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1 Mitochondrial Voltage-Dependent Anion Channel 1
2 (VDAC1)-Hexokinase-II (HK-II) Complex-Targeted
3 Strategy for Melanoma Inhibition Using Designed
4 Multi-Block Peptide Amphiphiles

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1 **KEYWORDS:** VDAC1-derived amphiphilic peptides, self-assembly, mitochondria-mediated
2 apoptosis, targeting VDAC1-HK-II complex, protein-protein interaction inhibition, cell-
3 penetrating peptides (CPP), human melanoma cells
4

5 **ABSTRACT:** Targeted therapies of melanoma are of urgent need considering the resistance of
6 this aggressive type of cancer to chemotherapeutics. The VDAC1-HK-II complex is an emerging
7 target for novel anti-cancer therapies based on induced mitochondria-mediated apoptosis. The
8 low cell membrane permeability of the anti-cancer 12-mer peptide N-Ter (RDVFTKGYGFGL)
9 derived from the N-terminal fragment of the VDAC1 protein impedes the intracellular targeting.
10 Here, novel multi-block VDAC1-derived cationic amphiphilic peptides (referred to as Pal-N-
11 Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT) are designed with self-assembly
12 propensity and cell-penetrating properties. The created multi-block amphiphilic peptides of
13 partial α -helical conformations formed nanoparticles of ellipsoid-like shapes and were
14 characterized by enhanced cellular uptake. The amphiphilic peptides can target mitochondria and
15 dissociate the VDAC1-HK-II complex at the outer mitochondrial membrane (OMM), which
16 results in mitochondria-mediated apoptosis. The latter was associated with decrease of the
17 mitochondrial membrane potential, Cytochrome c release, and changes of the expression levels
18 of the apoptotic proteins in A375 melanoma cells. Importantly, the mitochondrial VDAC1-
19 derived amphiphilic peptides have a comparable IC_{50} value for melanoma cells as the sorafenib
20 small molecule drug, which has been previously used in clinical trials for melanoma. These
21 results demonstrate the potential of the designed peptide constructs for efficient melanoma
22 inhibition.

1

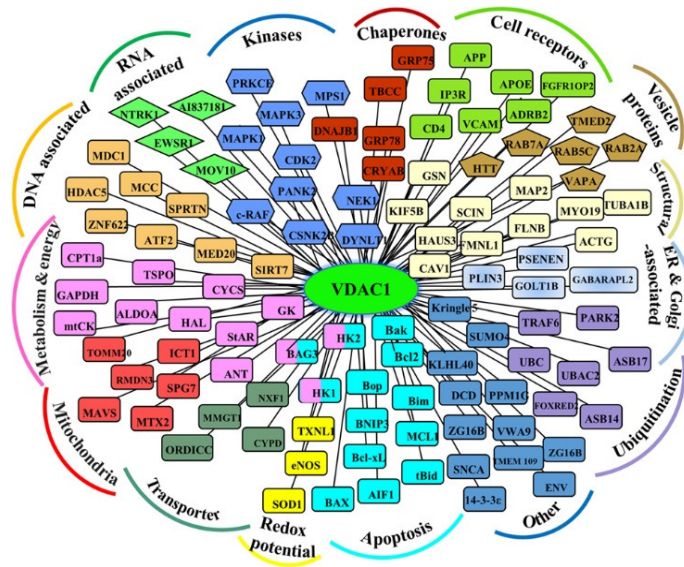
2 1. INTRODUCTION

3 Mitochondrial targeting receives increasing significance in the research for more efficient
4 strategies for melanoma inhibition and treatment.¹⁻² Mitochondria play a key role in regulating
5 cancer cell survival and apoptosis.³⁻⁵ Mitochondria-mediated apoptosis is provoked by changes
6 in the mitochondrial membrane permeability, translocation of Cytochrome c from the
7 mitochondrial intermembrane space to the cytosol, impaired cell metabolism, triggering of
8 caspase activation and cell death signaling.⁶⁻¹⁰ Mitochondria-mediated apoptosis can be activated
9 also via targeting the B-cell lymphoma-2 (Bcl-2) family proteins using small molecules or
10 peptidomimetic-based strategies,¹¹⁻¹³ hexokinase (HK) inhibitors,¹⁴ VDAC1 targeting drugs,¹⁵⁻¹⁹
11 growth factor withdrawal, increased production of reactive oxygen species (ROS), hypoxia or
12 DNA damage.²⁰⁻²¹

13 VDAC1, located at the outer mitochondrial membrane (OMM), is a multi-functional barrel
14 protein.^{17,22} It regulates mitochondrial energy metabolism by transporting various ions, active
15 substances, and metabolites into and out of the mitochondria.²³ VDAC1 plays a key role in
16 mitochondria-mediated apoptosis pathway via interactions with a variety of proteins with
17 different functions (Figure 1).²³⁻²⁴ VDAC1 protein-protein interaction inhibition offers emergent
18 opportunities for development of more efficient anti-cancer therapies.

19 The N-terminal domain of VDAC1 plays a significant role in regulating cell metabolism and
20 apoptosis.²³ Previous studies have suggested that the mobility of the N-terminal region (N-Ter)
21 of VDAC1 is involved in channel gating, VDAC1 dimer formation,²⁵ and interaction with the
22 anti-apoptotic proteins (*i.e.* HK).¹⁴ N-Ter serves also as the interaction site for the apoptosis-
23 regulating proteins of the Bcl-2 family (*i.e.* Bax, Bcl-2, and Bcl-xL).²⁶ VDAC1 binding to the

1 pro-apoptotic proteins (Bax) and oligomerization of VDAC1 into a channel pore triggers
 2 Cytochrome c release, followed by apoptosis. At variance, the association of VDAC1 with anti-
 3 apoptotic proteins (HK-II, Bcl-2 and Bcl-xL) protects cells against apoptosis.²⁷ Therefore,
 4 approaches to prevent the anti-apoptotic activity of the anti-apoptotic proteins will be required to
 5 induce cancer cell apoptosis.



6
 7 **Figure 1.** Mitochondrial VDAC1 protein-protein interactome, which can be explored for design
 8 of novel anti-cancer peptide therapeutics (figure adapted from reference 24 under open-access
 9 CC BY license with permission from Frontiers, 2017)²⁴.

10
 11 Hexokinase-II (HK-II) bound to mitochondria is overexpressed in aggressive tumors, like
 12 melanoma, as compared to its limited expression in normal tissues.²⁸⁻²⁹ The interaction between
 13 HK-II and VDAC1 is the molecular basis of the HK-II-mediated cell survival. Thus, dissociation
 14 of HK-II from mitochondria and disruption of the VDAC1-HK-II interaction have emerged as a
 15 very promising strategy for cancer treatment.²⁷ Anti-cancer agents that directly act on the
 16 VDAC1-HK-II complexes have been studied in different cancer models.¹⁷⁻¹⁹ Such apoptotic

1 compounds comprise enzymes that cleave the N-terminus of HK-II,³⁰ VDAC1-based peptides,³¹
2 hexokinase (HK) inhibitors (*e.g.* glucose-6-phosphate and its analogs)³² as well as the antifungal
3 drugs clotrimazole³³ and bifonazole.³⁴ Recently, a novel HK-II protein-derived amphiphilic
4 peptide Pal-pHK-pKV with self-assembly and cell-penetrating properties has shown a potential
5 for targeting the VDAC1-HK-II complex and triggering lung cancer cell apoptosis with certain
6 selectivity for lung cancer *vs.* normal cells.¹⁵

7 The Shoshan-Barmatz's research group has intensively investigated the anti-cancer activity of
8 a series of N-terminal derived VDAC1-based peptides with different aminoacid sequence lengths
9 modified by the cell-penetrating peptide ANTP (RQIKIWFQNRRMKWKK).^{23,35} The screening
10 studies with a short fragment RDVFTKGYGFGL (N-Ter) from VDAC1 have established the
11 anti-cancer activity of the VDAC1-based peptide and have revealed that the GYGFG
12 (GlyTyrGlyPheGly) sequence in the N-terminal of the protein is required for inducing cell
13 death.³⁵ Motivated by these reports on the anticancer activity of the N-Ter fragment of
14 VDAC1,³⁵ we have chosen the 12-amino acid peptide N-Ter as the template peptide for creation
15 of new anticancer agents with amphiphilic properties (Table 1). We hypothesize that the new
16 peptide derivatives may target the VDAC1-HK-II complex at the OMM of melanoma cells,
17 dissociate the HK-II protein from the mitochondrial membrane, and trigger mitochondrial-
18 mediated melanoma cell apoptosis.

19

20

21

1 **Table 1.** Conceived sequences of amphiphilic mitochondrial VDAC1-derived peptides
 2 comprised by two, three or four building blocks and involving a fluorescent dansylglycine
 3 residue.

Peptide	Sequence	MW	Charge at pH7
N-Ter ^a	RDVFTKGYGFGL	1360	+1
N-Ter-TAT ^b	RDVFTKGYGFGL- GRK(Dansylglycine)KRRQRRRPQ	3254	+8
Pal ^c -N-Ter-TAT	Pal-RDVFTKGYGFGL- GRK(Dansylglycine)KRRQRRRPQ	3492	+8
pFL ^d -N-Ter-TAT	FPWWPFL-RDVFTKGYGFGL- GRK(Dansylglycine)KRRQRRRPQ	4414	+8
Pal-pFL-N-Ter-TAT	Pal-FPWWPFL-RDVFTKGYGFGL- GRK(Dansylglycine) ^e KRRQRRRPQ	4652	+8

4
 5 ^aN-Ter: N-terminal 12-mer peptide fragment of the VDAC1 protein with a propensity of
 6 detaching the hexokinase (HK-II) from the OMM. ^bTAT: a positively charged cell penetrating
 7 peptide (GRKKRRQRRRPQ) derived from the transactivation of transcription protein of the
 8 human immunodeficiency virus (HIV). ^cPal: a hydrophobic lipid chain of palmitic acid. ^dpFL: a
 9 hydrophobic peptide fragment (FPWWPFL) rich in tryptophan (W). ^eK(Dansylglycine): a
 10 fluorescent amino acid residue located in the side chain of lysine (K).

11
 12 In a preliminary study, we found that the N-Ter peptide has poor ability to internalize into
 13 melanoma cancer cells, which is likely due to its low net charge and poor amphiphilicity. For this
 14 reason, we propose here a self-assembly strategy exploiting designed amphiphilic peptides with
 15 multiple building blocks. In recent years, synthetic peptides have found broad applications in
 16 development of antibacterial materials,³⁶ biocatalyst,³⁷⁻³⁹ and drug delivery systems.⁴⁰ However,
 17 the clinical use of the peptide therapeutics has been hampered by their poor stability and short
 18 half-life in the circulation. Strategies using amino acid substitution, cyclization, acetylation at the
 19 N-terminus, amidation at the C-terminus of the peptide sequence, or loading into carriers,⁴¹⁻⁴⁵ are

1 expected to increase the peptide residence time at the target site. Self-assembly with amphiphilic
2 compounds has also been employed.^{41-44,46} The self-assembly process is driven by various weak
3 interactions between the molecules including hydrogen bonding, electrostatic, hydrophobic, π - π
4 and van der Waals interactions.^{45,47-49} Various types of nanostructures formed by amphiphilic
5 peptides, such as nanovesicles, nanofibers, and nanotubes, may affect the outcome of the
6 biological experiments with amphiphilic peptide.^{46,50-53} Therefore, designing peptides with
7 specific structures and functions is an important trend towards advances in anti-cancer
8 nanomedicine.

9 Here, three-block amphiphilic peptides Pal-N-Ter-TAT and pFL-N-Ter-TAT were conceived
10 and custom synthesized by anchoring a 12-mer positively charged peptide (TAT) building block
11 at the C-terminus of the 12-mer N-Ter fragment. In this design, a hydrophobic palmitic acid
12 moiety or a short hydrophobic peptide (8-mer) building block (FPWWPFL), rich in
13 tryptophan, was anchored at the N-Ter terminus. In order to further increase the amphiphilicity
14 of the construct, its self-assembly propensity, and interaction with lipid membranes, we created a
15 peptide of four building blocks. The peptide Pal-pFL-N-Ter-TAT (Table 1) was constructed by
16 modifying the pFL-N-Ter-TAT sequence with a hydrophobic palmitic acid moiety at the N-
17 terminus.

18 We hypothesize that the achieved hydrophobic-hydrophilic balance and structural features of
19 the obtained amino acid sequences may be crucial for the cytotoxicity of the designed
20 amphiphiles to melanoma cells. We consider that major challenges that limit the therapeutic
21 efficacy of peptide agents to cancer cells are the lack of amphiphilicity and the low α -helical
22 content in the peptide conformation, which is determinant for the weak peptide interactions with
23 the lipid membranes.¹⁵

1 The purpose of the present work is to investigate the self-assembly behavior of the new multi-
2 block amphiphilic peptides (Pal-N-Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT) (Table
3 1) and the targeting of the VDAC1-HK-II complex at the OMM in A375 cancer cells by these
4 amphiphilic peptides. Synchrotron Bio-SAXS, cryo-TEM imaging, and CD spectroscopy are
5 employed to characterize the structures of the self-assembled peptides. As the multi-block
6 amphiphilic peptides are designed to amplify cancer cell apoptosis based on disruption of the
7 VDAC1-HK-II contacts at the OMM, several bioassays are performed in order to assess the
8 mitochondria-mediated apoptosis process that may be triggered upon dissociation of the
9 VDAC1-HK-II complex.

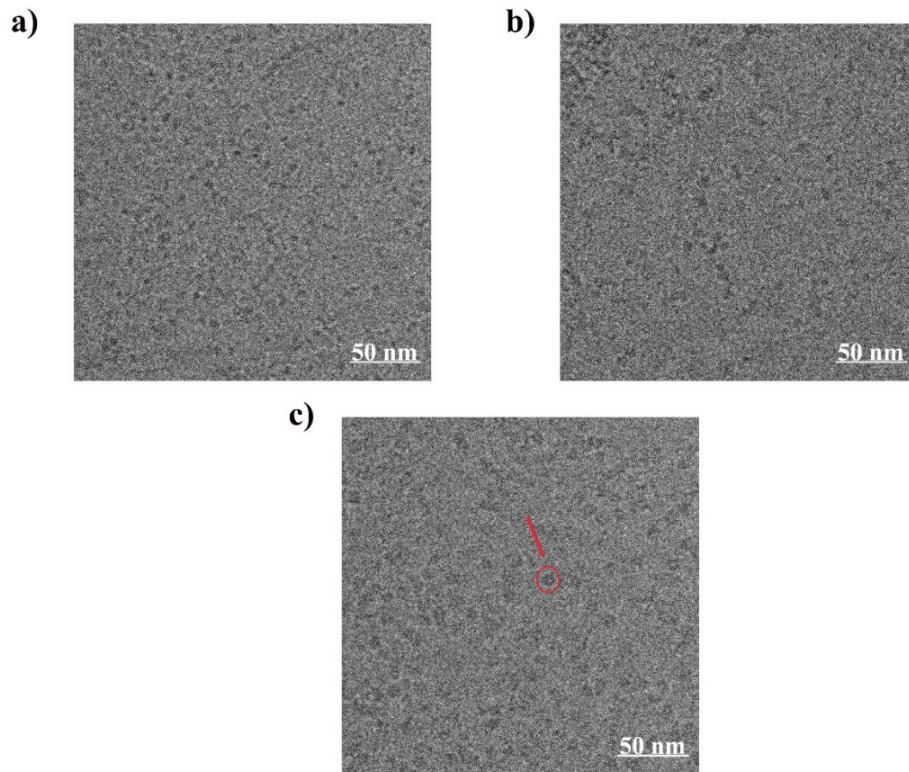
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11 **2. RESULTS AND DISCUSSION**

12 **2.1. Self-assembly behavior of novel mitochondrial VDAC1-based peptide amphiphiles**

13 The amino acid sequences of N-Ter and N-Ter-TAT (Table 1) did not exhibit amphiphilic
14 properties, owing to their hydrophobic/hydrophilic balance, and therefore did not form self-
15 assembled nano-objects in the studied concentration range from 0.1 to 1.5 mM. The self-
16 assembly behavior of the mitochondrial VDAC1-derived peptides Pal-N-Ter-TAT, pFL-N-Ter-
17 TAT and Pal-pFL-N-Ter-TAT into nanoscale objects was demonstrated by cryo-TEM
18 experiments at concentration 1.5 mM (Figure 2). The image contrast was relatively low due to
19 the lack of electron-rich atoms in the peptide molecules. Upon magnification, the cryo-TEM
20 images showed that Pal-N-Ter-TAT formed ellipsoid-like assemblies with an average length of
21 about 6 nm (Figure 2a). pFL-N-Ter-TAT formed ellipsoid-like assemblies with an average
22 length of about 8 nm (Figure 2b), whereas Pal-pFL-N-Ter-TAT formed ellipsoid-like assemblies
23 with an average length of about 12 nm (Figure 2c). The white dots in the center of other self-

1 assembled particles indicated the formation of hollow structures as shown by the arrow in Figure
2 2c.

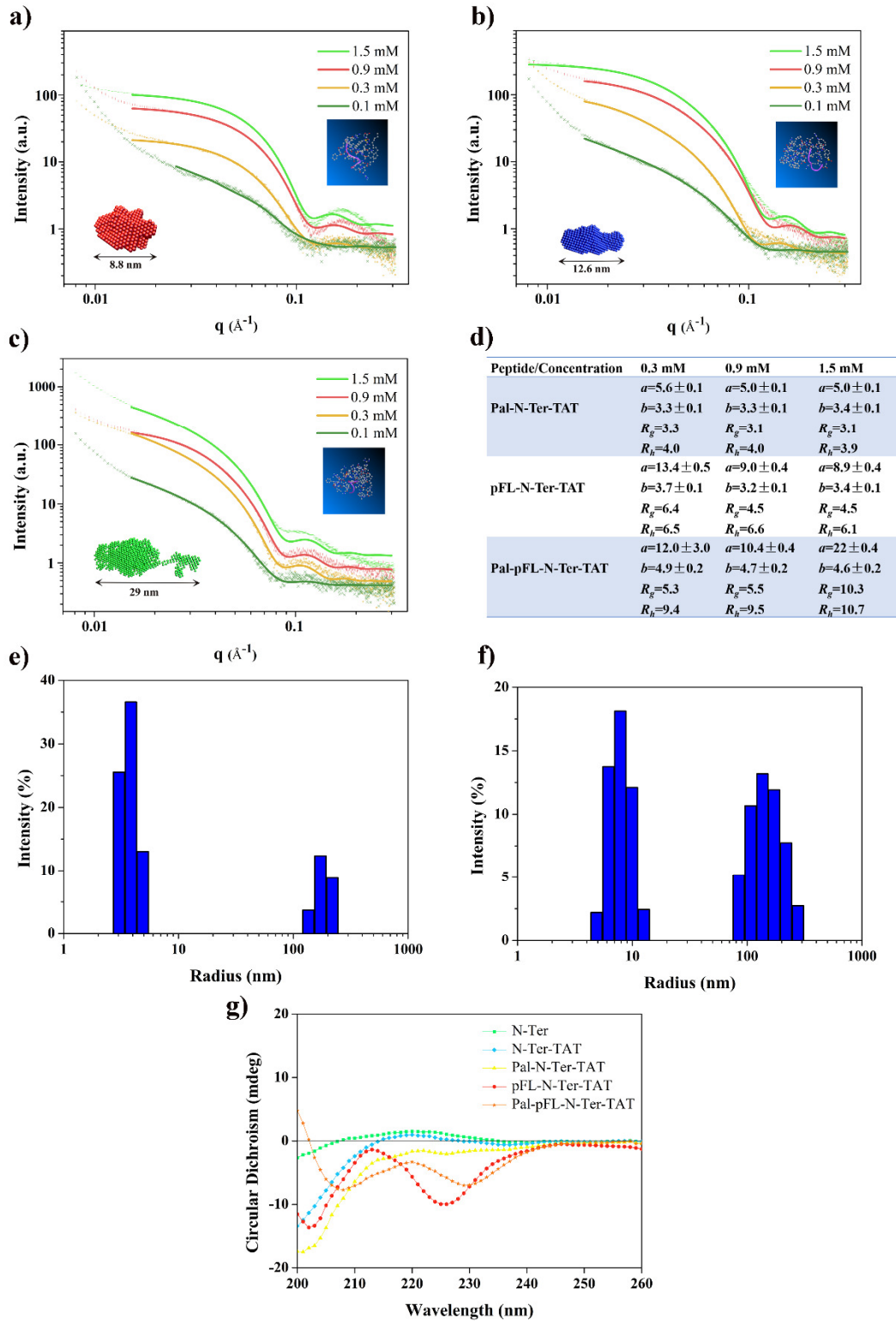


3
4 **Figure 2.** Cryo-TEM images of self-assembled nanoarchitectures formed by the designed
5 mitochondrial VDAC1-derived amphiphilic peptides: a) Pal-N-Ter-TAT (1.5 mM); b) pFL-N-
6 Ter-TAT (1.5 mM); c) Pal-pFL-N-Ter-TAT (1.5 mM). The majority of the assemblies displays
7 ellipsoid-like shapes. Minor fractions illustrate formation of hollow structures.

8
9 The self-assembly behavior of the Pal-N-Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT
10 peptides was further investigated by synchrotron BioSAXS as a function of the solution
11 concentration (Figure 3a, 3b and 3c). All peptide assemblies showed scattering in the
12 concentration range of 0.1–1.5 mM. This implies that the amphiphilic peptides are organized into
13 supramolecular nano-objects.

1 The SAXS data did not show a plateau at the lowest q -vector range except for pFL-N-Ter-TAT
2 at 1.5 mM concentration (Figure 3b). The shape of the scattering curves and the significant
3 deviation from Guinier's behavior $I(q) \sim \exp(-q^2 R_g^2/3)$, $q^2 R_g^2/3 \ll 1$ (where R_g is radius of
4 gyration of particle) is connected with the formation of larger structures. This points on the
5 presence of aggregated structures in the system, the sizes of which are outside the observation
6 window of the present SAXS experiments with a threshold $2\pi/q_{min}$ (~100 nm). Indeed, the
7 performed size measurements by quasi-elastic light scattering showed the presence of at least 2
8 populations of particles (Figure 3e and 3f). The small particles had a mean hydrodynamic radius
9 around 4-10 nm, whereas the fraction of larger particles was with a hydrodynamic radius about
10 100-200 nm.

11 The outer shapes of the obtained peptide assemblies were determined by the DAMMIN
12 method.⁵⁴ The objects are displayed in red (Figure 3a), blue (Figure 3b) and green (Figure 3c)
13 with indicated extended nanoscale dimensions determined from the *ab initio* modeling. This *ab*
14 *initio* analysis was performed for the highest concentration of the peptides as the fraction of the
15 compact small aggregates was predominant according to the particle size measurements.



1

2 **Figure 3.** Synchrotron SAXS patterns of VDAC1-derived peptides in aqueous solutions a) Pal-
 3 N-Ter-TAT, b) pFL-N-Ter-TAT, and c) Pal-pFL-N-Ter-TAT at varying concentrations. The

1 insets show the shapes of the self-assembled peptide objects (red, blue and green) with the
2 corresponding sizes obtained by the DAMMIN method as well as the monomer peptide
3 modeling by molecular dynamics (insets with a blue background). d) Structural morphological
4 parameters [semi axes of ellipsoids of revolution (a , b , b) and gyration radius (Rg)] of VDAC1-
5 derived peptide assemblies deduced by BioSAXS analysis and hydrodynamic radii (Rh) for the
6 small-particle population. The values are presented in [nm] units. Hydrodynamic radii of
7 mitochondrial VDAC1-derived peptides assembly e) Pal-N-Ter-TAT and f) Pal-pFL-N-Ter-TAT
8 determined at 0.9 mM solution concentration by quasi-elastic light scattering (DLS). g)
9 Conventional CD spectra of 0.1 mM N-Ter, N-Ter-TAT, Pal-N-Ter-TAT, pFL-N-Ter-TAT and
10 Pal-pFL-N-Ter-TAT in aqueous phase.

11
12 The analysis of the SAXS results indicated that the slope of the scattering intensities at the
13 lowest q -vector range ($I(q) \sim q^{-\alpha}$) became smaller at increasing concentration of the peptides.
14 This implies that the large aggregates become more compact at higher peptide concentrations. In
15 the intermediate q -vector range, the scattering intensities corresponded to the presence of small
16 aggregates, which entered in the observation window of the SAXS experiments (1-100 nm). The
17 quantitative SAXS analysis was performed for the intermediate and large q -vector intervals,
18 where the effect of coexisting bigger aggregates should be minimal. As a matter of fact, the
19 shapes of all SAXS curves were similar in the concentration range 0.3–1.5 mM in the
20 intermediate and large q -vector range (Figure 3a, 3b and 3c). This indicates that the shapes of the
21 peptide aggregates negligibly change with the variation of the concentration under these
22 experimental conditions. We performed fitting of the experimental scattering intensities by a
23 model scattering from ellipsoids of rotation with semi axes a , b , b . The model considers excluded
24 volume interactions among the aggregates for the interval of intermediate and large q -vector. It
25 was not possible to completely reproduce the broad maximum around 0.15 \AA^{-1} by the ellipsoid of
26 rotation model. This was most probably due to contributions of ions around the surface of the

1 aggregates, which require transformation to few shell models and additional a prior information
2 for stable fitting procedure. The absence of maxima for pFL-N-Ter-TAT sample is connected
3 with length polydispersity of formed aggregates, which has not been taken into account in
4 modelling for better comparison with other samples.

5 The details of the formal analysis were analogous to those previously reported.¹³ The fitting
6 parameters (semi axes of ellipsoid of revolution (a , b , b) and radius of gyration (R_g)) are given in
7 Figure 3d. The results showed that the length of the peptide aggregates increased upon dilution.
8 The peptide particles were more compact at higher concentrations, but this should be considered
9 with caution due to possible interference of the larger aggregates on the obtained parameters.
10 The ratio R_g/R_h gives the important information of shape, compactness, and structure of
11 hydration layer of the particles. It can be noted in Figure 3d that the ratio is always below 1 and
12 in some cases, it is above 0.774 (hard sphere ratio), which points on some elongated and/or
13 noncompacted structures. The first column in Figure 3d indicates that the shape of the Pal-N-
14 Ter-TAT assemblies is characterized by a ratio between the ellipsoid axes of around 1.5 at
15 concentration 1.5 mM. This result is confirmed by the obtained 3D model derived by the
16 DAMMIN method⁵⁴ (Figure 3a, red color object in inset). Considering the monomer mass of the
17 Pal-N-Ter-TAT peptide ($3492.15 \text{ g mol}^{-1}$), it can be deduced from the scattering data that the
18 small aggregates contain about 22-23 monomer peptides. Both the gyration radius and the
19 hydrodynamic radius values support the conclusion for a compact structure of the aggregates.

20 The pFL-N-Ter-TAT assemblies, in which the peptide monomer mass is $4414.11 \text{ g mol}^{-1}$, is
21 slightly elongated in shape with an axis ratio slightly higher than 2. The aggregates are composed
22 of around 52-53 monomer peptides. The shape of the assembly is also confirmed by the 3D
23 model obtained by DAMMIN (Figure 3b, blue color object in inset).

1 The more hydrophobic Pal-pFL-N-Ter-TAT derivative (with a monomer peptide mass 4652.52
2 g mol⁻¹) appears to be strongly aggregated. The axis ratio is bigger than 2 (Figure 3d) and the
3 number of peptide molecules in one aggregate is estimated to be around 186-187 monomer
4 peptides based on the scattering data. The 3D model shows an elongated structure (Figure 3c,
5 green color object in inset), but the results should be considered with caution due to the
6 overlapping of the scattering signal from aggregates of different sizes.

7 The secondary structures of the mitochondrial VDAC1-derived peptides were investigated by
8 CD spectroscopy (Figure 3g). The secondary structure content of the peptides in 0.1 mM
9 solutions is shown in Table 2. The acquired data indicated that the helicity of N-Ter-TAT was
10 29.2%, whereas the helicity for Pal-N-Ter-TAT and pFL-N-Ter-TAT varies from 19.8% to
11 23.4%. Among the amphiphilic derivatives Pal-N-Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-
12 Ter-TAT, the helicity for Pal-pFL-N-Ter-TAT was highest (29.8%). It may be expected that the
13 α -helical conformation has a higher propensity for interaction and insertion into lipid membranes
14 as well as for overcoming the biological membrane barriers.^[15]

15

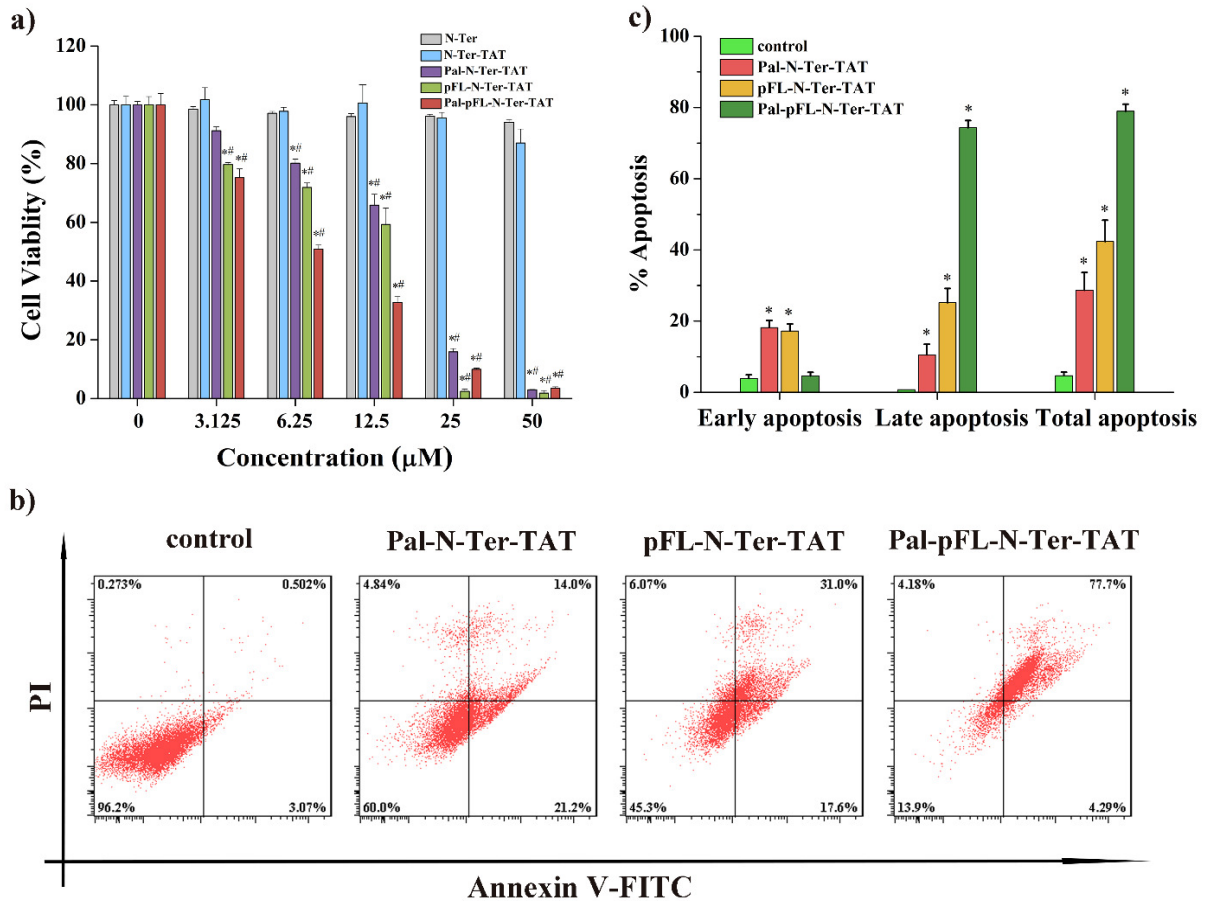
16 **2.2. Cytotoxicity and flow-cytometry cell apoptosis assays with mitochondrial VDAC1-** 17 **derived amphiphilic peptides**

18 The *in vitro* biological experiments with the novel multi-block peptides examined whether the
19 established amphiphilicity is a key factor affecting the cytotoxicity results. In the preliminary
20 work, we used the peptides Pal-N-Ter-TAT and Pal-pFL-N-Ter-TAT to respectively treat A549,
21 MCF-7 and A375 cells for 24 h. The results indicated that Pal-pFL-N-Ter-TAT displays
22 strongest anticancer activity in the three cancer cell lines and has the strongest toxicity to
23 melanoma A375 cells. Therefore, we have chosen the melanoma A375 cells as the research

1 objective in this work (Table S1). In addition, we studied whether the increased hydrophobicity
2 of the multi-block peptide constructs, for instance that of the Pal-pFL-N-Ter-TAT amphiphilic
3 peptide, may be advantageous for inhibition of melanoma cancer as compared to the small
4 molecule drug sorafenib,⁵⁵ which has been previously used in clinical trials for melanoma.

5 Human melanoma A375 cell viability was evaluated via MTT assays after treatment by
6 varying concentrations of N-Ter, N-Ter-TAT, Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-
7 Ter-TAT peptides for 24 hours (Figure 4a). The results showed that N-Ter and N-Ter-TAT
8 peptides had negligible toxicity. At 50 μM concentration, cell viability after treatment by N-Ter
9 and N-Ter-TAT peptides were 94.08% and 87.00%, respectively. It can be concluded that the N-
10 Ter and N-Ter-TAT peptides have low amphiphilicity, owing to low hydrophobicity, and
11 therefore cannot easily penetrate into the cancer cells. In contrast, the anti-proliferative activity
12 of the amphiphilic peptides is notably enhanced.

13 The half maximal inhibitory concentration (IC_{50}) values of Pal-N-Ter-TAT, pFL-N-Ter-TAT
14 and Pal-pFL-N-Ter-TAT peptides for A375 cancer cells after 24 h treatment were determined to
15 be $15.2 \pm 0.7 \mu\text{M}$, $11.1 \pm 0.1 \mu\text{M}$ and $5.5 \pm 1.1 \mu\text{M}$. Therefore, the amphiphilic amino acid sequences
16 appear to interact more strongly with the cellular membranes. The fact that the Pal-pFL-N-Ter-
17 TAT conjugate shows an improved anti-cancer effect as compared to sorafenib ($\text{IC}_{50} = 7.0 \pm 0.2$
18 μM), which is used in clinical trials for melanoma, suggests that the new peptide has a greater
19 potential for melanoma inhibition.



1
2 **Figure 4.** a) Determination of cellular viability with MTT assays. Cancer A375 cells were
3 treated with various concentrations of N-Ter, N-Ter-TAT, Pal-N-Ter-TAT, pFL-N-Ter-TAT or
4 Pal-pFL-N-Ter-TAT peptides for 24 h. Untreated cells were measured as a control. *P<0.01
5 compared to N-Ter and #P<0.01 compared to N-Ter-TAT. Data were presented as mean ± SD
6 (n=3). Determination of cellular apoptosis of A375 cells that were treated with 12.5 µM Pal-N-
7 Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT peptides for 4 h. b) FACS analysis after
8 Annexin V-FITC/PI staining. c) A summary of the incidence of early apoptosis, late apoptosis
9 and total apoptosis in A375 cancer cells. *P < 0.01 compared with controls. Data were presented
10 as mean ± SD (n = 3).

11
12 The IC₅₀ values of the mitochondrial VDAC1-derived peptides, determined from the
13 cytotoxicity assays with A375 cancer cells, are compared in Table 2. The results indicated that

1 the amphiphilic peptides Pal-N-Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT displayed
 2 enhanced A375 cell growth inhibition as compared to the non-amphiphilic peptide N-Ter-TAT.
 3 Among the amphiphilic peptides, Pal-pFL-N-Ter-TAT exhibited the most potent anti-cancer
 4 effect and this outcome was consistent with the increased α -helical content (Table 2). These
 5 results imply that amphiphilicity and α -helical structure play an important role for the peptide
 6 activity towards melanoma cell growth inhibition. A correlation can be suggested between the
 7 cytotoxicity and the designed molecular structures composed of multiple (hydrophobic and
 8 hydrophilic) building blocks (Table 1).

9
 10 **Table 2.** Correlation between the designed multi-block peptide molecular structures and the
 11 cytotoxicity against melanoma cancer cells. The α -helicity content and the IC₅₀ values obtained
 12 from cytotoxicity assays are indicated for every peptide.

Peptides	Helicity (%)	IC₅₀ (μM)
N-Ter	8.1	>50.0
N-Ter-TAT	29.2	>50.0
Pal-N-Ter-TAT	19.8	15.2 \pm 0.7
pFL-N-Ter-TAT	23.4	11.1 \pm 0.1
Pal-pFL-N-Ter-TAT	29.8	5.5 \pm 1.1
Sorafenib	-	7.0 \pm 0.2

13
 14 An Annexin V-FITC/PI apoptosis detection kit was used to examine by flow cytometry the
 15 ability of Pal-N-Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT peptides to induce
 16 melanoma cell apoptosis (Figure 4b and 4c). The A375 cells were exposure to 12.5 μ M Pal-N-
 17 Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT peptide derivatives, respectively, for 4 h. The

1 results indicated that the total apoptosis for A375 cells treated with Pal-N-Ter-TAT and pFL-N-
2 Ter-TAT were 35.2% and 48.6%, respectively, whereas the total apoptosis for cells treated with
3 Pal-pFL-N-Ter-TAT was 81.99%. These data demonstrate that Pal-pFL-N-Ter-TAT is
4 significantly more efficient to induce melanoma cell apoptosis. In all cases, the apoptosis
5 pathway is determinant for the cancer cell death triggered by the Pal-N-Ter-TAT, pFL-N-Ter-
6 TAT and Pal-pFL-N-Ter-TAT constructs.

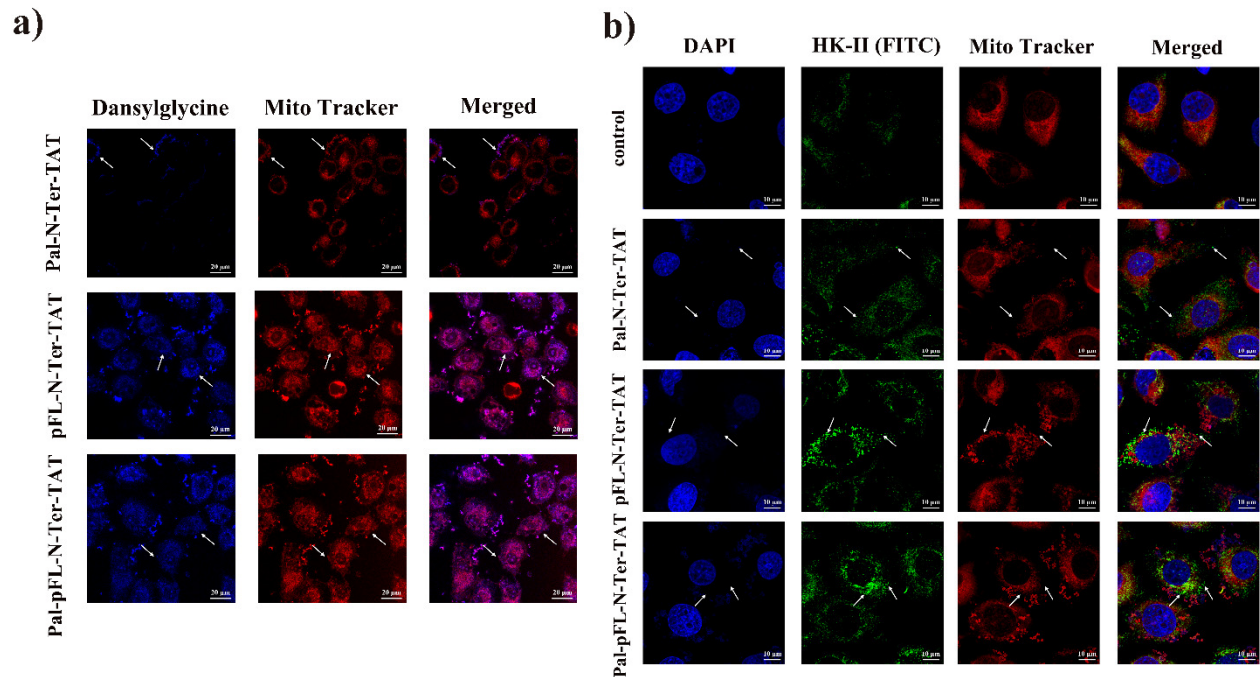
7 The flow cytometry results revealed that the amphiphilic Pal-pFL-N-Ter-TAT peptides are
8 characterized by a highest capacity to induce dissociation of HK-II from VDAC1 at the OMM
9 and trigger melanoma A375 cancer cell death. This opens new opportunities for the development
10 of mitochondria-targeting peptide amphiphiles in anti-cancer strategies.

11

12 **2.3. Intracellular localization of mitochondrial VDAC1-derived amphiphilic peptides in** 13 **melanoma cells followed by detachment of mitochondria-bound HK-II**

14 The intracellular localization of the novel amphiphilic peptides upon uptake by A375 cancer
15 cells was investigated by confocal fluorescence microscopy imaging. As noted above, the
16 investigated peptide constructs contain the fluorescent dansylglycine residue (Table 1). The
17 detected fluorescence of the internalized peptides is displayed in blue color in Figure 5a. The
18 A375 cells were exposed to 12.5 μ M Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT
19 for 4 h. The fluorescence signals from the A375 cells treated with Pal-N-Ter-TAT were weaker
20 as compared to those observed with pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT. This may be due
21 to insufficient uptake of Pal-N-Ter-TAT. In contrast, intensive fluorescence signals were
22 recorded with A375 cancer cells incubated with pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT
23 peptides, which are characterized by increased cellular uptake. These results were consistent with

1 the flow cytometry data for the cellular uptake of the dansylglycine-labelled peptides (Figure
 2 S1). Thus, the fact that the observed intracellular uptake is related to the amphiphilic structure of
 3 the peptide conjugates appears to be in agreement with the flow cytometry results.



4 **Figure 5.** a) Confocal microscopy study of intracellular localization of the investigated peptides.
 5 The A375 cells were incubated with 12.5 μM Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-
 6 Ter-TAT for 4 h. The blue color indicates the fluorescence due to the uptake of the
 7 dansylglycine-labeled peptide. The red color is associated with the mitochondrial staining by 100
 8 nM MitoTracker Deep Red dye. The arrows indicate the co-localization established by the
 9 merged images. b) Confocal microscopy study of detachment of mitochondria-bound HK-II by
 10 immunofluorescence. The A375 cells were incubated with 12.5 μM Pal-N-Ter-TAT, pFL-N-Ter-
 11 TAT or Pal-pFL-N-Ter-TAT for 4 h. The blue color corresponds to the location of the cell
 12 nucleus. The green color is associated with HK-II by immunostaining using anti-HK-II antibody.
 13 The red color shows the fluorescence of MitoTracker Deep Red dye of stained mitochondria.
 14 The arrows indicate HK-II dissociated from mitochondria.

16

1 Figure 5a (merged images) demonstrates the co-localization between the mitochondria of the
2 treated melanoma cells and the amphiphilic peptides internalized in the cells. The comparison of
3 the images evidenced the distribution of the dansylglycine-labeled fluorescent peptides (blue
4 color) and the fluorescence location of the mitochondria-targeting MitoTracker dye (red color).
5 The obtained merged confocal fluorescence images confirmed that mitochondrial membranes are
6 indeed the target site for the studied VDAC1-derived amphiphilic peptides, which was further
7 demonstrated by the results of cell co-localization of peptide-treated A375 cells for 2 h (Figure
8 S2).

9 The detachment of mitochondria-bound HK-II was studied by confocal fluorescence
10 microscopy imaging (Figure 5b). The A375 cells were incubated with 12.5 μ M Pal-N-Ter-TAT,
11 pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT for 4 h. The co-localization of green [HK-II (FITC)]
12 and red fluorescence (MitoTracker) of control cells indicated the mitochondrial distribution of
13 HK-II. In contrast, part of the green fluorescence signal of peptide-treated cells essentially did
14 not co-localize with the red fluorescence signals, pointing to the detachment of HK-II from
15 mitochondria. Moreover, mitochondrial blebbing in peptide-treated A375 cells revealed the
16 occurrence of cell apoptosis.

17 Therefore, it can be suggested that the new VDAC1-derived peptides target the mitochondria,
18 detach HK-II from the mitochondria, and trigger the A375 cells apoptosis. The mitochondria-
19 mediated A375 cancer cells apoptosis is further studied in the next section.

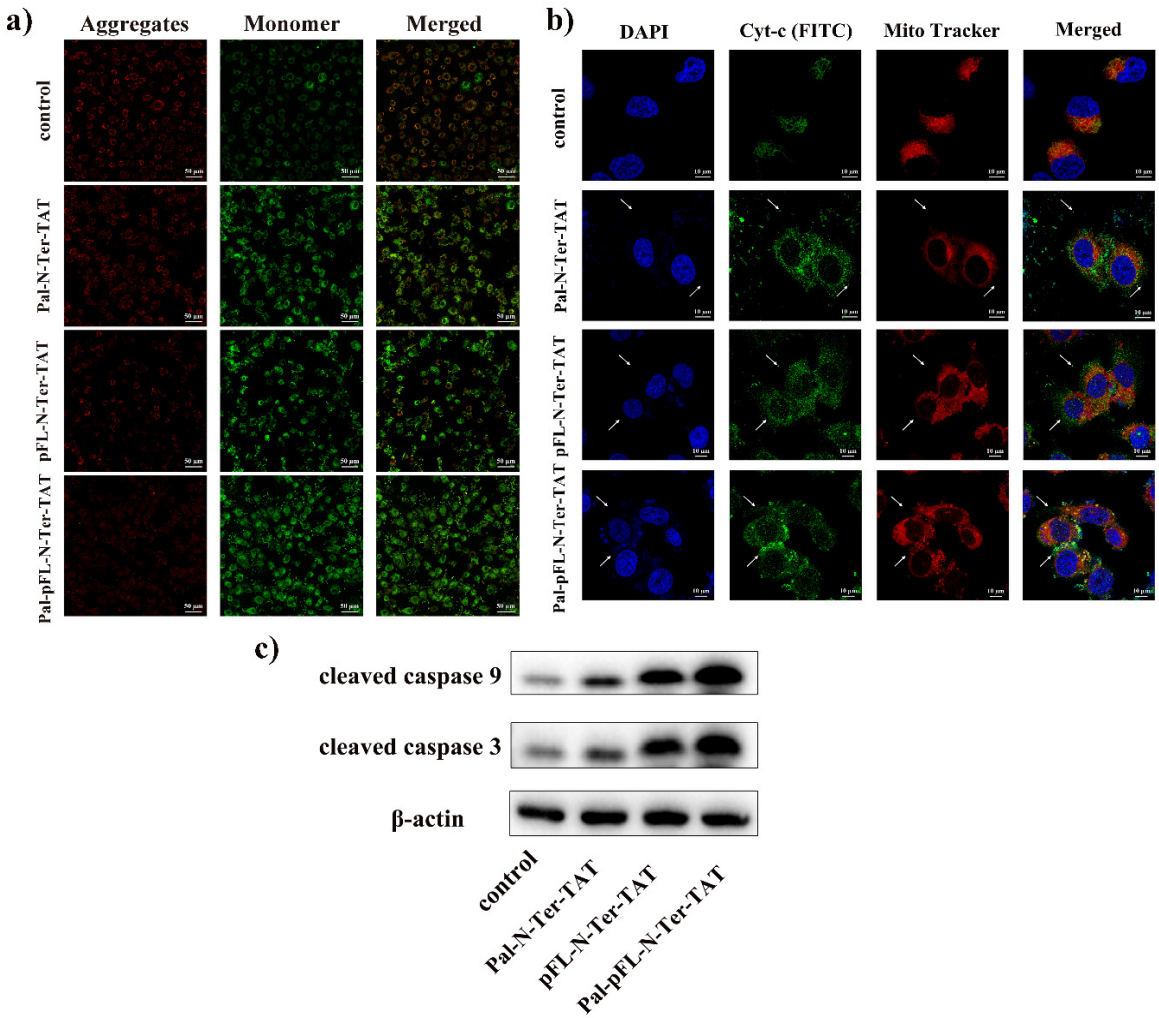
20

21 **2.4. Mitochondrial membrane potential changes, immunofluorescence and western blot**
22 **analysis evidencing the mitochondria-mediated A375 cancer cell apoptosis triggered by**
23 **VDAC1-derived amphiphilic peptides**

1 Fluorescence microscopy with JC-1 staining was used to detect the mitochondrial membrane
2 potential changes of A375 cells treated by peptide constructs (Figure 6a). The decrease of the
3 mitochondrial membrane potential is a marker of early apoptosis. In healthy mitochondria, the
4 JC-1 dye can form aggregates generating red fluorescence. At variance, the JC-1 dye is in a
5 monomeric form, generating green fluorescence in unhealthy cells, in which the mitochondria-
6 mediated apoptosis pathway is triggered. Compared with a control group, the A375 cells
7 incubated with 12.5 μ M Pal-N-Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT for 4 h
8 showed increased green fluorescence and decreased red fluorescence, which indicates the
9 decrease of the mitochondrial membrane potential. These effects reveal that the mitochondria-
10 mediated apoptosis is initiated. The red fluorescence intensity of the Pal-N-Ter-TAT-treated
11 group was comparable with the red fluorescence intensity of the pFL-N-Ter-TAT-treated group.
12 The red fluorescence intensity of the Pal-pFL-N-Ter-TAT-treated group was the weakest, which
13 agrees with the flow cytometry results.

14

15



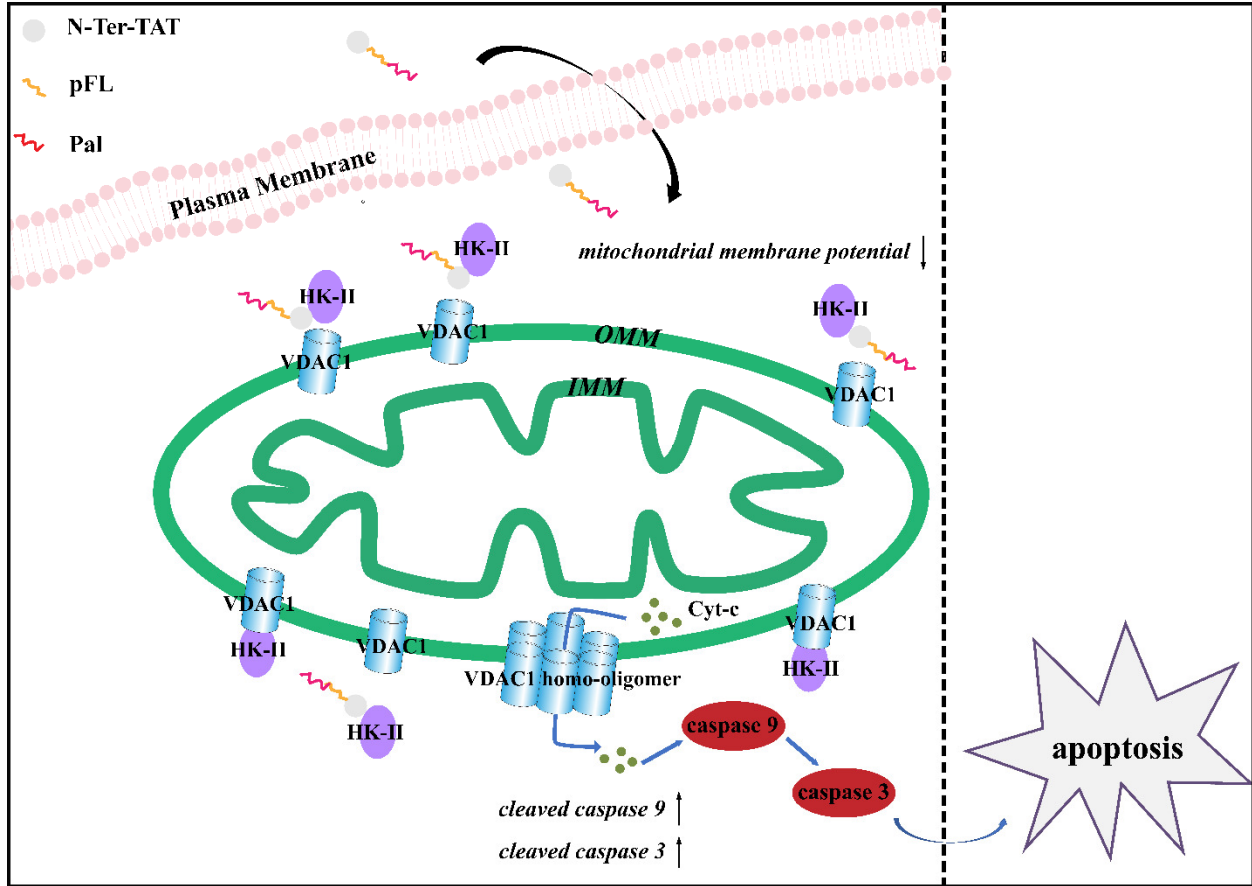
1
2 **Figure 6.** a) Changes in the mitochondrial membrane potential of A375 cells that were treated
3 with 12.5 μM Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT amphiphilic peptides for
4 4 h (fluorescence microscopy detection by the JC-1 kit). Mitochondria-mediated apoptosis of
5 melanoma A375 cells incubated with 12.5 μM Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-
6 Ter-TAT evidenced by immunofluorescence and Western blot analysis. b) Detection of
7 Cytochrome c release in A375 cells treated by peptides for 4 h. The blue color indicates the
8 location of the cell nucleus. The green color is associated with Cytochrome c by immunostaining
9 using an anti-Cytochrome c antibody. The red color shows the fluorescence of MitoTracker Deep
10 Red dye in the stained mitochondria. The arrows indicate Cytochrome c release. c) Changes in
11 the expression levels of apoptosis-associated proteins in A375 cells that were treated by peptides
12 for 24 h.

1
2 Cytochrome c release was investigated via immunofluorescence experiments. A375 cells were
3 incubated with 12.5 μ M Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT for 4 h.
4 Representative images of control cells showed co-localization of most green fluorescence signals
5 and red fluorescence signals, suggesting mitochondrial distribution of Cytochrome c. In peptide-
6 treated cells, part of the green fluorescence signals did not co-localize with the red fluorescence
7 signals, pointing to the release of Cytochrome c (Figure 6b).

8 The expression levels of apoptosis-associated proteins, as evidence for mitochondria-mediated
9 apoptosis, were determined using Western blot technology (Figure 6c). Cellular proteins were
10 extracted from melanoma A375 cells treated with 12.5 μ M Pal-N-Ter-TAT, pFL-N-Ter-TAT or
11 Pal-pFL-N-Ter-TAT for 24 h using Column Tissue and Cell Protein Extraction Kits. The results
12 in Figure 6c showed that the expression levels of cleaved caspase 3 and cleaved caspase 9
13 proteins were significantly upregulated as compared to a control group. This reveals that
14 mitochondria-mediated apoptosis is indeed triggered. For pFL-N-Ter-TAT, the expression levels
15 of three apoptosis-associated proteins were higher in the treated group than for the Pal-N-Ter-
16 TAT-treated group. However, the expression levels of the three apoptosis-associated proteins
17 were highest for the melanoma treatment by Pal-pFL-N-Ter-TAT. These results agree with the
18 flow cytometry results and the other biological tests.

19 The decrease of the mitochondrial membrane potential, Cytochrome c release, and the
20 increased expression levels of cleaved caspase 3 and cleaved caspase 9 proteins in A375 cells
21 treated with amphiphilic peptides prove that the cancer cell death is induced via mitochondria-
22 mediated apoptosis pathway. Evidently, the Pal-pFL-N-Ter-TAT multi-block peptide has an
23 enhanced activity in inducing mitochondria-mediated apoptosis of melanoma

1 cells.



2

3 **Figure 7.** Proposed model of mitochondrial VDAC1-derived amphiphilic peptide action. Once in
4 the cytosol, Pal-pFL-N-Ter-TAT peptides target the mitochondrial membrane, where they can
5 detach HK-II from VDAC1 at the outer mitochondrial membrane. The disruption of the VDAC1-
6 HK-II interaction leads to the decrease of the mitochondrial membrane potential, Cytochrome c
7 release and increased expression levels of cleaved caspase 3 and cleaved caspase 9 proteins, and
8 finally to apoptosis. The mechanism of action of the Pal-N-Ter-TAT and pFL-N-Ter-TAT
9 peptides is likely the same as the Pal-pFL-N-Ter-TAT peptide.

10

11 In summary, the mitochondrial VDAC1-derived amphiphilic peptides Pal-N-Ter-TAT, pFL-N-
12 Ter-TAT and Pal-pFL-N-Ter-TAT target the cancer cell mitochondrial membrane and dissociate

1 HK-II from VDAC1 at the OMM (Figure 7). Inducing the mitochondria-mediated apoptosis
2 pathway and the disruption of the VDAC1-HK-II interaction results in the decrease of the
3 mitochondrial membrane potential, release of Cytochrome c and increased expression levels of
4 cleaved caspase 3 and cleaved caspase 9 proteins. Taken together, Pal-N-Ter-TAT, pFL-N-Ter-
5 TAT and Pal-pFL-N-Ter-TAT are novel amphiphilic CPP with potent anticancer properties.
6 Further to the published work on the VDAC1 protein and VDAC1-derived peptides,^{56-XX} these
7 amphiphilic constructs may serve for development of alternative therapeutic tools.

8

9 **3. CONCLUSIONS**

10 VDAC1 is overexpressed in many cancer types including human melanoma. In this study, we
11 designed novel mitochondrial VDAC1-derived peptide amphiphiles for melanoma inhibition via
12 minimization of the self-defense mechanisms of the cancer cells. Induction of apoptosis was
13 achieved through protein–protein interaction inhibition using rationally designed amphiphilic
14 peptide structures. Pal-N-Ter-TAT, pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT derivatives were
15 created from a 12-mer fragment of the N-terminal of VDAC1 (N-Ter), which was anchored to
16 two kinds of hydrophobic building blocks and a positively charged cell-penetrating peptide TAT.
17 The outcome of the peptide amphiphile design was investigated by high resolution structural
18 methods, which revealed supramolecular peptide self-assembly into ellipsoid-like aggregates
19 with nanometric dimensions. The secondary structures of the novel peptide amphiphiles involved
20 partial α -helical conformations. Notably, Pal-pFL-N-Ter-TAT displayed a higher helicity, which
21 implied stronger interaction with the lipid membranes. It is suggested that the amphiphilicity is
22 essential for the cytotoxicity to melanoma cells. The obtained peptide amphiphiles had a lower
23 IC_{50} value in experiments on A375 cancer cell inhibition as compared to the soluble N-Ter and

1 N-Ter-TAT peptides. The IC₅₀ value of the Pal-pFL-N-Ter-TAT peptide was found to be lower
2 than that of sorafenib, which has been tested in clinical trials for melanoma. All Pal-N-Ter-TAT,
3 pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT constructs were found to (i) co-localize with
4 mitochondria, (ii) detach HK-II from mitochondria, and (iii) induce mitochondria-mediated
5 A375 cancer cell apoptosis. Among them, the Pal-pFL-N-Ter-TAT peptide has shown an
6 increased bioactivity against melanoma cells as evidenced by the significantly upregulated levels
7 of the apoptosis-associated proteins. It is concluded that the designed amphiphilic peptide
8 structure with two hydrophobic building blocks and a positively charged TAT anchor is
9 determinant for the efficacy of the peptide internalization and the exerted activity at the OMM
10 target site in melanoma cells. Therefore, the Pal-pFL-N-Ter-TAT peptide amphiphile appear to
11 be promising for the development of more efficient anti-cancer therapeutic strategies based on
12 targeting of the mitochondrial VDAC1-HK-II complex.

13

14 **4. MATERIALS AND METHODS**

15 **4.1. Reagents and materials.** The designed peptides sequences (shown in Table 1) were
16 custom synthesized by Nanjing Peptide Biotech Ltd (China). Dimethyl sulfoxide (DMSO) was
17 purchased from Aladdin Chemical Reagent Co. Ltd (China). Dulbecco's modified Eagle's
18 medium (DMEM), Fetal Bovine Serum (FBS) and 0.25% trypsin–EDTA were purchased from
19 Gibco (USA). An annexin V-FITC/PI apoptosis detection kit was purchased from Boster
20 Biological Technology Co., Ltd (China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
21 bromide (MTT) kits, JC-1 kits and MitoTracker Deep Red FM were purchased from MKBio
22 (China). Sorafenib was purchased from CSNpharm (USA). Caspase 3 rabbit pAb and caspase 9
23 rabbit pAb, β-actin rabbit mAb, HRP goat anti-rabbit IgG (H+L) and FITC goat anti-Rabbit IgG

1 (H+L) were purchased from ABclonal (China). HK-II rabbit pAb and Cytochrome c rabbit pAb
2 were purchased from Proteintech Group, Inc Rosemont, IL 60018 (USA). Antifade solution
3 (DAPI) was purchased from meilun Biotechnology Co., Ltd (China). Omni-PAGETM Hepes-
4 Tris Gels, PVDF membranes and Omni-ECLTM Femto Light Chemiluminexscent Kit were
5 purchased from EpiZyme (China).

6 **4.2. Custom peptide synthesis.** The studied peptides were synthesized by Standard Fmoc
7 Solid Phase Techniques. Reverse-phase high performance liquid chromatography (Hanbon,
8 NP7005C, China) with a Kromasil C18 column was used to isolate and purify the crude peptides.
9 Mobile phases of HPLC were consist of solvent A (0.1% TFA in acetonitrile) and solvent B
10 (0.1% TFA in water) and a liner gradient was from 20% to 100% solvent A. Purity and mass
11 were respectively determined by high performance liquid chromatography -mass spectrometry
12 (SHIMADZU, LCMS-2020, Japan). The purity of all peptides was $> 98\%$ and peptide
13 lyophilized powder was stored at $-20\text{ }^{\circ}\text{C}$ until need.

14 **4.3. Small-angle X-ray scattering (SAXS) experiments.** SAXS experiments were performed
15 at the BL19U2 beamline of the Shanghai Synchrotron Radiation Facility (Shanghai, China). In
16 order to reduce radiation damage, samples were loaded into flow cells made of cylindrical quartz
17 capillary. The X-ray beam wavelength was 1.033 \AA . A Pilatus 1M detector (DECTRIS Ltd) was
18 employed to measure the intensity of X-ray scattering. A beam size of $0.40\text{ mm} \times 0.15\text{ mm}$ was
19 adjusted to pass through the center of the capillary in every measurement. The detection range of
20 the scattering vector q was from 0.007 to 0.35 \AA^{-1} . The exposure time of each sample was 1 s . The
21 software BioXTAS RAW was used to average the similar data, then subtract the background,
22 and finally convert the two-dimensional scattering images into a one-dimensional SAXS curve.
23 The *ab initio* shapes were determined using DAMMIN.

1 **4.4. Cryogenic transmission electron microscopy (cryo-TEM).** Cryo-TEM experiments
2 were performed at National Center for Protein Science Shanghai (Shanghai, China). peptide
3 samples (3 μ L) were placed on a carbon-coated holey copper grid with a diameter of 3 mm that
4 had been hydrophilized, blotted with a filter paper leaving a thin liquid film, instantly immersion
5 into liquid ethane at -180 °C to vitrified and cooled at liquid nitrogen. The sample copper grid
6 was transferred into a Tecnai G2 F20 transmission electron microscopy which was operated at an
7 acceleration voltage of 200 kv. The structural image of the sample was taken with a 4 k \times 4 k
8 CCD camera system.

9 **4.5. Circular dichroism (CD) analysis.** CD spectra of all peptides (0.1 mM) were measured
10 at room temperature on a Jasco J-810 spectrometer (Jasco Co., Japan) employing a quartz cell
11 with a pathlength of 1 mm. The spectra were recorded in the UV range of 190-250 nm at a scan
12 rate of 60 nm min⁻¹. The measurements were performed three times. The content of various
13 secondary structures of the peptide in PBS buffer was calculated by the BESTSEL tool
14 (<http://bestsel.elte.hu/index.php>).

15 **4.6. Cell culture growth.** The human Melanoma A375 cells were cultured in DMEM medium
16 containing 10% (v/v) fetal bovine serum, penicillin (100 U mL⁻¹), streptomycin (100 g mL⁻¹) in a
17 sterile incubator at 37 °C, in 5% CO₂ saturated humidity atmosphere.

18 **4.7. Cytotoxicity assays.** Cell viability was determined via MTT assay. A375 cells in
19 logarithmic growth phase were seeded in 96-well plates at a cell density of 5 \times 10⁴ cells per well.
20 Cells were incubated for 24 h to grow fully adherent. After removing the medium and washing
21 the cells with PBS, DMEM medium that contained N-Ter, N-Ter-TAT, Pal-N-Ter-TAT, pFL-N-
22 Ter-TAT or Pal-pFL-N-Ter-TAT peptide at concentrations of 0, 3.125, 6.25, 12.5, 25, 50 μ M
23 were added to the wells (100 μ L per well). The cells were incubated at 37 °C for 24 h. Then,

1 MTT solution (5 mg mL^{-1} , $10 \text{ }\mu\text{L}$) was added to each well and incubate for 4 h at $37 \text{ }^\circ\text{C}$. The
2 solution was removed and DMSO ($100 \text{ }\mu\text{L}$) was added to each well. OD values were measured at
3 492 nm and 630 nm . The formula:

4
$$\text{Viability (\%)} = (\text{Mean OD value of treatment group-blank}) / (\text{Mean OD value of control group-}$$

5
$$\text{blank}) \times 100\%$$

6 was used to calculate cell viability. The IC_{50} values were analyzed by GraphPad Prism 7
7 software.

8 **4.8. Cell apoptosis assays.** The annexin V-FITC/PI apoptosis detection kit was used to detect
9 the rate of apoptosis cells in accordance with the manufacturer's protocol on a BD LSRFortessa
10 Flow Cytometer (BD Biosciences, USA). Briefly, A375 cells were seeded in a 6-well plate at a
11 density of 1×10^5 cells per well for 24 h followed by incubation with Pal-N-Ter-TAT, pFL-N-
12 Ter-TAT or Pal-pFL-N-Ter-TAT peptide ($12.5 \text{ }\mu\text{M}$) for 4 h. The cells were detached with the
13 EDTA-free trypsin solution, centrifugated at 1000 rpm for 5 min, washed twice with cold PBS
14 and resuspended in $1 \times$ binding buffer ($195 \text{ }\mu\text{L}$). Then, in the dark, the cells were incubated with
15 annexin V-FITC ($5 \text{ }\mu\text{L}$) for 15 min and washed with $1 \times$ binding buffer ($200 \text{ }\mu\text{L}$). The cells were
16 collected, resuspended in $1 \times$ binding buffer ($190 \text{ }\mu\text{L}$) and incubated with PI ($10 \text{ }\mu\text{L}$) in the dark.
17 In order to avoid fluorescence decay, all samples were tested within 4 h. The fluorescence
18 measurements were conducted at excitation wavelengths of 488 nm and 561 nm and emission
19 wavelengths of 530 nm and 575 nm for the FITC-conjugated annexin V and PI, respectively.

20 **4.9. Intracellular co-localization study.** The intra-cellular distribution of the Pal-N-Ter-TAT,
21 pFL-N-Ter-TAT and Pal-pFL-N-Ter-TAT peptides were viewed under a Zeiss LSM 710
22 confocal laser scanning microscope (Zeiss, Germany). Briefly, the A375 cells were seeded into a
23 20 mm glass bottom dish at a density of 4×10^4 cells per cm^2 for 24 h, incubated with Pal-N-Ter-

1 TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT peptide (12.5 μ M) for 4 h. Thereafter, in order to
2 stain the mitochondria, the cells were treated with 100 nM MitoTracker Deep Red FM for 25
3 min at 37 $^{\circ}$ C. Finally, the cells were fixed with 4% paraformaldehyde for 20 min at 37 $^{\circ}$ C. The
4 fluorescence measurements were conducted at excitation wavelengths of 405 nm and 633 nm
5 and emission wavelengths of 451 nm and 697 nm for the dansylglycine-conjugated peptides and
6 MitoTracker Deep Red FM, respectively. ZEN software was used to process image.

7 **4.10. Detachment of mitochondria-bound HK-II and Cytochrome c release assay by**
8 **immunofluorescence experiment.** Mitochondria-bound HK-II detachment and Cytochrome c
9 release were investigated on Zeiss LSM 710 confocal laser scanning microscope (Zeiss,
10 Germany). Briefly, the A375 cells were seeded into a 96-well glass bottom plates for 24 h,
11 followed by incubation with the Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT
12 peptides (12.5 μ M, 4 h). Then, They were washed with PBS, paraformaldehyde-fixed (4%, 10
13 min), washed with PBS, permeabilized with 0.1% Triton X-100 in 3% BSA (30 min), incubated
14 with anti-HK-II antibodies or anti-Cyt-c antibodies (1:200, 2 h), washed with PBS-0.5% Triton
15 X-100, and then incubated with secondary FITC-conjugated anti-rabbit antibodies (1:200, 30
16 min), washed with PBS-0.5% Triton X-100 and stained with MitoTracker Deep Red FM (45
17 min). Finally, the cells were washed with PBS, treated with antifade solution (DAPI). The
18 fluorescence measurements were conducted at excitation wavelength of 405 nm and emission
19 wavelength of 451 nm for the DAPI. ZEN software was used to process image.

20 **4.11. Measurement of the mitochondrial membrane potential.** The mitochondrial
21 membrane potential was measured by JC-1 kit according to manufacturer's instructions. Briefly,
22 the A375 cells were seeded into a 20 mm glass bottom dish at a density of 4×10^4 cells per cm^2
23 for 24 h, treated with Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-TAT peptide (12.5

1 μM) for 4 h. The cells were washed twice with cold PBS and incubated with JC-1 working
2 buffer at 37 °C for 20 min in the dark. Thereafter, the cells were washed twice with JC-1 staining
3 buffer and observed under a Zeiss LSM 710 confocal laser scanning microscope (Zeiss,
4 Germany) (magnification, x20). JC-1 monomers and JC-1 aggregates were excited at 488 nm
5 and 561 nm, respectively. Fluorescence emission were recorded at 530 nm and 590 nm for JC-1
6 monomers and JC-1 aggregates, respectively. Image processing was done using ZEN software.

7 **4.12. Protein extraction and Western blot analysis.** Cellular proteins were extracted by the
8 Column Tissue and Cell Protein Extraction Kit and the expression level of apoptosis proteins
9 was analyzed by western blot. Briefly, the A375 cells were seeded into a 100 mm dish for 24 h,
10 subjected to different treatments (12.5 μM Pal-N-Ter-TAT, pFL-N-Ter-TAT or Pal-pFL-N-Ter-
11 TAT peptide) for 24 h. The cells were washed twice with cold PBS, added denature lysate (250
12 μL) supplemented with protease inhibitor, collected into the cold purification column sleeve,
13 centrifuged at 16000 rpm for 30 s and immediately placed the collection tube on ice. Total
14 proteins were extracted successfully. The total protein content was determined by the Instant
15 BCA Protein Assay Kit. Electrophoresis with samples (20 $\mu\text{g}/\text{lane}$) was done on a 4-20% SDS
16 polyacrylamide gel. Then, they were transferred to a PVDF membrane, and were incubated
17 overnight with rabbit anti-human caspase 3, caspase 9 pAb or β -actin mAb, followed by
18 incubation for 30 min with HRP-conjugated goat anti-rabbit IgG Ab. Samples were then
19 visualized using Bio-Rad ChemiDoc MP imaging System (Bio-Rad, USA).

20

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6 **Author Contributions**

7 F. Zhang, A. Angelova, A. Zou and N. Li designed the study; F. Zhang performed the
8 experiments; F. Zhang, A. Angelova, A. Zou and N. Li analyzed the data; V. M. Garamus did
9 ellipsoids of rotation simulation and SAXS analysis; B. Angelov did molecular dynamic
10 simulation and SAXS analysis; S. Tu guided immunofluorescence and WB experiments; L.
11 Kong performed the cryo-TEM experiment; X, Zhang helped with biological experiments; F.
12 Zhang, A. Angelova, V. M. Garamus, B. Angelov, A. Zou and N. Li wrote the manuscript.

13

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20 **Notes**

21 The authors declare no conflicts of interest.

22

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5 immunofluorescence and WB results.

6

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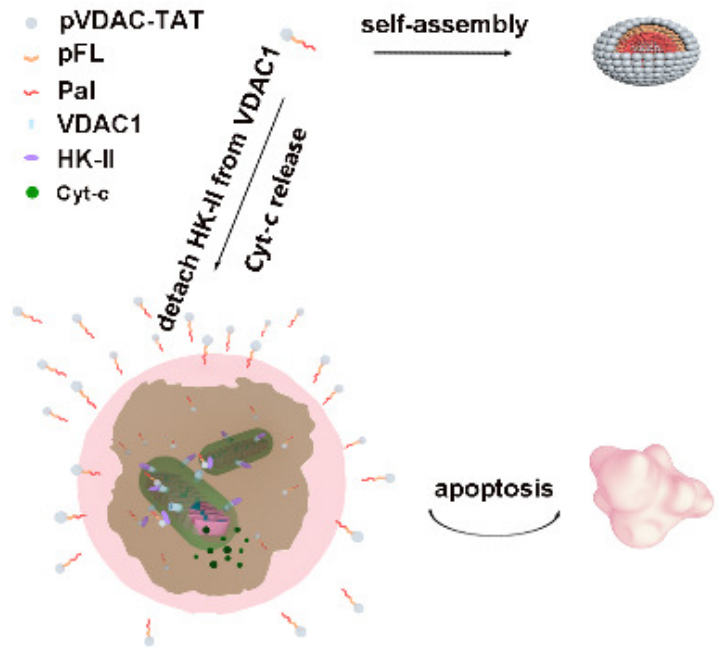
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