A Cardiolipin-Targeting α-Helical Bundle Peptide forms Self-Assembled Trimer to Facilitate Cell Penetration and Stabilizes Inner Membrane to Rejuvenate Mitochondrial Dysfunction

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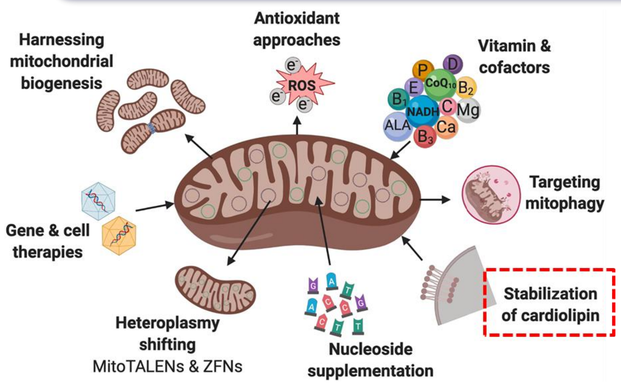
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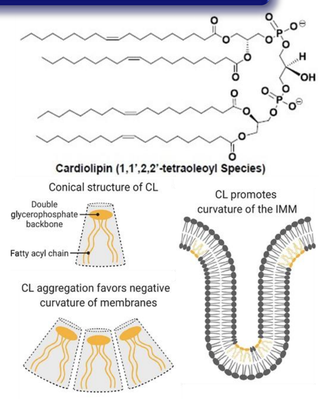
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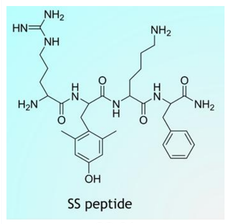
Cardiolipin as a Therapeutic Target in Mitochondria



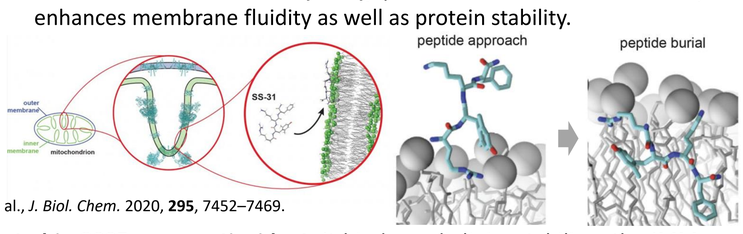


Rahman et al., *Epilepsy Behav.* 2019, **101B**, 106546.

Falabella M et al., *Trends in Endocrinology & Metabolism*, 2021; **32**, 224-237



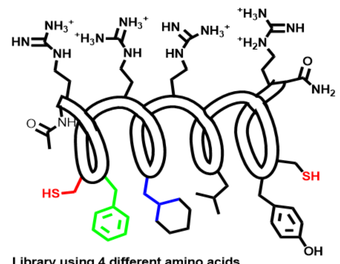
- **Cardiolipin** accounts for approximately 20% of the lipids in the **inner mitochondrial membrane (IMM),** promotes membrane curvature, and enhances membrane fluidity as well as protein stability.



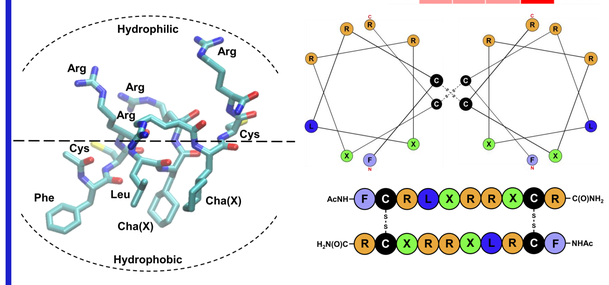
Mitchell et al., *J. Biol. Chem.* 2020, **295**, 7452–7469.

- **SS-31 was first reported in 2005 as an antioxidant**, It binds cardiolipin, stabilizes the IMM, and suppresses pathological remodeling. **Approved by the FDA for Barth Syndrome in September 2025**, **SS-31 is the first-in-class mitochondria-targeting peptide therapeutic.**

Discovery of 3029 Peptide

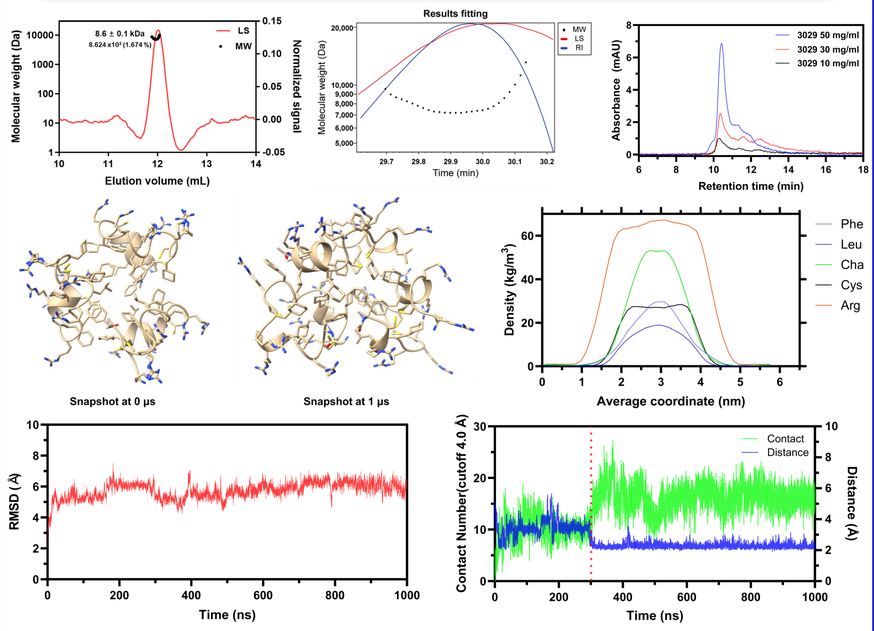


N-	hdf1	hdf2	hdf3	hdf4	-C
Ac-	Phe	Hdf	Hdf	Hdf	-amide
Ac-	Leu	Hdf	Hdf	Hdf	-amide
Ac-	Tyr	Hdf	Hdf	Hdf	-amide
Ac-	Cha	Hdf	Hdf	Hdf	-amide
Ac-	Hdf	Phe	Hdf	Hdf	-amide
Ac-	Hdf	Leu	Hdf	Hdf	-amide
Ac-	Hdf	Tyr	Hdf	Hdf	-amide
Ac-	Hdf	Cha	Hdf	Hdf	-amide
Ac-	Hdf	Hdf	Phe	Hdf	-amide
Ac-	Hdf	Hdf	Leu	Hdf	-amide
Ac-	Hdf	Hdf	Tyr	Hdf	-amide
Ac-	Hdf	Hdf	Cha	Hdf	-amide
Ac-	Hdf	Hdf	Hdf	Phe	-amide
Ac-	Hdf	Hdf	Hdf	Leu	-amide
Ac-	Hdf	Hdf	Hdf	Tyr	-amide
Ac-	Hdf	Hdf	Hdf	Cha	-amide



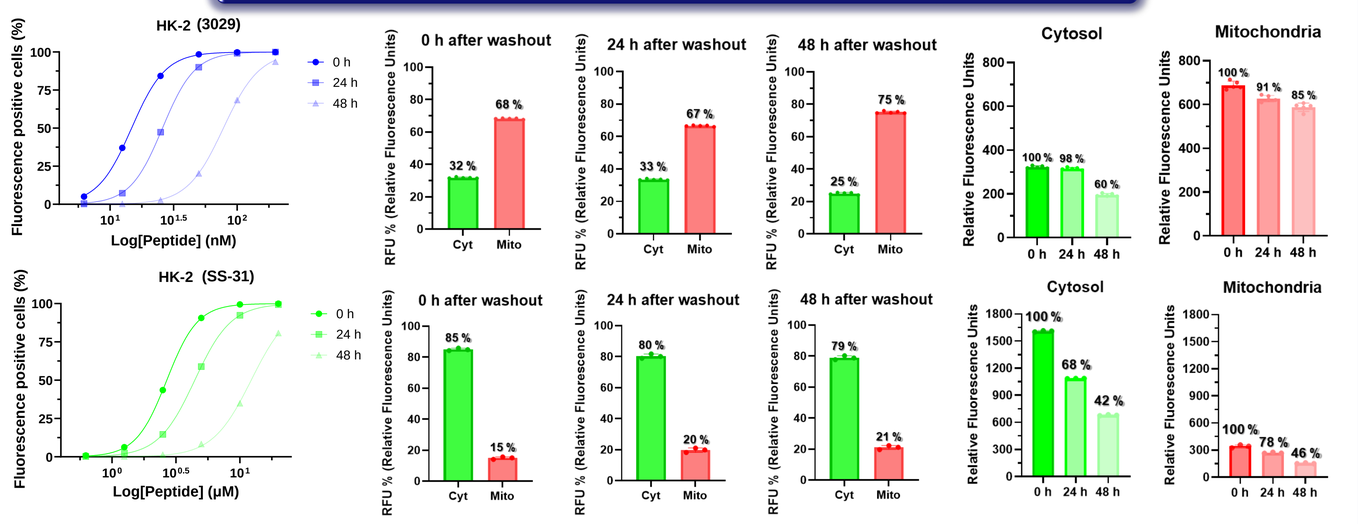
- Using a mixture of known **mitochondrial specific hydrophobic AA** (Phe, Leu, Tyr & Cha), **Each hydrophobic AA was position-scanned** to make 16 sub-libraries (each sub-library contains 64 kinds, total 1,024 kinds of peptides).
- 16 sub-libraries were screened for **the inhibition of ROS production, 3029 was selected.**

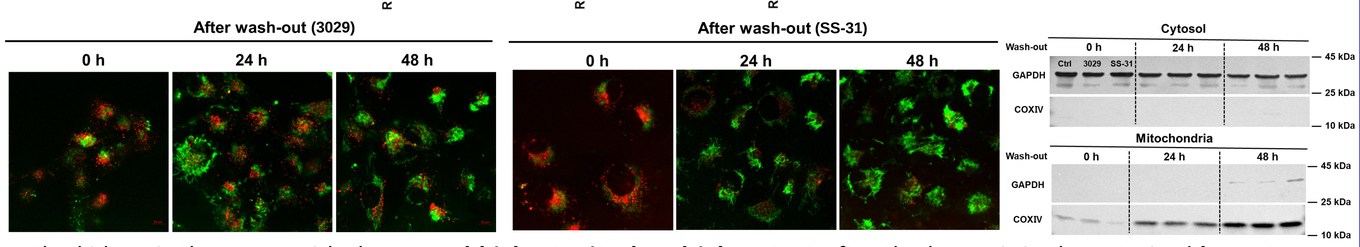
Trimerization of 3029 Confirmed by SEC-MALS and MD



- **Cyclohexylalanine (Cha) residues** are preferentially located in inter-region of 3029, **forming oligomeric status with compact hydrophobic core.**
- 3029 is **self-assembled into a discrete trimer,** The measured molecular wight (8,624 g/mol ±1.674 %) **corresponded to a trimeric assembly** (8,729 g/mol) of the cyclic peptide. MD simulations of the trimeric assembly further supported its **structural stability**, since the trimer maintains a compact bundle structure over 1 μs timescale.

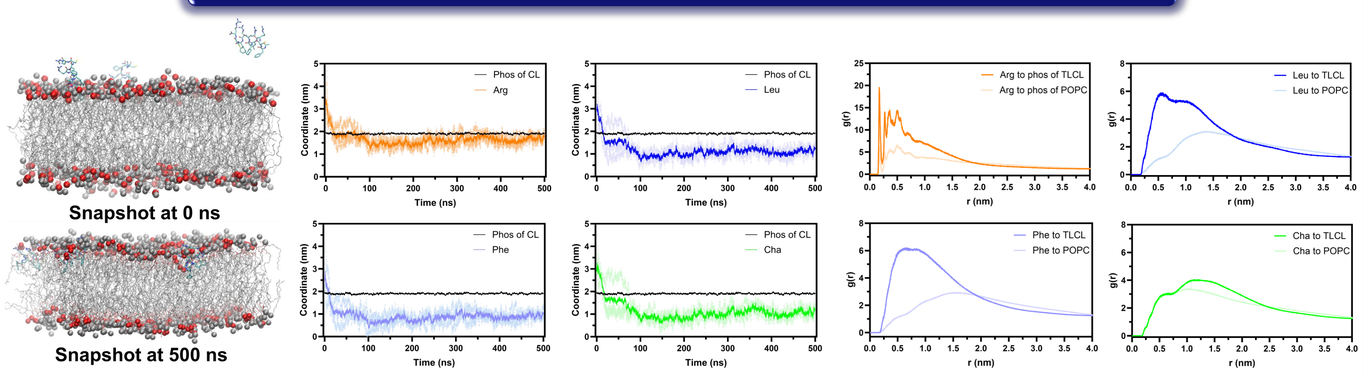
3029 Persists in Cells and Accumulates in Mitochondria





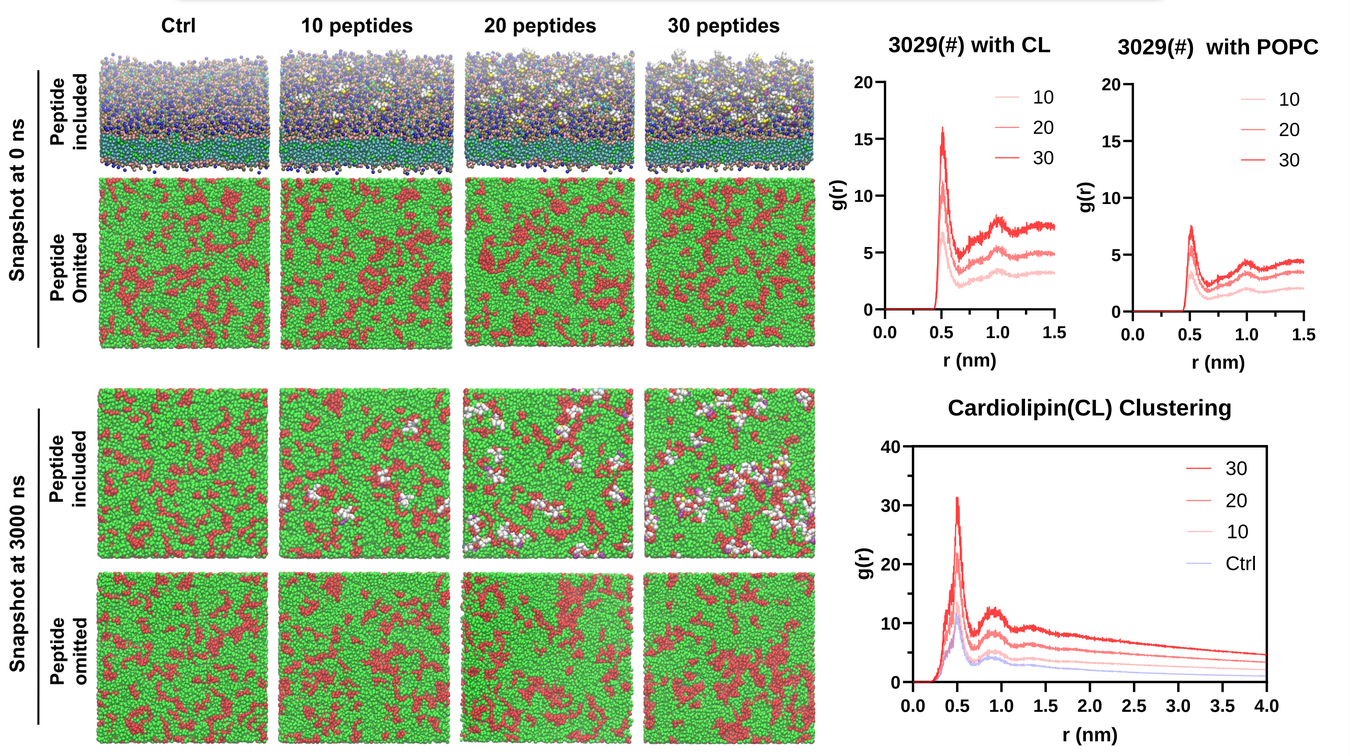
- The kidney is the organ with the **second-highest mitochondrial content** after the heart. it is characterized **by a strong dependence on mitochondria for ATP production.**
- The intracellular EC_{50} at 48 h was 78 nM, indicating that 3029 persistently are **within cells at nanomolar scales even after 48 h**.
- These results suggest that **the preferential accumulation of 3029 in mitochondria** after cell penetration lasts for long period of time.
- **3029 is preferentially going to mitochondria, remaining there**. In contrast, small portion of 3029 in cytosol is removed from cell.

All-Atom MD Reveals Cardiolipin-Selective Membrane Insertion



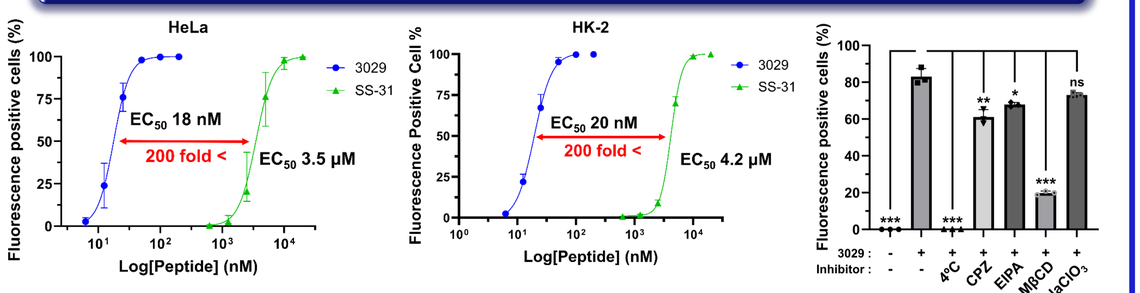
- **MD simulations in a 20% cardiolipin (CL)-containing IMM model** demonstrate that 3029 initially binds to TLCL phosphate groups before flipping its hydrophobic residues into the membrane interior for structural stabilization.
- The radial distribution function (RDF) analysis of key residues—Arg, Cha, Leu, and Phe—further confirms the high selectivity of 3029 for TLCL over POPC, **demonstrating its specific anchoring mechanism within the mitochondrial inner membrane.**

3029-Induced Cardiolipin Clustering in Martini CG Simulations



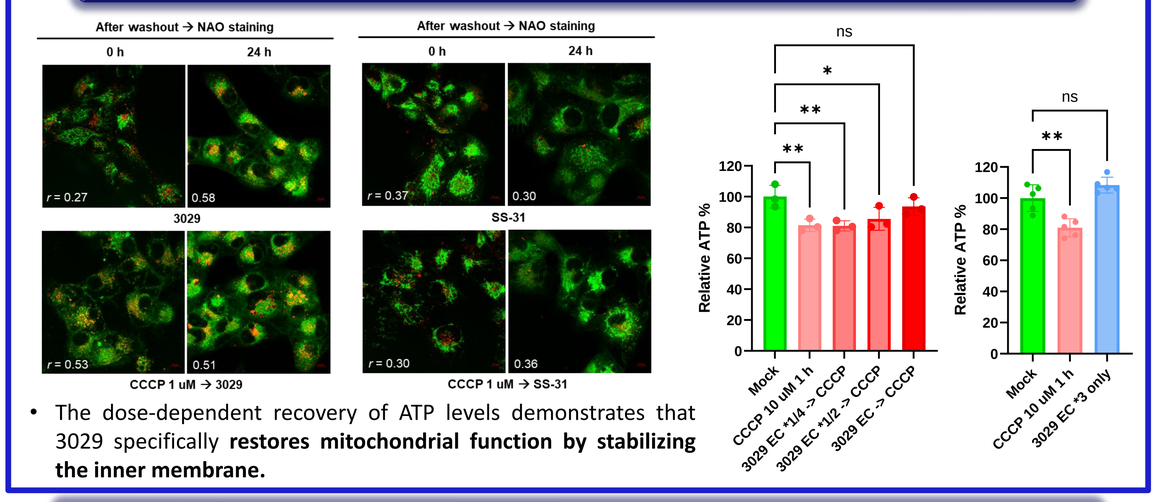
- Martini CG MD simulations reveal that **3029 induces concentration-dependent clustering of cardiolipin (CL),** with RDF analysis confirming its superior selectivity for CL over POPC. These results highlight **the critical role of 3029 in restoring mitochondrial structural and functional integrity. Curvature structural stability of the inner mitochondrial membrane (IMM)**, in which cardiolipin (CL) is a major phospholipid, is **essential for the recovery of mitochondrial dysfunction.**

3029 Efficiently Penetrates Cells via Endocytosis Compared with SS-31



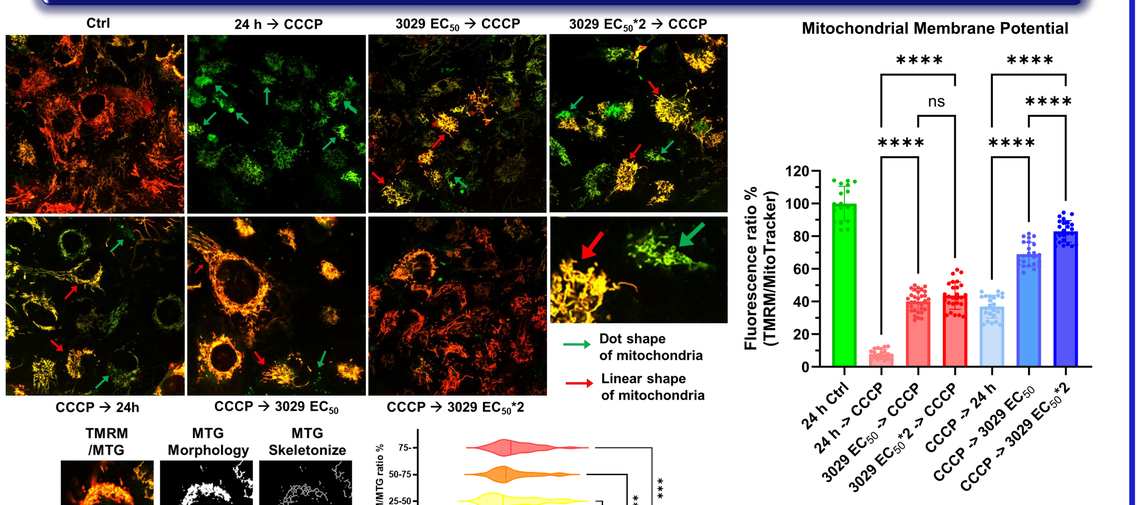
- 3029 showed more than **200-fold greater cellular penetration** than SS-31.
- TAMRA-labeled 3029 penetrates into HeLa and HK-2 cells in 3 h, at EC_{50} **values as 18 nM and 20 nM**, respectively. 3029 penetrates into cells through a clearly defined **energy- and cholesterol-dependent endocytosis mechanism.**

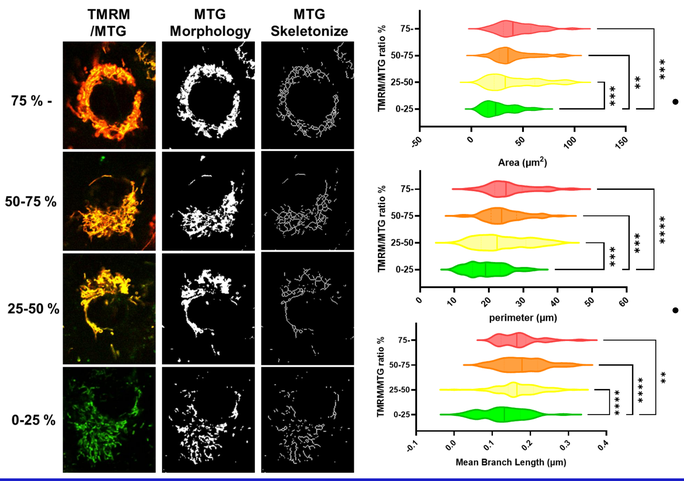
3029 Restores Mitochondrial Function by Targeting Cardiolipin



- The dose-dependent recovery of ATP levels demonstrates that 3029 specifically **restores mitochondrial function by stabilizing the inner membrane.**

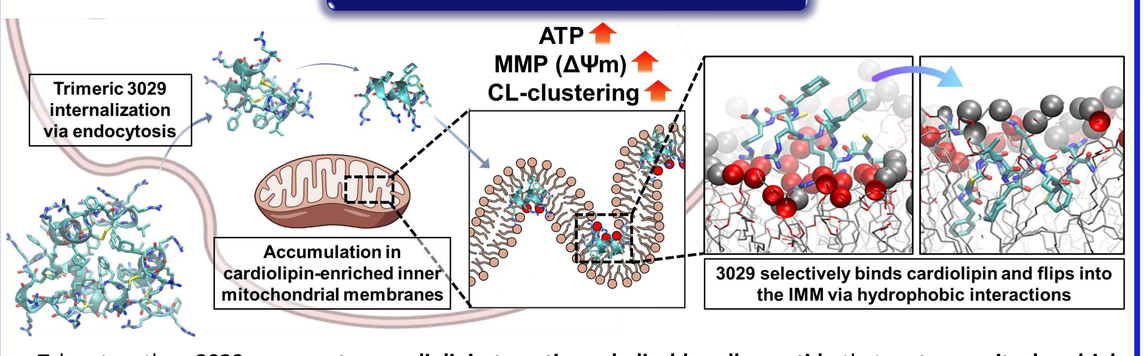
3029 Rejuvenates Mitochondrial Membrane Potential and Morphology





- Indicating **sustained association of the 3029 with cardiolipin-rich regions under oxidative stress conditions.** In contrast to the gradual increase observed in CCCP untreated cells, these results suggest that **mitochondrial dysfunction triggers rapid targeting of 3029 to cardiolipin**.
- MMP and Morphological analysis reveals that while **CCCP induces dysfunctional 'dot-shaped' mitochondria**, 3029 treatment maintains or **rejuvenates the healthy 'linear-shape' structure**, indicating its protective and therapeutic potential.

Conclusion



- Taken together, **3029 represents a cardiolipin-targeting α-helical bundle peptide** that **restores mitochondrial function** through **selective membrane interaction and lipid reorganizatio**n. This strategy provides a promising framework for the **development of mitochondria-targeted therapeutics.**