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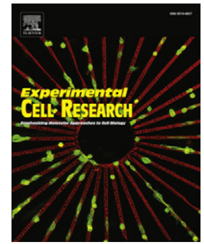

Research Article

The elimination of P-glycoprotein over-expressing cancer cells by antimicrobial cationic peptide NK-2: The unique way of multi-drug resistance modulation

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ABSTRACT

Most chemotherapeutics harm normal cells causing severe side effects and induce the development of resistance in cancer cells. Antimicrobial peptides (AMPs), recognized as anti-cancer agents, may overcome these limitations. The most studied mechanism underlying multi-drug resistance (MDR) is the over-expression of cell membrane transporter P-glycoprotein (P-gp), which extrudes a variety of hydrophobic drugs. Additionally, P-gp contributes to cell membrane composition and increases the net negative charge on cell surface. We postulated that NK-lysin derived cationic peptide NK-2 might discriminate and preferentially eliminate P-gp over-expressing cancer cells. To test this hypothesis, we employed MDR non-small cell lung carcinoma (NCI-H460/R) and colorectal carcinoma (DLD1-TxR) cell lines with high P-gp expression. MDR cancer cells that survived NK-2 treatment had decreased P-gp expression and were more susceptible to doxorubicin. We found that NK-2 more readily eliminated P-gp high-expressing cells. Acting in 'carpet-like' manner NK-2 co-localized with P-gp on the MDR cancer cell membrane. The inhibition of P-gp reduced the NK-2 effect in MDR cancer cells and, vice versa, NK-2 decreased P-gp transport activity. In conclusion, NK-2 could modulate MDR in unique way, eliminating the P-gp high-expressing cells from heterogeneous cancers and making them more vulnerable to classical drug treatment.

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Introduction

Current anti-cancer chemotherapies are heterogeneous in their mode of action, and most of them harm human normal cells causing, consequently, severe side effects [1]. In addition, cancer cells usually evade immune surveillance making therapeutic manipulation harder [2]. In order to act against and kill cancer cells, chemotherapeutics need to penetrate the cell and reach

Abbreviations: NK-2, NK-lysin derived peptide; NK11, inactive form of NK-2; PS, phosphatidylserine; MDR, multi-drug resistance; P-gp, P-glycoprotein; AMP, antimicrobial peptide; Dex-VER, R+Verapamil; TQ, tariquidar; PI, propidium iodide; MTT, 3-(4,5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium bromide; FITC, fluorescein isothiocyanate; Rho123, Rhodamine 123

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specific molecular targets. In many cases, cancer cells develop the resistance in response to treatment by effluxing the drugs using membrane transporter proteins [3]. These are the main limitations for the efficient chemotherapy.

Antimicrobial peptides (AMPs), also referred to as innate immunity peptides, seem to overcome these limitations via a unique mechanism of cancer cell killing that involves membrane lysis [4]. They are gene-encoded peptides, which normally provide a first line of defense against invading microbes on epithelial tissues. Interaction of AMPs with target cell membranes eventually leads to the disturbance or destruction of membrane potential and its barrier function [5]. Most AMPs specifically target bacteria and are almost non-toxic for host cells. Moreover, it has been shown that some cancer cells are killed by certain AMPs, whereas normal cells, such as blood lymphocytes, endothelial cells and fibroblasts were not affected [6–8]. This differential sensitivity is driven by electrostatic interactions with the negatively charged lipids of the bacterial membranes [9]. The human cell plasma membrane surface consists of the zwitterionic choline phospholipids and sphingomyelin and almost lacks anionic phospholipids, while the bacterial cytoplasmic membrane is characterized by a significant amount of negatively charged phospholipids, mainly phosphatidylglycerol [10,11]. Negatively charged phosphatidylserine (PS), a constituent of the inner layer of human cytoplasmic membranes, can be translocated to the outer leaflet during the loss of membrane asymmetry [12]. Surface exposed PS then serves as a marker for the clearance of pathological or aged erythrocytes and apoptotic cells from the bloodstream by monocytes and macrophages [13–15].

Though it is not well recognized, it has been shown that various cancer cells have elevated surface levels of negatively charged phospholipids, i.e., PS [16–19]. It has been suggested that surface-exposed PS makes these cells susceptible to the cationic, membranolytic peptides [18]. There are evidences that P-glycoprotein (P-gp) expressed on plasma membrane of many cells (normal and pathologically changed) and involved as drug-efflux transporter in the development of multi-drug resistance (MDR) in cancer [20], also transports endogenous PS [21]. The increase of negatively charged PS on the surface of MDR cells, due to P-gp activity, could make these cells more vulnerable to AMPs than other cancer cells that possess less P-gp molecules.

In the present study, we tested this hypothesis regarding the anticancer effects of peptide NK-2, which is an internal fragment of porcine NK-lysin consisting of 27 amino acid residues with an overall positive net charge and which adopts an amphipathic, α-helical secondary structure upon membrane interaction [22]. There are studies that support the ‘carpet mechanism’ for membrane lysis by this group of peptides [23,24]. NK-2 is similar in size and structure to the bee venom melittin that is toxic to bacteria and both mammalian cancer and non-cancer cells [25]. However, NK-2 is characterized by different degrees of target selectivity and hydrophobicity in comparison to melittin. The selectivity of NK-2 could be assigned to differences in the membrane phospholipid composition of the target cells [22]. We used MDR non-small cell lung carcinoma (NCI-H460/R) and colorectal carcinoma (DLD1-TxR) cell lines as target cells, established in our laboratory from their sensitive counterparts [26,27].

Many authors speculate that AMPs are not affected by MDR [28–30] and that they can be useful in overcoming the resistance mechanism [31–33]. To our knowledge, herein we present an exclusive study on this subject that has not been conducted so far.

Materials and methods

Drugs

R+Verapamil (Dex-VER) was purchased from Sigma-Aldrich Chemie Gmbh, Germany. Tariquidar (TQ) was a generous gift from Dr. Sven Rottenberg from The Netherlands Cancer Institute. Doxorubicin (DOX) solution was obtained from EBEWE Arzneimittel GmbH, Vienna, Austria. Dex-VER was diluted in sterile water to a concentration of 1 mM and kept at room temperature. TQ was diluted in DMSO and 10 μM aliquots were thawed from −20 °C before use. DOX was diluted in sterile water and 1 mM aliquots were thawed from −20 °C before use.

Chemicals

RPMI 1640 medium, DMEM, penicillin–streptomycin solution, antibiotic–antimycotic solution, L-glutamine, Fetal bovine serum (FBS) and trypsin/EDTA were purchased from PAA, Vienna, Austria., MTT, rhodamine 123 (Rho123) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemie GmbH, Germany. Annexin-V-FITC (AV) was purchased from Abcam, Cambridge, UK. SYTOX® Green and DAPI Nucleic Acid Stains were obtained from Invitrogen Life Technologies, CA, USA. Propidium iodide (PI) was obtained from R&D Systems GmbH, Wiesbaden, Germany.

Culture and harvesting of human cell lines

NCI-H460 and DLD1 were purchased from American Type Culture Collection (Rockville, MD, USA). HaCaT cell line (normal human keratinocytes) was obtained from CLS – Cell Lines Service, Eppelheim, Germany. NCI-H460 and DLD1 cells were maintained in RPMI 1640 containing 10% heat inactivated FBS, 2 mM L-glutamine, 4.5 g/l glucose, 10,000 U/ml penicillin, 10 mg/ml streptomycin, 25 μg/ml amphotericin B solution at 37 °C in a humidified 5% CO_2 atmosphere while HaCaT cell line was cultured in DMEM supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 4.5 g/l glucose, 10,000 U/ml penicillin and 10 mg/ml streptomycin at 37 °C in a humidified atmosphere at 5% CO_2. NCI-H460/R cells were originally selected from NCI-H460 cells and cultured in a medium containing 100 nM DOX [26]. DLD1-TxR cells were selected from DLD1 cells after continuous exposure to stepwise increasing concentrations of paclitaxel (PTX, 60–600 nM) [27]. All three cell lines were sub-cultured at 72 h intervals using 0.25% trypsin/EDTA and seeded into a fresh medium at the following densities: 8000 cells/cm^2 for NCI-H460 and DLD1 cell lines, 16,000 cells/cm^2 for NCI-H460/R and DLD1-TxR cell lines, and 32,000 cells/cm^2 for HaCaT cell line.

Peptides

NK-2 (KILRG VCKKI MRTFL RRISK DILTG KK), NK11 (KISKR ILTGK K), melittin (GIGAV LKVLT TGLPA LISWI KRKRQ Q), as well as the fluorescent analogs of NK-2 and NK11were synthesized with an amidated C terminus by the Fmoc solid-phase peptide synthesis technique on an automatic peptide synthesizer (model 433A; Applied

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