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Electrical potentiation of the membrane permeabilization by new peptides with anticancer properties

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ABSTRACT

New polycationic peptides were designed on the basis of 16-mer and 14-mer fragments of the peptide BTM-P1, derived from the Cry11Bb protoxin. The peptides caused mitochondrial, but not red blood cell membrane permeabilization. Conjugation of the cell penetrating hepta-arginine vector to their N- or C-termini through two glycine residues resulted in more active peptides, which also permeabilized the red blood cells with a relatively high plasma membrane potential generated in the presence of valinomycin. The efficiency of the peptides was remarkably higher in the lower ionic strength media. The capability of the plasma membrane permeabilization of the normal red blood cells by the designed conjugated peptides and by known anticancer peptide R7-KLA was also strongly potentiated by the external electrical pulses applied to the cell suspension. These results open the new avenues of the local destruction of solid tumors using the combined "peptide-–electrical pulses" synergistic treatment. The designed peptides were active against the human leukemia Jurkat cells but not against the normal wild type CHO cells.

1. Introduction

Many natural polycationic amphipathic peptides with antimicrobial activity have also been found to kill mammalian cancer cells [1–7]. Similarly, the artificial antimicrobial polycationic peptides enriched in KLA sequences [8,9] have been shown to induce apoptosis in cultured cancer cells and solid tumors [10–12].

The anticancer activity of the 14-residue peptide $(KLAKLAK)_2$ has been significantly enhanced by its conjugation to a cancer-cell binding peptide LTVSPWY [13], to a protein transduction domain PTD-5 [11,14], to a "tumor-homing" cyclic peptide CNGRC or double-cyclic peptide ACDCRGDCFC [10], to a peptide CPIEDRPMC specifically detecting HT29 colon carcinoma cells [15], to monoclonal antibodies recognizing malignant hematopoietic cells [16], to an antibody of the prostate-specific membrane antigen [17], or to the hepta-arginine cell penetrating peptide [12].

The peptide RRRRRRR-GG-KLAKLAKKLAKLAK (named as R7-KLA) [12], formed by conjugation of the peptide $(KLAKLAK)_2$ to the cell penetrating hepta-arginine vector (R7) [18,19] through two additional unstructured glycine residues, has demonstrated the activity against cancer cells that was two orders of magnitude higher than that of the un-conjugated KLA peptide [12]. It has been assumed that the R7 fragment increased cell delivery of the fragment $(KLAKLAK)_2$ to specifically target and damage mitochondria [12], because it has been demonstrated that 10 μM $(KLAKLAK)_2$ decreased light dispersion in the suspension of isolated rat liver mitochondria causing their swelling [10].

Further studies of the mechanism of action of R7-KLA peptide and of its D-amino acid analog r7-kla, designed and synthesized in the Massachusetts General Hospital of the Harvard Medical School, have demonstrated that R7 fragments of these peptides increased not only cell delivery of the peptides, as demonstrated in [12], but also remarkably enhanced their capacity to directly permeabilize isolated mitochondria [20]. Most importantly, a significant increase in the permeabilization of the red blood cells (RBCs) by the peptides R7-KLA or r7-kla was observed when a high plasma membrane potential (minus inside) was artificially increased in the presence of valinomycin [20].

This phenomenon of potential-dependent permeabilization of mitochondrial and plasma membranes has been earlier observed for the peptide BTM-P1 [20–23] derived from the Cry11Bb protoxin. This peptide has demonstrated more than one order of magnitude higher membrane permeabilizing activity than R7-KLA or r7-kla [20]. It has been observed that BTM-P1 at the concentration of 0.36 μM induced fast oxidation of endogenous NAD(P)H, known to depend on the inner membrane potential, and caused swelling of isolated mitochondria [20]. Meanwhile, the peptide $(klaklak)_2$, a fragment of the peptide r7-kla [12], was inactive even at the concentration of 3.6 μM [20]. These observations allowed us to hypothesize [24] that new anticancer polycationic peptides might be designed on the basis of even shorter fragments of highly active BTM-P1. Such Cry toxin fragments,

Abbreviations: RBCs, red blood cells; DNP, 2,4-dintrophenol; P7-27, IYLATALAKWALKQGF; P7-26, IYLATALAKWALKQ; P7-4, RRRRRRR-GG-IYLATALAKWALKQGF; P7-5, IYLATALAKWALKQGF-GG-RRRRRRR; P7-6, RRRRRRR-GG-IYLATALAKWALKQ; P7-7, IYLATALAKWALKQ-GG-RRRRRRR; R7-KLA, RRRRRRRGGKLAKLAKKLAKLAK; KLA-R7, KLAKLAKKLAKLAK-GG-RRRRRRR

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of natural or slightly modified sequences, might be used instead of the artificial peptides to conjugate them to cell penetrating vectors or to "tumor-homing" fragments with the aim to obtain new peptides with selective anticancer activity.

In the present work, we designed four polycationic peptides. Two peptides were designed on the basis of the 16-mer fragment KYLAT ALAKWALKQGF and the other two were designed on the basis of the 14-mer fragment KYLATALAKWALKQ of BTM-P1. These fragments were slightly modified by replacing the first N-terminus lysine residue to isoleucine. The cell penetrating vector R7 was conjugated to each of these modified fragments at their N- or C-termini through two glycine residues, similar to that described by Law et al. for the peptide R7-KLA [12]. The peptides demonstrated toxicity for human leukemia Jurkat cells that was higher than that observed for the peptides R7-KLA or KLA-R7. No measurable toxicity was detected in the case of the normal wild type CHO cells. The designed peptides permeabilized isolated rat liver mitochondria but did not induce hemolysis of the normal RBCs. Remarkable permeabilization of the cell plasma membrane, due to the formation of peptide pores, was observed when a relatively high plasma membrane potential was artificially generated in the presence of valinomycin, and this effect was even higher in the low ionic strength media. The capability of permeabilization of the normal RBCs by the peptides was also strongly potentiated by the application of external electrical pulses. The electrical pulses alone did not cause a significant permeabilizing effect. The obtained results open the possibility of development of novel "peptide–electrical pulses" synergistic technologies to locally damage cancer tissues.

2. Materials and methods

2.1. Materials

The peptides were designed in our laboratory on the basis of two relatively short fragments KYLATALAKWALKQGF and KYLATALAKWALKQ of BTM-P1 derived earlier from the Cry11Bb protoxin [21,22]. The first lysine residue of these fragments was replaced by the hydrophobic isoleucine residue and the R7 cell-delivery vector was conjugated to each of these fragments at the N- or C-terminus through two glycine residues (Table 1). All peptides were synthesized by GenScript Corporation (NJ, USA) according to our orders. For comparison, the known anticancer peptide R7-KLA [12] and its C-terminus-R7 version KLA-R7 (Table 1) were synthesized by the same company. The chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and valinomycin was purchased from Merck.

2.2. Isolation of rat liver mitochondria

Mitochondria from liver of male white rats (starved overnight) were isolated by the method of differential centrifugation as described earlier [25], following the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Local Ethics Committee of the National University of Colombia, Medellin Branch. The cooled liver was homogenized in medium containing 210 mM mannitol, 70 mM sucrose, 2.5 mM $MgCl_2$, 1 mM EGTA–KOH, 0.3 mg/ml bovine serum albumin (BSA, free fatty acid fraction V), and 10 mM HEPES–KOH, pH 7.2, at 0–4 °C. The homogenate was centrifuged at 600 ×*g* for 10 min at 0–4 °C and the obtained supernatant was centrifuged at 10 000 ×*g* for 10 min. The pellet was resuspended in the medium containing 210 mM mannitol, 70 mM sucrose, 50 μM EGTA–KOH, 0.3 mg/ml BSA and 10 mM HEPES–KOH, pH 7.2, and mitochondria were sedimented again at 10 000 ×*g* for 10 min. This stage of mitochondrial washing was repeated and mitochondria were finally resuspended in 1 ml of the same medium without BSA. The mitochondria obtained with this procedure were characterized by the respiratory control ratio higher than 4.5 using 5 mM succinate as substrate of oxidation.

Table 1
Polycationic peptides used in the work.

Name	Amino acid sequence and peptide purity
P7-27	IYLATALAKWALKQGF (93%)
P7-26	IYLATALAKWALKQ (98%)
P7-4	RRRRRRR-GG-IYLATALAKWALKQGF (97%)
P7-5	IYLATALAKWALKQGF-GG-RRRRRRR (95%)
P7-6	RRRRRRR-GG-IYLATALAKWALKQ (94%)
P7-7	IYLATALAKWALKQ-GG-RRRRRRR (99%)
R7-KLA	RRRRRRR-GG-KLAKLAKKLAKLAK (93%)
KLA-R7	KLAKLAKKLAKLAK-GG- RRRRRRR (95%)

2.3. Isolation of red blood cells

RBCs were isolated from the blood of white rats as described in [26] with slight modifications. Approximately 20% blood in medium composed of 120 mM NaCl, 10 mM EDTA, 5 mM sodium citrate, and 5 mM Tris–HCl, pH 7.4, was centrifuged at 3000 rpm for 10 min (Jouan centrifuge RM1812). The cells were washed three times in medium of 150 mM NaCl, 5 mM Tris–HCl, pH 7.4, and finally resuspended in the same medium supplemented with 10 mM glucose to the final hematocrit of 20%.

2.4. Monitoring of the redox state of mitochondrial pyridine nucleotides

The level of reduced forms of mitochondrial pyridine nucleotides, NAD(P)H, was monitored by fluorescence at 450 nm using the Aminco-Bowman Series 2 Luminescence Spectrometer as described earlier [27]. Mitochondria, at the final concentration of 0.5 mg protein/ml, were added to the incubation medium composed of 100 mM sucrose, 75 mM KCl, 10 mM potassium phosphate, 50 μM EGTA, and 5 mM HEPES, pH 7.2 (SKPH medium). Where indicated, 5 mM succinate was added to energize the mitochondria. The peptides were added at the final concentrations of 2 μM or 5 μM, thus observing NAD(P)H oxidation induced by the inner membrane permeabilization.

2.5. Measuring of the ATPase activity of mitochondria

The ATPase activity of the mitochondria was registered as a pH change resulting of protonic dissociation of the liberated inorganic phosphate $H_2PO_4^{1-}$ in the low buffered incubation medium composed of 100 mM sucrose, 75 mM KCl, 4 mM HEPES–KOH, pH 7.4 (SKH medium). The pH trace was registered using the equipment composed of a pH electrode, the ML165 pH Amp amplifier and the ML866 PowerLab 4/30 high-performance data acquisition system (ADInstruments) connected to the computer through the USB port. Mitochondria (0.5 mg protein/ml) were added to the SKH medium supplemented with 1 mM ATP. In some experiments, the SKH medium was also supplemented with 1 mM $MgCl_2$. To uncouple the mitochondria, and thus to accelerate ATP hydrolysis, 2,4-dintrophenol (DNP) was added to the final concentration of 75 μM. DNP is a weak acid known to cross biomembranes in protonated and un-protonated forms thus strongly increasing membrane permeability to protons and abolishing the proton electrochemical gradient across the inner mitochondrial membrane that allows H^+-ATPase functioning with the maximal rate. At the end of pH registration, the pH value was shifted to an initial state by appropriate addition of KOH, and 0.2 mM KH_2PO_4 was added to demonstrate acidification of the suspension resulting of protonic dissociation of the appeared $H_2PO_4^{1-}$ anions. The peptides were added to final concentrations of 2 μM, 3 μM or 5 μM.