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Mitoparans: mitochondriotoxic cell penetrating peptides and novel inducers of apoptosis

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Introduction

The amphipathic helical peptide mastoparan (MP; *H*-INLKALAALAKKIL-NH_2) inserts into biological membranes to modulate the activity of heterotrimeric G proteins and other targets. Moreover, whilst cell free models of apoptosis demonstrate MP to facilitate mitochondrial permeability transition and release of apoptogenic cytochrome c, MP-induced death of intact cells has been attributed to its non-specific membrane destabilising properties (necrotic mechanisms). However, MP and related peptides are known to activate other signalling systems, including p42/p44 MAP kinases [1] and could therefore, also modulate cell fate and specific apoptotic events.

The ability of MP to facilitate mitochondrial permeability in cell free systems has lead to proposals that MP could be of utility in tumour therapeutics provided that it conferred features of cellular penetration and mitochondrial localization [2]. We have recently reported that our highly potent amphipathic MP analogue mitoparan (mitP; [Lys^{5,8}Aib^{10}]MP; Aib = α-aminoisobutyric acid) [3,4], specifically promotes apoptosis of human cancer cells, as was confirmed by *in situ* TUNEL staining and activation of caspase-3 [4] . Moreover, we have also demonstrated that mitP penetrates plasma membranes and redistributes to co-localize with mitochondria [4]. Complementary studies, using isolated mitochondria, further demonstrated that mitP, through co-operation with a protein of the permeability transition pore complex voltage-dependent anion channel (VDAC), induced swelling and permeabilization of mitochondria, leading to the release of the apoptogenic factor cytochrome c [4].

An expanding field of peptide and cell penetrating peptide (CPP) research has focussed on the selective targeting of tumours by engineering constructs that incorporate cell-specific or tissue–specific *address* motifs. Peptidyl *address* motifs could enhance the selectivity of drug delivery whilst the improved cellular uptake offered by CPP enhances bioavailability. Thus and as a potential therapeutic strategy, we extended our findings to design target-specific mitP analogues. The integrin-specific *address* motif RGD and a Fas ligand mimetic WEWT [5] were incorporated by N-terminal acylation of mitP to produce novel tandem-linked chimeric peptides.

Results and Discussion

Design. The ability of MP to form an amphipathic α–helix is a major determinant of its biological activity. Lysyl residues at positions 4, 11 and 12, together with the amino terminus, contribute to a cationic hydrophilic face [6]. In accordance with this observation, chimeric mitP analogues were rationally designed to maintain the amphipathicity of mitP. Z-Gly-RGD*f*-mitP conferred a cationic charge distribution that was compatible with an amphipathic α-helix. N- terminal blockade with Z-Gly and the inclusion of a D isomer of Phe(*f*), were additional modifications likely to improve the biostability of Z-Gly-RGD*f*-mitP (Table 1). The control peptide Z-Gly-RGE*f*-mitP was also included in this study; since the conservative substitution of Glu for Asp in the core RGD sequence prevents integrin binding. N-terminal extension of mitP with the Fas ligand mimetic peptide WEWT was predicted to be incompatible with the formation of an extended amphipathic α-helix. Therefore, aminohexanoic acid was used as a flexible linker in the chimeric sequence of WEWT(Ahx)mitP.

Table 1. Mitoparans: sequences and cytotoxic potencies

Peptide	Sequence	LD_{50}[a]
MP	H-INLKALAALAKKIL-NH_2	30.26 + 0.05
MitP	H-INLKK LAKL(Aib)KKIL-NH_2	6.81 + 0.05
Z-Gly-RGD *f*-mitP	Z-Gly-RGD*f*INLKKLAKL(Aib)KKIL-NH_2	1.40 + 0.13
Z-Gly-RGE *f*-mitP	Z-Gly-RGE*f*INLKKLAKL(Aib)KKIL-NH_2	4.71 + 0.05
WEWT(Ahx)mitP	H-WEWT(Ahx)INLKKLAKL(Aib)KKIL-NH_2	4.44 + 0.04

Cytotoxicity was calculated by changes in ECV304 cell viability, assessed by MTT conversion. [a]LD_{50} values are expressed in μM as mean ± S.E.M. from 3 separate experiments

Cytotoxicity profiles. MTT conversion was used to evaluate the cytotoxic potencies of our chimeric mitP analogues. Table 1 shows LD_{50} values of all peptides tested using ECV304 bladder cancer carcinoma cells. All chimeric mitP analogues produced enhanced cytotoxic potencies compared to mitP alone and most dramatically so with the RGD containing chimeric peptide. Previous reports [2] that advocate MP as a potential anti-tumour therapeutic by delivery of the peptide in a target-selective cell penetrant liposomal complex, have shown such constructs to be effective at concentrations of 25μM. In contrast, we report that mitP is a highly potent

mitochondriotoxic CPP and a structure that easily incorporates into a tissue specific construct without impacting on the bioactivity of the parent cytotoxin. Moreover, the enhanced potency of Z-Gly-RGD*f*-mitP of 1.4μM is a concentration readily achievable *in vivo*.

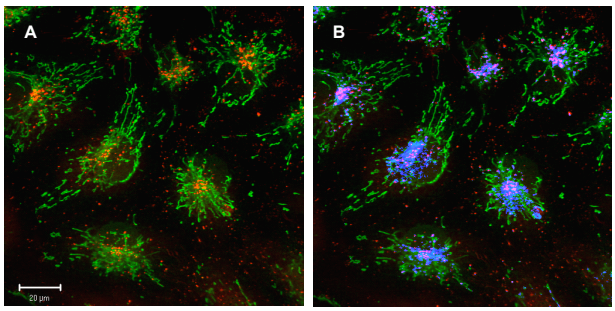

Fig. 1. Co-localization of mitP with mitochondria

ECV304 cells were treated with 5μM rhodamine-labelled mitP and 200nM Mitotracker Green (Molecular Probes, Invitrogen) and viewed by confocal live cell imaging (A). Co-localizing pixels are shown here in blue (B)

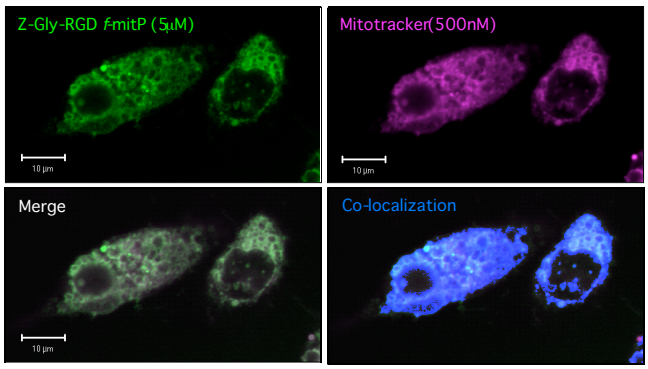

Fig. 2. Z-Gly-RGDf-mitP co-localizes with mitochondria

ECV304 cells were treated with 5μM fluorescein-labelled Z-Gly-RGD*f*-mitP and 500nM Mitotracker Deep Red (Molecular Probes, Invitrogen) and viewed by confocal live cell imaging.

Mechanisms. The sequence variant Z-Gly-RGE*f*-mitP, demonstrated a decreased potency to that of Z-Gly-RGD*f*-mitP (Table 1) and suggests that our chimeric analogue is indeed binding to integrin receptors. Further investigations were performed to ascertain whether our novel-tandem linked chimerae shared the same cell penetrant and mitochondriotoxic properties as mitP. Fig. 1 indicates that following 1 hour, mitP (5μM) assumes a distinct co-localization with mitochondrial membranes in ECV304 cells. Similarly, live confocal cell imaging was also used to determine the intracellular distribution of our chimeric analogues. Fig. 2 clearly shows that Z-Gly-RGD*f*-mitP (5μM) assumes a predominant mitochondrial co-localization in ECV304 cells following 1 hour exposure to the peptide. Finally and in accordance with our previous observations for mitP [4], our chimeric analogues induced cell death by apoptotic mechanisms as evidenced by *in situ* TUNEL staining (Fig.3).

We conclude that, our target-selective chimeric mitP analogues provide utility for the therapeutic induction of apoptosis.

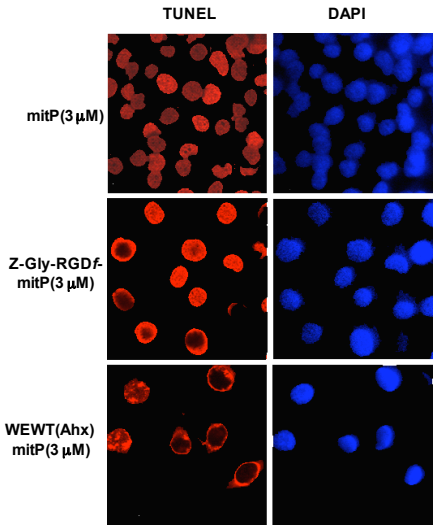

Fig. 3. Chimeric mit-P analogues induce apoptosis

Inter-nucleosomal DNA fragmentation, a feature of apoptosis was detected by TUNEL *in situ* cell detection assay (Roche). Cells were counterstained with DAPI to visualize nuclear DNA. The presence of TMR red fluorescence located in the nuclei is indicative of apoptosis.

Acknowledgments

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