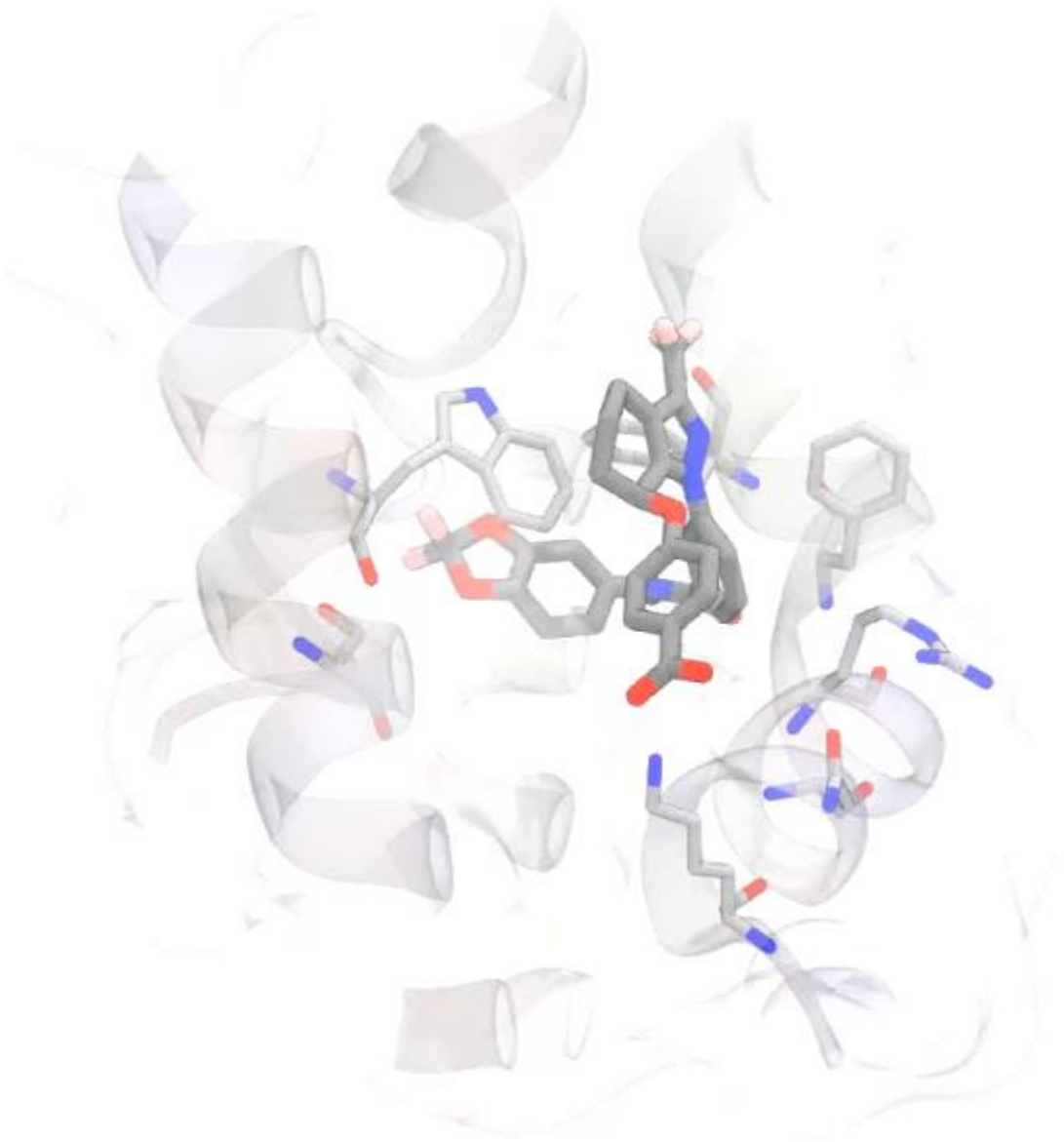
Effect of single-point mutations on F508del-CFTR maturation



HEK-293 cells transfected with F508del-CFTR double mutants and treated with *ARN23765* (10 nM) or with a positive control [3151 (10μM)+VX-445 (3 μM)] for 24h at 30° C.
Protein maturation evaluated with WB using an anti-CFTR-specific antibody [band C abundance = C/C+B, and expressed as fold change of DMSO negative control]

MD simulation of CFTR-ARN23765 complexes



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Conclusions

ARN23765 primary target (wt- and F508del-CFTR), mechanism of action and putative binding site were identified.

- ✓ PAL technology applied to the identification of *ARN23765* biological target(s). **wt- and F508del-CFTR demonstrated as ARN23765 biological target in live cells**.
- ✓ Functional studies on single or multiple CFTR domains allowed identifying the domains involved in ARN23765-induced correction.
- ✓ Computational docking analyses, along with MD calculations predicted and highlighted the key molecular interactions for ARN23765 binding.
- ✓ Structure-function studies through site-directed mutagenesis experiments proved ARN23765 putative binding site to CFTR in cells.

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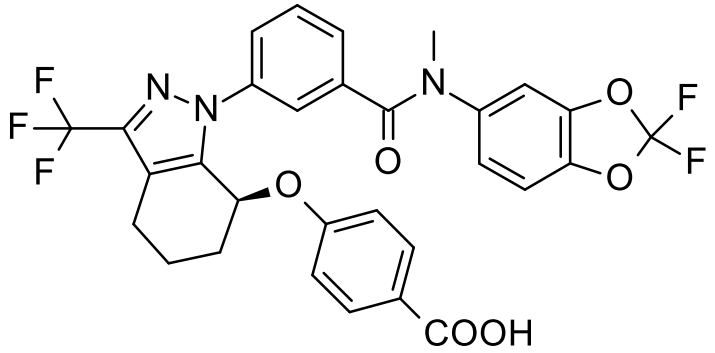
(FFC#4-2020) ***(FFC#2-2022)***

ARN23765: Target/mechanism of action ID …to be continued



Identification of **ARN23765** target protein(s) and mechanism/site of action **in live cells**

(wt- and F508del-CFTR CFBE41o-)

Discovery of other proteins related and/or unrelated (i.e., off-targets) to the CFTR interactome will represent an important finding

Thanks a lot for your kind attention