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Targeting lipodisks enable selective delivery of anticancer peptides to tumor cells

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Abstract

Issues concerning non-specificity, degradation and hemolysis severely hamper the development of membranolytic amphiphilic peptides into safe and efficient anticancer agents. To increase the therapeutic potential, we have previously developed a strategy based on formulation of the peptides in biocompatible nanosized lipodisks. Studies using melittin as model peptide show that the proteolytic degradation and hemolytic effect of the peptide are substantially reduced upon loading in lipodisks. Here, we explored the possibilities to increase the specificity and boost the cytotoxicity of melittin to tumor cells by use of targeting lipodisk. We demonstrate that small (~20 nm) EGF-targeted lipodisks can be produced and loaded with substantial amounts of peptide (lipid/peptide molar ratio >7) by means of a simple and straightforward preparation protocol. *In vitro* cell studies confirm specific binding of the peptide-loaded disks to tumor cells and suggest that cellular internalization of the disks results in a significantly improved cell-killing effect.

Key words: Nanocarrier; Melittin; PEG-stabilized lipid disk; EGFR; Selective targeting

Membranolytic peptides, such as cationic amphiphilic peptides (CAPs) derived from, or inspired by, natural antimicrobial peptides, have received considerable attention as potential anticancer agents.[1] The high interest in these peptides originates mainly from their wide-spectrum antitumor activity and their nonspecific mode of action. Upon association with cell membranes the peptides destabilize the phospholipid membrane through mechanisms that ultimately lead to pore formation and loss of membrane integrity. This detergent-like action of the peptides is coupled to a reduced likelihood of resistance development.

Melittin, a 26 amino acid CAP derived from bee venom, belongs to the group of membranolytic peptides with well-established anticancer effect.[2] Although identified as a promising cancer therapeutic agent, melittin's *in vivo* application is hampered by its low serum stability, significant off-target toxicity (*e.g.*, hemolysis), and insufficient selectivity to tumor cells. Formulation of melittin in biocompatible nanocarriers has been suggested as a viable means to overcome these issues. Attempts along these lines include loading of melittin in phospholipid stabilized perfluorocarbon emulsion particles,[3] and complexation of melittin with a thiolated zwitterionic glycol chitosan to form polymer-based nanoparticles.[4] While both these approaches have been shown to significantly attenuate melittin's hemolytic effect, the comparatively large size (>200 nm) of the nanocarriers may prevent them from penetrating deep into solid tumors.[5] Considerably smaller (~20 nm) particles have been produced by linking melittin to an apolipoprotein mimetic peptide and subsequently utilizing the hybrid melittin for stabilization of well-defined emulsion particles.[6] Merging CAPs with synthetic peptides may, however, affect their antitumor activity. Moreover, since several studies point towards synergistic effects of melittin with conventional chemotherapeutic agents,[7] it is noteworthy that none of the above-mentioned nanocarriers allow for straightforward encapsulation of such drugs.

We have chosen an alternative formulation approach with potential to successfully combine the advantageous

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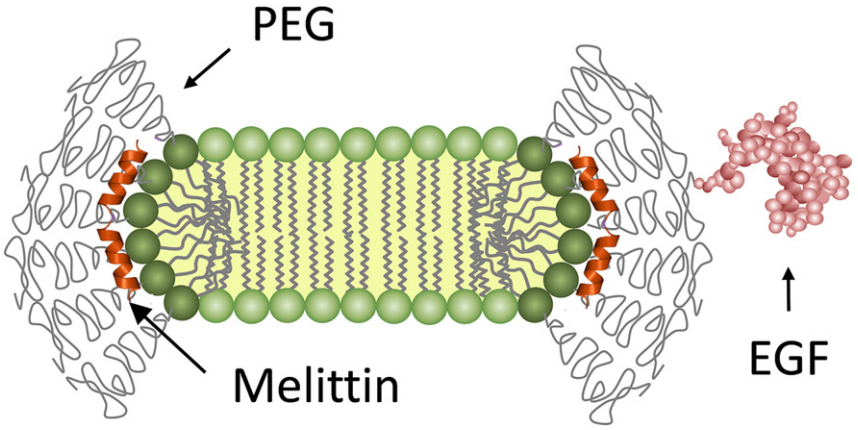

Figure 1. The lipodisk consist of a flat, circular lipid bilayer surrounded by a highly curved rim composed of PEGylated lipids. Targeting agents can be conjugated to distal end of the PEG-polymers. Melittin binds with high affinity to the lipodisk edge.

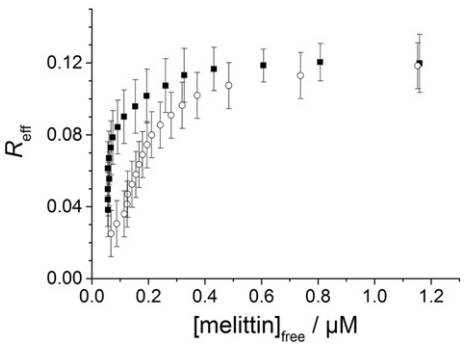

Figure 2. Association isotherms describing the binding of melittin to EGF-targeting (○) and non-targeting (■) lipodisks.

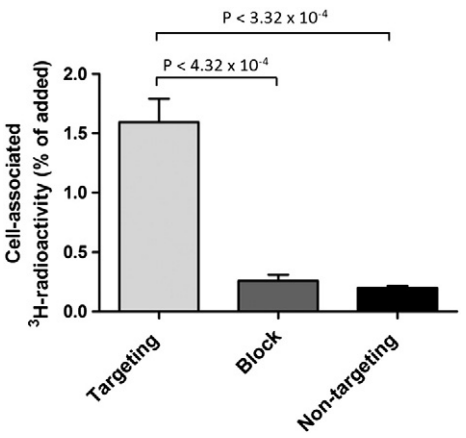

Figure 3. Cellular binding specificity of ^{3}H-labeled melittin-loaded lipodisks (3 μM lipid, 0.36 μM melittin) to A-431 cells. Two groups of cells were incubated for 2 h with EGF-targeting lipodisks at 37 °C. One group of cells was pre-incubated with 100 nM EGF to block the EGF receptors. A third group of cells was incubated 2 h at 37 °C with non-targeting lipodisks. Data presented as mean ± range, n = 3.

characteristics of the above strategies, while offering greater opportunities for functionalization and adaption of the nanocarriers. Previous studies have confirmed that melittin, and several related CAPs, bind with high affinity to the edge of PEG-stabilized lipodisks (Figure 1).[8–10] When formulated in lipodisks melittin is well protected from proteolytic degradation.[11,12] Further, as confirmed by *in vitro* and *in vivo* studies, its antibacterial and anticancer activity is preserved while the hemolytic effect is substantially reduced.[11,12] Recent studies demonstrate moreover that the lipodisk hydrophobic core offers means for synergistic loading of conventional chemotherapeutics.[13,14] The lipodisks have long blood circulation time and have been shown to spontaneously accumulate in subcutaneous xenografts.[12,13] These properties, together with the fact that their size and composition can be easily tailored for different applications, make lipodisks well suited as carriers for delivery of melittin to solid tumors.

The membranolytic action of CAPs is not limited to the cell membrane but can be extended also to the membranes of organelles. In case of mellitin, an important part of the cytotoxic activity originates from the peptide's ability to permeabilize mitochondria and thereby induce the release of several pro-apoptopic factors.[15] Thus, it can be anticipated that nanocarriers capable of mediating intracellular delivery of mellitin would be advantageous. In many cases, such as when the aim is to co-deliver a chemotherapeutic drug or an imaging agent, strategies that allow for internalization of the entire nanocarrier would moreover be preferable. We have in previous studies shown that epidermal growth factor (EGF)-conjugated lipodisks bind selectively to EGF receptor expressing tumor cells,[14,16] and, importantly, that the binding results in cellular internalization of the disks.[14] We hypothesized that it could prove beneficial to utilize EGF-targeting lipodisks for the formulation and delivery of melittin.

Methods

Lipodisks consisting of distearoyl phosphatidylcholine (DSPC) and PEG-linked distearoyl phosphatidylethanolamine (PEG-DSPE), molar ratio 8:2, were prepared by BioBead-assisted detergent depletion from micellar octyl glucoside solutions. In case of EGF-targeting lipodisks ~2.5% of the PEG-lipids were replaced by EGF-conjugated PEG-lipids. The lipodisks were characterized by cryo-transmission electron