Mitochondrial VDAC1 porin as a therapeutic target in demyelination process : investigation of the interaction sites between Hexokinase-I and VDAC1

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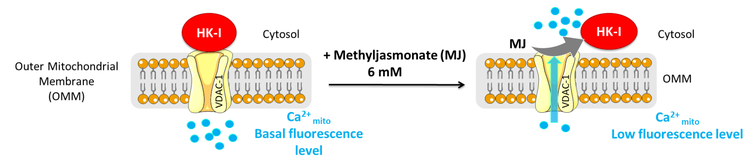
INTRODUCTION

Voltage-Dependent Anion Channel 1 (VDAC1) is a transmembrane β-barrel protein essential for cell metabolism as in the regulation of proteins and metabolites traffic in and out of the mitochondria. VDAC1 displays binding sites for numerous proteins with various functions in cellular activities, such as pro- and anti-apoptotic proteins of the Bcl-2 family proteins and Hexokinases-I and -II (HK-I/II), thus playing a key role in mitochondria-mediated apoptosis. Hexokinase-I (HK-I) is highly expressed in brain and binds to VDAC1 through its N-terminal domain (NHK1) structured in α-helix. Upon apoptotic stimuli, several phenomenon occur : HK dissociates from VDAC1, followed by mitochondrial calcium efflux to the cytosol, VDAC1 oligomerization, and finally release of cytochrome c.[1] [2] Therefore, this protein-protein interaction has attracted much attention in the fight against cancer, neurodegenerative and demyelinating diseases such as Alzheimer, Parkinson, or Diabetic Peripheral Neuropathy, among others. Several interaction models of HK-I/II binding to VDAC1 have been proposed, however they show to be very different in terms of binding association. Herein, we aim to develop new biologically active peptides derived from N-terminal region of HK-I (NHK1 peptides) which are able to interact with VDAC1. Then we ensured to structure NHK1 peptides into an α-helix after several modifications, and finally we determined the stability of the NHK1 peptides towards proteases degradation.

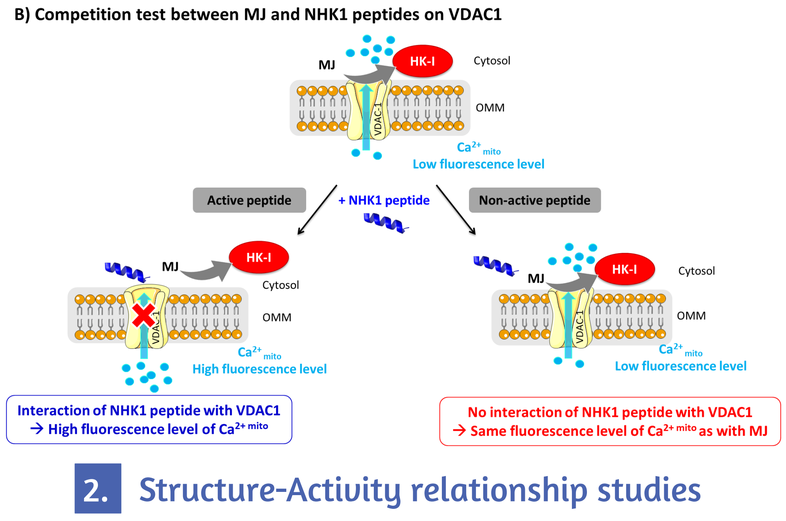
1. *In-vitro* competition assay

- **Characteristics**
  - HEK cells transfected with mito-GCaMP2 probe
  - NHK1 sequence coupled to TAT for internalization delivery
    - ➢ Methyljasmonate (MJ) induces demyelination at a concentration of 6mM
    - ➢ NHK1 peptides ED_{50} (Ca^{2+}_{mito}) = 10µM

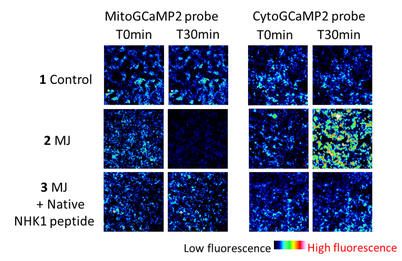
A) Disruption of HK-I : VDAC1 binding using Methyljasmonate (MJ)



B) Competition test between MJ and NHK1 peptides on VDAC1



C) Fluorescence levels of mitoGCaMP2 and cytoGCaMP2 probes with MJ and native NHK1 peptide



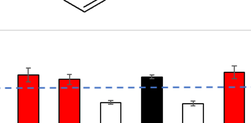
Principle of the *in-vitro* assay of NHK1-TAT peptides to VDAC1 in presence of methyljasmonate (MJ).
Quantification of the fluorescence levels of the mitoGCaMP2 (Ca^{2+}_{mito}) inside the mitochondria, and cytoGCaMP2 within the cytosol and used as control. Active NHK1 peptide would result in fluorescence level of mitochondrial calcium comparable to basal fluorescence level without MJ, while with a non-active peptide, low fluorescence level of mitochondrial calcium would be observed. Experiments were performed by B. Gautier from INM.

2. Structure-Activity relationship studies

- **Ala-scan and deletion studies of native NHK1 sequence** :
  - NHK1 minimal active sequence : [1]MIAAQLLAYYFTELK[15]
    - ➢ High activity of coumpound**1** for VDAC1
    - ➢ Identification of key residues involved in the interaction with VDAC1 : [4]AQLLAYYF[11]

- **Interaction optimizations of NHK1-TAT peptides :**
  - Leucine replacement by hydrophobic aminoacids
    - ➢ High activity of compound **2** for VDAC1

- **Reinforcement of helical binding of NHK1-TAT peptides :**
  - Introduction of α-aminoisobutyric acid (Aib, U) to NHK1 peptides
    - ➢ As AUAU patch at N-terminus
    - ➢ By replacing Alanine at 8th position
  - Introduction of a hydrophobic tag [3] : 3-F_3C-Ph[Tz]U



Cpd	NHK1-TAT peptide sequence																		
	(n+1)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
NHK1	Ac-			M	I	A	A	Q	L	L	A	Y	Y	F	T	E	L	K	TAT	NH_2
1	Ac-						A	Q	L	L	A	Y	Y	F	T	E	L	K	TAT	NH_2
1a	Ac-						A	Q	L	L	**U**	Y	Y	F	T	E	L	K	TAT	NH_2
1b	Ac-				**A**	**U**	A	Q	L	L	**U**	Y	Y	F	T	E	L	K	TAT	NH_2
1c	Ac-	**A**		**U**	**A**	**U**	A	Q	L	L	A	Y	Y	F	T	E	L	K	TAT	NH_2
1d	Ac-	**A**		**U**	**A**	**U**	A	Q	L	L	**U**	Y	Y	F	T	E	L	K	TAT	NH_2
2	Ac-						A	Q	W	W	A	Y	Y	F	T	E	W	K	TAT	NH_2
2a	Ac-	**A**		**U**	**A**	**U**	A	Q	W	W	A	Y	Y	F	T	E	W	K	TAT	NH_2
2b		**3-CF_3Ph[Tz]**			**U**		A	Q	W	W	A	Y	Y	F	T	E	W	K	TAT	NH_2

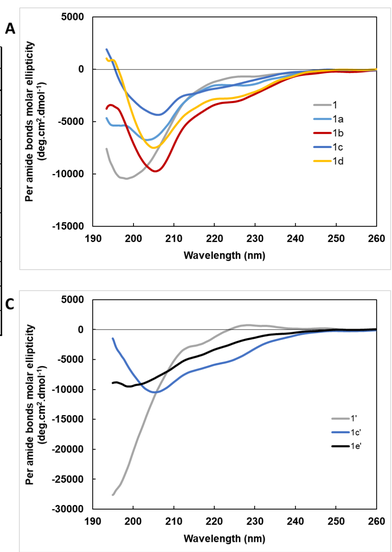
Mean of fluorescence level of mitochondrial calcium. (N=4-6 tests, triplicate/test). Methyljasmonate (MJ) at 6mM has a mean of fluorescence of 0,55. A) NHK1 peptides tested at a 10µM concentration. B) NHK1 peptides tested at a 3µM concentration. Experiments were performed by B. Gautier from INM.

3. Circular dichroism studies

- **NHK1-TAT & NHK1 peptides in phosphate buffer :**
  - Compound **1d** : tendency to fold into α-helix
  - Compounds **2a**, **2b** : the most folded into α-helix
- **NHK1 peptides in methanol :**
  - Compound **1d'** is the most structured into α-helix

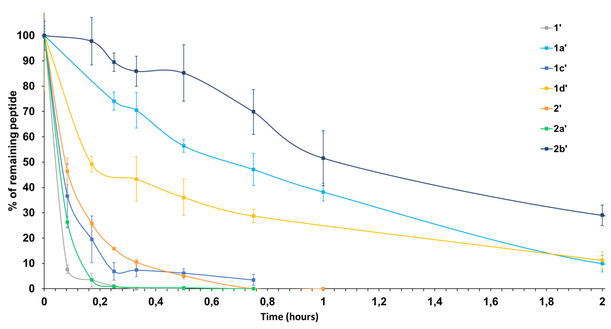
Cpd	NHK1 peptide sequence																
	(n+1)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1'	Ac-				A	Q	L	L	A	Y	Y	F	T	E	L	K	NH_2
1a'	Ac-				A	Q	L	L	**U**	Y	Y	F	T	E	L	K	NH_2
1b'	Ac-			**A**	**U**	A	Q	L	L	**U**	Y	Y	F	T	E	L	K	NH_2
1c'	Ac-	**A**		**U**	**A**	**U**	A	Q	L	L	A	Y	Y	F	T	E	L	K	NH_2
1d'	Ac-	**A**		**U**	**A**	**U**	A	Q	L	L	**U**	Y	Y	F	T	E	L	K	NH_2
1e'	Ac-			**A**	**U**	A	Q	L	L	A	Y	Y	F	T	E	L	K	NH_2
2'	Ac-					A	Q	W	W	A	Y	Y	F	T	E	W	K	NH_2
2a'	Ac-	**A**		**U**	**A**	**U**	A	Q	W	W	A	Y	Y	F	T	E	W	K	NH_2
2b'		**3-CF_3Ph[Tz]**			**U**		A	Q	W	W	A	Y	Y	F	T	E	W	K	NH_2
2c'		**3-CF_3Ph[Tz]**			**U**		A	Q	W	W	**U**	Y	Y	F	T	E	W	K	NH_2

Circular dichroism studies. Conditions A, B, D in DPBS solution pH=7, E in methanol solution. All peptides were dissolved at a 100 µM concentration. Compounds **1-2b** refer to NHK1-TAT peptides, whereas compounds **1'-2c'** refer to NHK1 peptides. Experiments were performed by B. Legrand from IBMM.

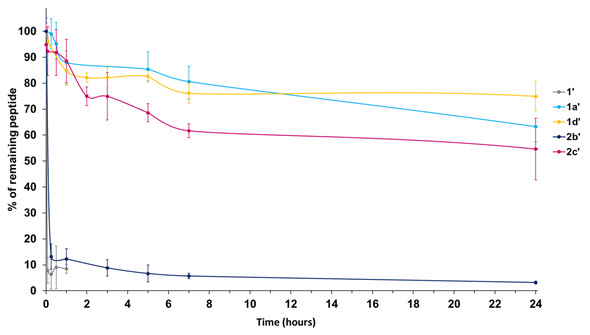


4. Proteolytic stability assays by HPLC-MS

- **NHK1 peptides stability towards Elastase**
  - Compounds **2b' >1a' >1d'** are the most stable against Elastase
- **NHK1 peptides stability towards rat serum**
  - Compounds **1d' >1a' > 2c'** are the most stable against serum proteases



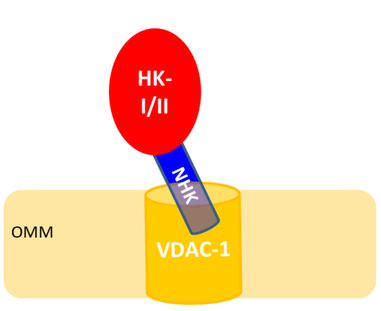
Stability profiles of NHK1 peptides towards Elastase.
All peptides were tested at a concentration of 66.6 µmol/L in presence of 9.4 µg/mL of Elastase in Tris.HCl buffer pH 8 after incubation at 370C for 2h. Assays were performed in triplicate.



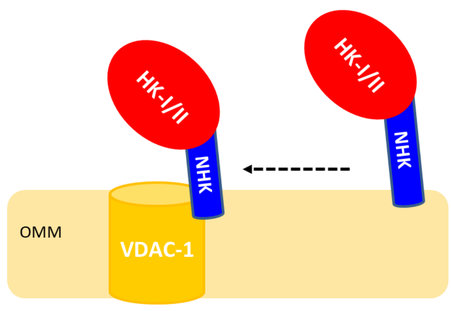
Stability profiles of NHK1 peptides towards rat serum.
All peptides were tested at a concentration of 66.6 µmol/L in presence of 25% (v/v) of rat serum and MilliQ water after incubation at 37° C for 24h. Assays were performed in triplicate.

5. Proposed interaction models of HK-I/II and VDAC1

- **Suggested model no.1 : NHK is inserted into the core of VDAC1** [4]
  - NHK is hydrophobic while VDAC's core is hydrophilic
- **Suggested model no.2 : NHK interacts on the side of VDAC1** [5]
  - Step 1: NHK binds to the Outer Mitochondrial Membrane
  - Step 2: NHK interacts with VDAC1 through hydrophobic residues



Model no. 1



Model no.2

CONCLUSION

- ✓ Synthesis of NHK1 peptides biologically active for VDAC1 blocking Ca^{2+}_{mito} efflux
- ✓ Optimization of helical folding of NHK1 peptides
- ✓ Optimized degradation kinetic of NHK1 peptides in rat serum
- ✓ The binding nature of NHK1 to VDAC1 occurs more preferentially via model no. 2

PERSPECTIVES

- ❑ Introduction of peptide bond surrogates in NHK1 peptides to increase peptide's half-life
- ❑ CD and NMR studies of NHK1 peptides in trifluoroethanol
- ❑ CD experiments on new NHK1 peptides with KUKU or KUAU patch at N-terminus
- ❑ Determination of the binding affinity of NHK1 peptides for VDAC1 by Microscale Thermophoresis technology
- ❑ Using NHK1 peptides as a tool for crosslinking studies with VDAC1

REFERENCES

NB : NHK1 coupled to TAT sequence : GRKKRRQRRRPPQ
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