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Anticancer Peptide SVS-1: Efficacy Precedes Membrane Neutralization

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Supporting Information

ABSTRACT: Anticancer peptides are polycationic amphiphiles capable of preferentially killing a wide spectrum of cancer cells relative to noncancerous cells. Their primary mode of action is an interaction with the cell membrane and subsequent activation of lytic effects; however, the exact mechanism responsible for this mode of action remains controversial. Using zeta potential analyses we demonstrate the interaction of a small anticancer peptide with membrane model systems and cancer cells. Electrostatic interactions have a pivotal role in the cell killing process, and in contrast to the antimicrobial peptides action cell death occurs without achieving full neutralization of the membrane charge.

Cancer is one of the leading causes of death globally, and as a result, much effort has been devoted to designing new molecules as potential therapeutics and molecular tools to further our understanding of the disease.[1,2] Although most effort has centered on small molecules and biologics, such as antibodies, recent reports indicate that a distinct class of peptides show anticancer activity.[3,4]

Antimicrobial peptides (AMPs) are produced by a variety of organisms as a defense mechanism against pathogens,[5] and although structurally diverse, most fold into amphiphilic structures due to their short size (<50 amino acid residues), positive charge, and high proportion of hydrophobic residues.[5,6] These peptides kill bacteria by associating with their negatively charged cell surface and subsequently disrupt their cell membranes via mechanisms that involve membrane thinning, formation of transient pores, or disruption of their lipid matrix, which impairs the membranes' barrier function.[5,7] Some AMPs pass through the lipid bilayer of the membrane to act on intracellular targets.[5] Because of their membrane perturbing activity, the bactericidal and fungicidal activity of AMPs has been explored. More recently, certain AMPs have been shown to be cytotoxic toward a wide spectrum of human cancer cells[8,9] and some have been shown to be tumoricidal.[10]

Cancer cells are characterized by a loss of lipid asymmetry that results in accumulation of up to 9% of negatively charged phosphatidylserine (PS) on the outer leaflet of their membrane.[1,5,9] In addition, changes in the glycosylation pattern of glycoproteins result in a high level of sialic acid on the cell's surface.[1,9] As a result, the surface of many types of cancer cells are negatively charged, a characteristic shared by bacterial cells.[8,9] Because of this similarity, many researchers speculate that the mechanism(s) of action for anticancer peptides (ACPs) might be similar to those of AMPs. However, only a subset of AMPs display anticancer activity, and there is active controversy as to whether the mechanism of action for AMPs and ACPs is truly the same.[9] Reports indicate that the killing mechanism of ACPs is preferentially a membrane-lytic effect; however, other mechanisms such as apoptosis triggering and angiogenesis inhibition might be involved.[5,8] Nevertheless, the rapid killing caused by ACPs implies that a nonreceptor mediated membranolytic killing mechanism is dominant.[11,12] Here we study the interaction of a de novo designed ACP with model membranes as well as cancer cell membranes to further our mechanistic understanding for this class of peptides. In particular, we investigate the role that surface electrostatics play in initial engagement of the peptide to the membrane surface.

SVS-1 ($KVKVKVKV^{D}P^{L}PTKVKVKVK\text{-}NH_2$) is an 18-residue cationic peptide (formal charge at neutral pH of +9) designed to adopt an ensemble of random coil conformations which are bioinactive when dissolved in solution. However, when SVS-1 encounters a negatively charged surface, it binds to the surface and folds into a bioactive amphiphilic β-hairpin structure capable of disrupting the lipid bilayer.[13] Peptide folding results from the formation of electrostatic interactions between the peptide's lysine side chains and the negatively charged components of the lipid membrane as the unfolded peptide engages the surface. Because SVS-1 only folds at negatively charged surfaces, it is capable of discriminating between cancer and noncancerous cells, whose membrane outer leaflets are largely zwitterionic. This behavior makes SVS-1 a good model ACP to investigate the role that electrostatics play in their mode of action.

We started by analyzing the effect of the SVS-1 peptide on the zeta potential values of model biomembrane systems composed of different phospholipids that mimic noncancerous and cancer cell membranes: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-

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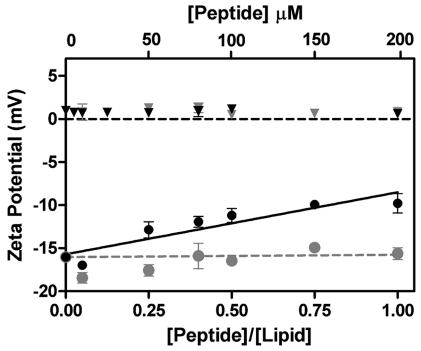

Figure 1. Zeta potential measurements of 200 μM POPC and POPC/POPS 40:60 mixture after 30 min incubation and stabilization with SVS-1 and SVS-2 at 37 °C. Triangles POPC, circles POPC/POPS 40:60, black symbols SVS-1, and gray symbols SVS-2. Error bars represent the standard deviation of at least two independent experiments.

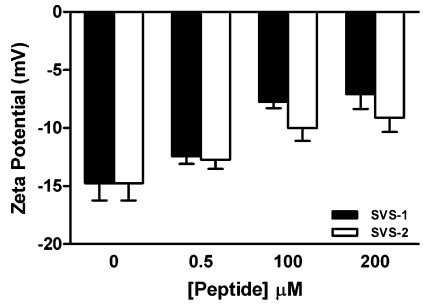

Figure 2. Zeta potential of A549 (lung carcinoma) cells in the presence of SVS-1 and SVS-2. At 2.6 × 10^5 cells/mL cells were incubated and stabilized for 30 min with different peptide concentrations and the potential was measured at 37 °C. Error bars represent the standard deviation of at least two independent measurements.

phosphocholine (POPC) and mixtures of POPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) 40:60 respectively. Zeta potential values report on the electrostatic potential existing at the shear plane of a particle[14,15] and have been used to describe cellular phenomena such as adhesion and agglutination.[15] Importantly, zeta potentials can report on the surface electrostatics of intact cells as a function of environmental conditions[16] and thus be used to assess the interactions of peptides with cell surfaces that result in a change of cell surface electropotential.[17] Here, we measure changes in zeta potential for neutral and negatively charged large unilamellar vesicles (LUVs) as well as A549 lung carcinoma cells as a function of added peptide. In addition to SVS-1, a control peptide was studied. SVS-2 (KVKVKVKV^{L}P^{L}PTKVKVKVK-NH_2) is similar in sequence to SVS-1 but contains a key stereochemical change. The D-proline at position 10 of SVS-1 was changed to L-proline. This change renders SVS-2 incapable of folding, even in the presence of a negatively charged surface. Unlike SVS-1, SVS-2 is unable to form a rich valine hydrophobic face and a lysine-rich face responsible for membrane engagement. SVS-2 was found to be noncytotoxic to cancer cells. Including SVS-2 in this study allows one to assess the importance of the β-hairpin folding event to the activity of SVS-1.[13]

Figure 1 shows the zeta potential values of POPC and POPC/POPS 40:60 LUVs titrated with SVS-1 and SVS-2.

In these measurements, peptide aliquots in BTP buffer were added to 200 μM LUVs suspensions. The addition of SVS-1 to POPC vesicles caused no significant change on the potential values, suggesting a weak or noninteraction with the zwitterionic phospholipid. On the contrary, the potential values of POPC/POPS LUVs are significantly dependent on added SVS-1 and become less negative as the concentration of SVS-1 increases. An overall change from −16.03 ± 0.38 mV to −9.79 ± 1.12 mV is realized after the addition of 200 μM SVS-1. This suggests that the positively charged SVS-1 is interacting with the negatively charged LUVs inducing a change in the membrane surface charge either by direct peptide binding and/or insertion in the membrane. However, the progressive neutralization of the LUVs suggests that the phospholipids' negatively charged polar head groups are being neutralized. This observation supports a mechanism that involves initial peptide engagement of the headgroup region prior to downstream lytic events. Concerning SVS-2, there was very little dependence of the zeta potential as a function of added peptide irrespective of the LUVs type. This is somewhat surprising given the fact that SVS-2, although incapable of folding at the electronegative surface, still contains nine positively charged functional groups in its sequence (eight lysines and an N-terminal amine). Even though not bioactive, one would expect this peptide to at least bind to the surface of the negatively charged LUVs. The fact that it does not underpins the importance of obtaining the folded state of the hairpin at the membrane surface. In the folded state, the peptide is capable of displaying its electropositive side chains at higher surface density per monomer than in the unfolded state, thus driving the association of peptide to the membrane surface. These results point to an electrostatic interaction of SVS-1 with the surface of negatively charged model membranes that is driven by peptide conformation.

Data in Figure 1 can be fit to derive partition coefficients (K_p), a quantitative measure of interaction between the SVS-1 and SVS-2 with the anionic lipid bilayer (see Supporting Information).[14] Partition coefficients K_p = 224.90 ± 9.92 and K_p = 31.98 ± 13.02 were obtained for SVS-1 and SVS-2, respectively. The K_p for SVS-1 is over 4-fold greater than SVS-2 even though their formal charge state is identical, again indicating that the manner in which the electropositive functionalities of the peptide are presented to the membrane is important. This is mirrored in other ACPs whose amphiphilic structures contain high densities of positively charged side chains.[9]

A similar zeta potential analysis was performed on A549 (lung carcinoma) cells. Figure 2 shows the zeta potential values obtained for the interaction of both SVS-1 and SVS-2 with the cancer cells. The measured zeta potential for the cells in the absence of peptide is −14.76 ± 1.49 mV, indicating that their surfaces are negatively charged. This is due to both lipid composition and negatively charged cell surface macromolecules, as described earlier. The addition of SVS-1 caused an increase of the zeta potential values toward neutralization indicating that the peptide is binding to the cell surface. Interestingly, the data also show that the control peptide also binds. Although it is impossible to determine exact partition coefficients with living cells, it is qualitatively clear that SVS-2 binds with significantly lower affinity.

Lastly, we assessed the ability of both SVS-1 and SVS-2 to kill A549 cells at peptide concentrations used in this study. The data in Figure 3 indicate that the IC_{50} for SVS-1 is around 5

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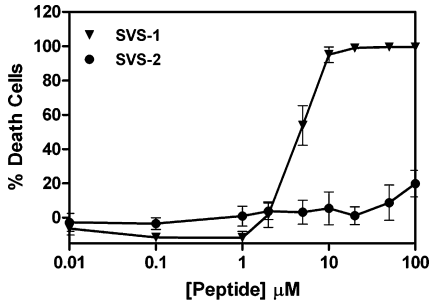

Figure 3. In vitro cytotoxicity of SVS-1 and SVS-2 peptide toward A549 lung carcinoma evaluated at 24 h after addition of the peptides. Data for SVS-1 were taken from ref 13. Error bars represent standard deviation of at least three independent experiments.

μM. Correlation with the data in Figure 2 suggests that this corresponds to a zeta potential of −11 mV, an increase in potential of only about 4 mV. Thus, full neutralization of the cell's surface potential by the peptide is not necessary for its cytotoxic action. This is in direct contrast to the mode of action of AMPs, which when introduced to bacteria, fully neutralize the cell's surface potential to effect their action. Figure 3 also shows that SVS-2, even at 200 μM, is not capable of killing the cells, even though the data in Figure 2 indicate that the control peptide is associating with the cell surface in some capacity. It may be that SVS-2 can bind to negatively charged cell surface components, such as GAG, but not strongly to the headgroup region of the bilayer as evident in Figure 1.

Taken together, these results suggest that SVS-1 partitions to negatively charged surfaces, and that although peptide binding to anionic, nonlipid components can occur, lipid composition is a seminal component defining the killing mechanism of the peptide. Data also suggest that proper presentation of positively charged side chains drives SVS-1 association with the headgroup region of the cancer cell membrane, and full cell neutralization is not a prerequisite for downstream lytic events. Recently, our group reported that the MIC for most lytic AMPs correlates with their ability to affect full cell surface charge neutralization.[18] A difference in the mechanisms of AMPs and the ACP, SVS-1, is the observation that full neutralization of the cell surface is not necessary for its killing action. In contrast to anionic lipid on the surface of bacteria, PS can segregate into patches on the surface of cancer cells. It is possible that cell death results from SVS-1 interacting with only a subset of these regions, and thus total surface neutralization is not necessary to elicit its anticancer action. For other ACPs that act through a lytic mechanism, it will be interesting to see if this is a general event.

■ ASSOCIATED CONTENT

Supporting Information

Supporting methodological details, K_p determination, and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AMPs, antimicrobial peptides; ACPs, anticancer peptides; PS, phosphatidylserine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; LUVs, large unilamellar vesicles; BTP, bis-tris-propane; K_p, partition coefficient; IC_{50}, half maximal inhibitory concentration; MIC, minimal inhibitory concentration

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