Membrane permeabilization of the African horse sickness virus VP5 protein is mediated by two N-terminal amphipathic α-helices

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Abstract

African horse sickness virus (AHSV) VP5 protein is cytotoxic when expressed in *Spodoptera frugiperda* (Sf-9) cells. Secondary structure analysis of the VP5 amino acid sequence of AHSV-9 identified two N-terminal amphipathic α-helices within the first 43 amino acids. Baculovirus expression of N- and C-terminal truncated VP5 proteins in Sf-9 cells indicated that the N-terminal 43 amino acids correlated with low levels of protein expression, and with increased membrane permeabilization and cytotoxicity. Exogenous addition of chemically synthesized VP5 peptides indicated that both N-terminal amphipathic α-helices are required for membrane permeabilization of Sf-9 cells. These findings suggest that AHSV VP5 is a membrane destabilizing protein.

African horse sickness virus (AHSV), a member of the *Orbivirus* genus in the family *Reoviridae*, is an arthropod-borne virus (*Culicoides* spp.) and the causative agent of African horse sickness (AHS), a highly infectious disease of equines with high mortality rates in horses [4]. Like BTV, the prototype orbivirus, AHSV consists of two concentric protein layers that encapsidate the genome of ten double-stranded (ds) RNA segments [14, 19]. The core particle is composed of two major (VP3 and VP7) and three minor (VP1, VP4 and VP6) structural proteins, and is surrounded by the outer capsid, composed of the two major structural proteins VP2 and VP5 [19]. Infected cell extracts containing AHSV VP2, in combination with VP5 and VP7, induces neutralizing antibodies in horses, and indicate that VP5 and VP7 may contain epitopes which could enhace the immune response to the virus [17, 18]. Other than its role in immunogenicity, no functional studies have been undertaken on the VP5 protein of AHSV. Nevertheless, expression of the AHSV VP5 protein in *Escherichia coli* [18] and *Spodoptera frugiperda* (Sf-9) cells [8] has been reported to be cytotoxic, causing rapid cell lysis and resulting in low levels of protein expression. The basis of the apparent cytotoxicy, however, has not yet been investigated. Here, through the characterization of a series of VP5 deletion mutants and relevant peptides, based on the predicted structural features of VP5, we report that the viral protein possess membrane permeabilizing activity. We furthermore show that this activity is linked to two N-terminal amphipathic α-helices, located within the first 43 amino acids of AHSV VP5.

The nucleotide sequence of the full-length cDNA copy of AHSV-9 genome segment M6, contained in recombinant plasmid pBSVP5 (kindly provided by Dr. W. Fick, Department of Genetics, University of Pretoria), was determined by automated sequencing procedures and the deduced amino acid sequence was used in secondary structure analyses. The hydrophobicity profile of the VP5 protein was predicted with the algorithm of Kyte and Doolittle [16], whilst the PredictProtein server (www.predictprotein.org) was used for secondary structure analysis. The hydrophobic profile of the 505 residues of the AHSV-9 VP5 protein indicated a clear partition between two domains; an N-terminal domain (amino acids 1 to 220) and a C-terminal domain (amino acids 280 to 505), separated by a hydrophobic hinge region (amino acids 220 to 280) that is rich in alanine and glycine residues (Fig. 1a). Two α-helices were also identified in the first 43 residues at the N-terminus of VP5, which are immediately followed by a stretch of hydrophobic residues. Helical wheel representation of amino acids 1 to 22 (α-helix 1) and amino acids 23 to 43 (α-helix 2) of VP5, using the BioEdit version 7.0.4.1 software program [12], revealed that both helices have a net-positive charge on their hydrophilic faces as a result of the clustering of positively charged lysine residues (K) (Fig.

1b). The latter may allow the helices to interact with negatively charged phospholipids present in cell membranes. Indeed, cationic amphipathic α-helices are motifs common to many polypeptides with membrane-destabilizing properties and have been implicated in the membrane-binding activities of viral fusion proteins [9, 23, 24].

Towards examining which domain(s) of the VP5 protein plays a role in its reported cytotoxicity [8, 18], full-length and several different truncated VP5 fragments were generated by PCR amplification with oligonucleotides complementary to the relevant AHSV-9 VP5 coding sequence (GenBank accession no. U74489). BTV VP5 glutathione-*S*-transferase (GST) fusion proteins are reportedly expressed at higher levels than that of native VP5 protein, suggesting that masking the amino terminus allows higher levels of protein to stably accumulate [13]. Therefore, the PCR amplicons were ligated to pGEM®-T Easy vector (Promega) and subsequently cloned into the GST baculovirus transfer vector pAcGHLT-B (BD Biosciences) using standard recombinant DNA methodologies [22]. The nucleotide sequence and orientation of cloned insert DNA was verified by automated sequencing procedures. Recombinant baculoviruses were obtained by co-transfecting Sf-9 cells with the respective recombinant baculovirus transfer vectors and linearized BaculoGold™ DNA, according to the specifications of the manufacturer (BD Biosciences). A representation of the full-length and truncated VP5 fusion proteins used in this study is shown in Fig. 2a. The resultant parental and recombinant baculoviruses were used to infect Sf-9 cell monolayers (1×10^7 cells) at a MOI of 10 PFU/cell. The recombinant fusion proteins were expressed at maximal levels between 24 to 48 h post-infection, following which the expression levels significantly declined (results not shown). Therefore, following incubation at 27°C for 48 h, mock-infected and baculovirus-infected Sf-9 cells were harvested and the respective cell lysates analyzed by 12% SDS-PAGE (Fig. 2b). Subsequent immunoblot analyses indicated that the recombinant fusion proteins were recognized by a both an anti-AHSV-9 antibody (results not shown) and a polyclonal anti-GST antibody (Fig. 2c). Whereas the full-length VP5 and truncated VP5 proteins VP5Δ221-505 and VP5Δ44-505 were expressed at low levels, the truncated VP5 proteins VP5Δ1-22, VP5Δ1-43, VP5Δ1-123 and VP5Δ1-279 were expressed at comparatively higher levels. A similar low level of protein expression has been reported for full-length and N-terminal VP5 fragments (amino acids 1 to 341 and amino acids 1 to 49) in *E. coli* cells [18]. We therefore conclude that the amino terminus of VP5 is responsible for the observed cytotoxicity and resultant low expression levels.

The cytopathic effects of several viruses may result from the altered membrane permeability of the host cell due to the expression of a single viral polypeptide [5, 7, 11]. Therefore, the ability of the recombinant baculovirus-expressed full-length and truncated VP5 fusion proteins to permeabilize Sf-9 cells was evaluated, and the cytotoxicity (cell leakage) determined using the Cytotoxicity Detection Kit (Roche Applied Science). This quantitative assay measures levels of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme that is released when the plasma membrane is damaged. The amount of LDH present in the supernatant is directly proportional to the amount of lysed cells. This assay reveals low-level damage to cell membranes, gives similar values to ^{51}Cr release assays [6, 15] and has been used successfully in other virus-induced cytotoxicity studies [13, 21]. Control samples were included in each experiment to correct for LDH in the culture medium (background control) and for spontaneous release of LDH from uninfected cells (low control). The maximum release of LDH from uninfected cells (high control) was measured after lysis of the cells with 2% (v/v) Triton X-100 in the medium. Three independent experiments, each consisting of triplicate samples, were performed. Sf-9 cells (1.5×10^4 cells/well) were infected with the respective recombinant baculovirus at a MOI of 10 PFU/cell. The cytotoxicity of each of the expressed full-length and truncated GST-VP5 proteins was determined by measuring the amount of released LDH at 30 h post-infection (p.i.), following the onset of recombinant protein expression, but prior to cell lysis. Expression of the GST-tagged full-length VP5 protein, as well as expression of VP5 fusion proteins with C-terminal deletions (*e.g.*, VP5Δ221-505 and VP5Δ44-505) induced substantial release of LDH, with VP5Δ44-505 exhibiting the highest activity (cytotoxicity of *ca.* 98%). In contrast, expression of VP5 fusion proteins with deletions from the N-terminus (*e.g.*, VP5Δ1-279, VP5Δ1-123, VP5Δ1-43 and VP5Δ1-22) resulted in comparatively low levels of LDH-release (Fig. 3a). Based on the low cytotoxicity (*ca*. 3.3%) associated with expression of the GST protein only and considering that GST is unable to associate with liposomes [5], these results therefore indicated that the cytotoxicity observed with the VP5 fusion proteins was mediated by the VP5 component. Notably, the presence of the two amphipathic α-helices at the N-terminus of VP5 correlated strongly with increased membrane permeabilization and thus cytotoxicity.

To ascertain whether the two predicted N-terminal amphipathic α-helices individually or in combination trigger LDH release, four synthetic VP5 peptides were generated (GenScript Corp.). Peptide VP5(1-43), encompassing both α-helices, had the sequence $_1$MGKFTSFLKRAGSATKKALTSD$_{22}$AAKRMYKMAGKTLQKVVESEV$_{43}$. Peptides

VP5(1-22) and VP5(23-43) were composed of amino acids 1 to 22 (α-helix 1) and amino acids 23 to 43 (α-helix 2), respectively, of the above peptide sequence. The fourth peptide, designated VP5(280-301), had the sequence PHIIEKAMLKDKIPDNELAMAI and comprised of residues at the VP5 C-terminal region (amino acids 280-301). This peptide was used as a control since baculovirus expression of the VP5 fusion protein VP5(280-505) resulted in neglible cytotoxicity (Fig. 3a). Sf-9 cell monolayers (1.5×10^4 cells) were incubated with 50 μM of each synthetic VP5 peptide at 27ºC, and observed until maximum cytopathic effect was observed in peptide-treated cells, whilst control cells remained unchanged. At 24h post-treatment, the cell culture supernatants were assayed for the amount of LDH released, and the results indicated that the VP5(1-43) peptide caused substantial release of LDH (cytotoxicity of *ca*. 100%), whilst none of the other three VP5 peptides assessed showed any such effect (Fig. 3b). This data therefore indicated that both N-terminal amphipathic α-helices of VP5 were required to permeabilize the plasma membrane of Sf-9 cells. It therefore appears that for AHSV VP5, α-helix 1 exerts its membrane permeabilizing activity in concert with α-helix 2 and that both these helices may be cooperatively involved in the formation of membrane-integral pores. These results are in contrast to those of BTV VP5, in which the most N-terminal α-helix (amino acids 1 to 20) exhibits a significantly higher permeabilizing activity than the adjacent α-helix (amino acids 22 to 41) [13].

In conclusion, our study indicates that the two putative amphipathic α-helices within the first 43 amino acids of AHSV-9 VP5 are responsible for mediating membrane permeabilization in Sf-9 cells. While the data represented here demonstrates that peptide VP5(1-43) induces membrane permeabilization when added exogenously to cultured cells, the importance of this domain in membrane destabilization in AHSV-infected cells remains to be determined. We hypothesize that the VP5 protein of AHSV, similarly to BTV VP5 [10, 13, 25], may be a membrane destabilizing protein.

Acknowledgements

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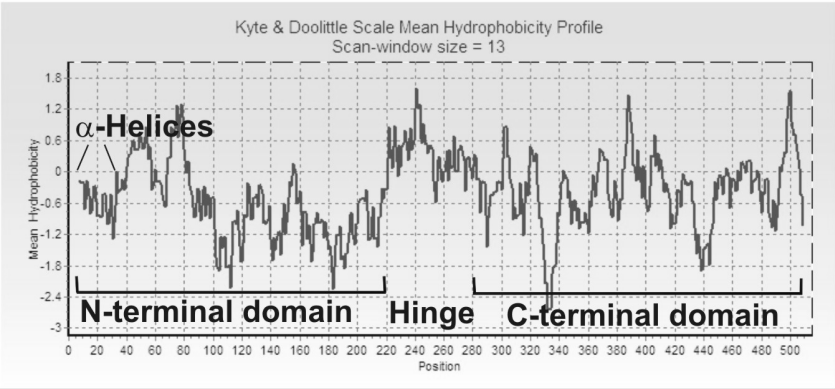
Figure captions

Fig. 1 Predicted secondary structure of the AHSV-9 VP5 protein. (a) Hydrophobicity profile of AHSV-9 VP5 predicted using the algorithm of Kyte and Doolittle [16] with a window setting of 13. (b) Helical wheel diagrams showing the amphipathic nature of each predicted α-helix at the N-terminus of AHSV-9 VP5. Each panel represents an α-helix viewed along the helix axis. Hydrophobic amino acids residues are indicated in blocks

Fig. 2 Baculovirus expression of full-length and truncated AHSV-9 VP5 proteins in Sf-9 cells. (a) Full-length and truncated VP5 proteins were expressed as GST fusion proteins in Sf-9 cells, and 48 h p.i., the cell lysates were resolved by (b) SDS-PAGE, and (c) immunoblot analysis was performed with polyclonal anti-GST antibodies. Molecular weight markers (kDa) are indicated to the left of the figure. Uninfected Sf-9 cells and GST-expressing baculoviruses were included as controls

Fig. 3 Membrane permeabilization of Sf-9 cells. (a) Cytotoxicity of baculovirus-expressed full-length and truncated AHSV VP5 proteins expressed in Sf-9 cells (1.5×10^4 cells) by recombinant baculoviruses (10 PFU/cell) at 30 h p.i. Sf-9 cells infected with a baculovirus expressing GST only served as a control. (b) Synthetic VP5 peptides were dissolved in UHQ water, and 50 μM of each peptide was added to 1.5×10^4 Sf-9 cells, followed by incubation at 27°C for 24 h. In both assays, the amount of LDH release was measured (OD_{492}) from triplicate wells, and used to calculate the percent cytotoxicity using the following equation: cytotoxicity (%) = [(experimental value) – (low control)/(high control – low control)] × 100. Bars show mean ± SD for three independent experiments.

Fig. 1a


Kyte & Doolittle Scale Mean Hydrophobicity Profile
Scan-window size = 13
Mean Hydrophobicity
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1.2
0.6
0
-0.6
-1.2
-1.8
-2.4
-3
α-Helices
N-terminal domain
Hinge
C-terminal domain
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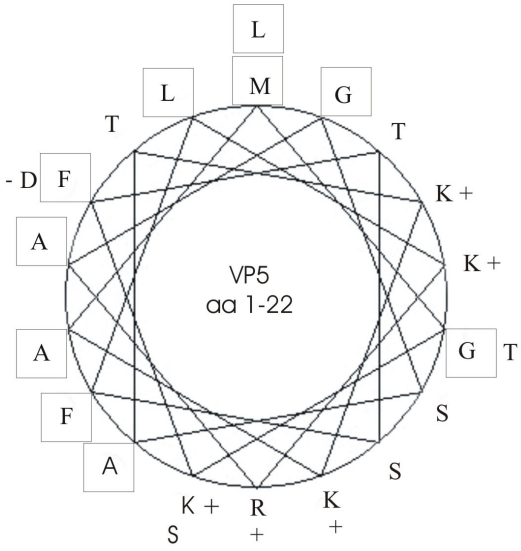
Position

Fig. 1b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- D
F
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K
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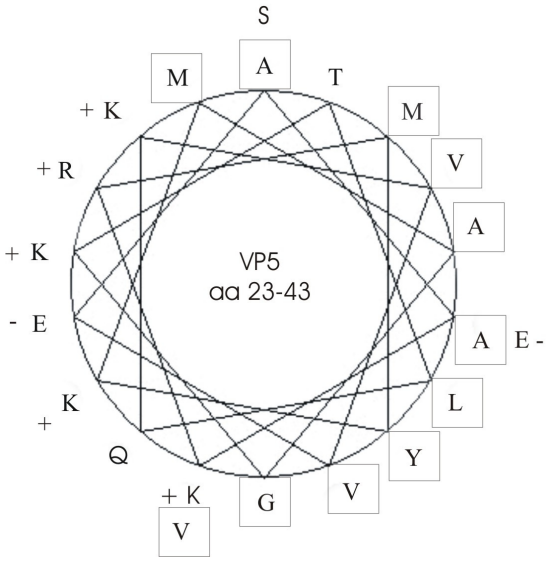
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M
T
+ K
M
+ R
V
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VP5
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L
Q
Y
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G
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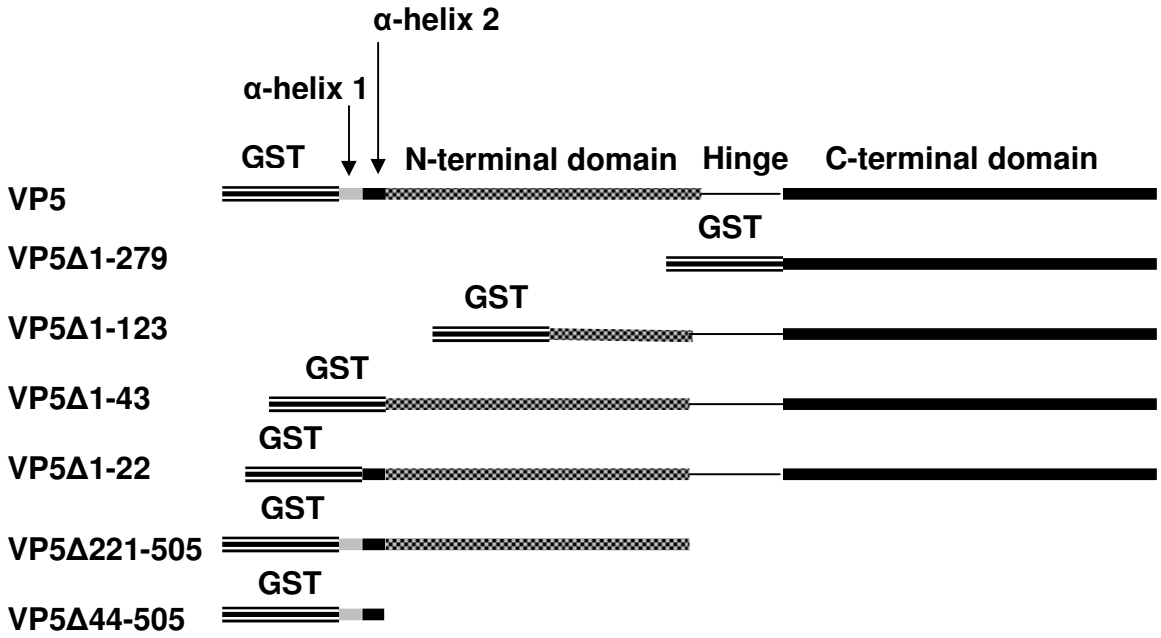
V

Fig. 2a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α-helix 1
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Hinge
C-terminal domain
VP5
GST
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VP5Δ1-22
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VP5Δ221-505
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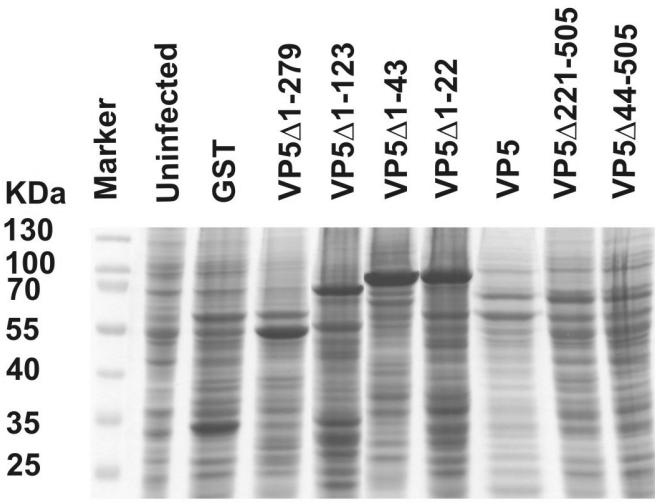
VP5Δ44-505

Fig. 2b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Uninfected
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Fig. 2c

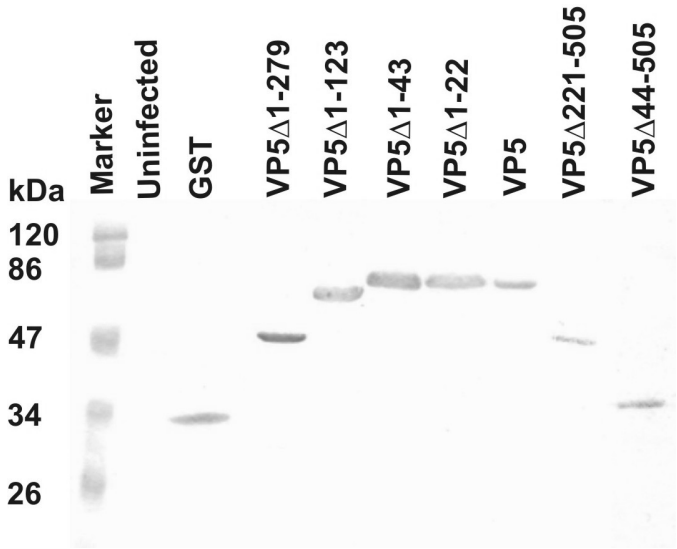
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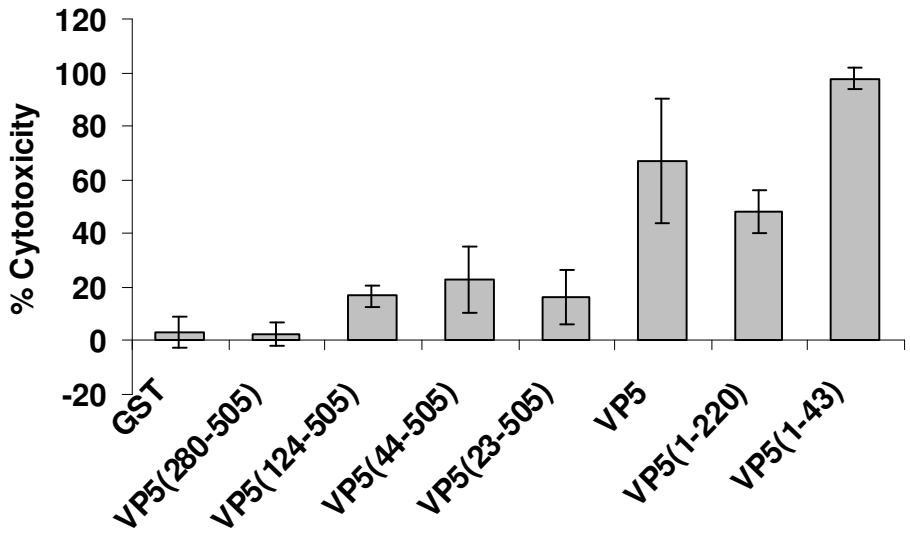
Fig. 3a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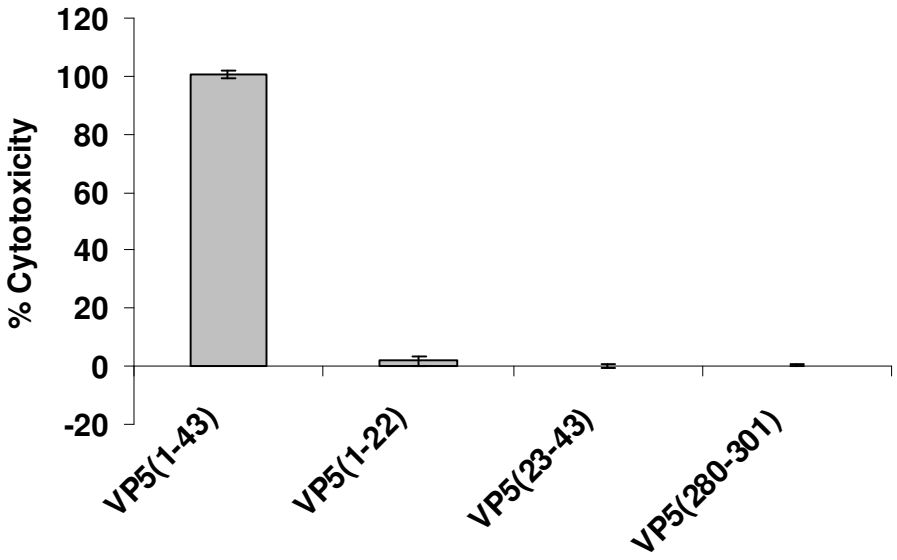
100
80
60
40
20
0
-20
GST
VP5(280-505)
VP5(124-505)
VP5(44-505)
VP5(23-505)
VP5
VP5(1-220)
VP5(1-43)

Fig. 3b


% Cytotoxicity
120
100
80
60
40
20
0
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VP5(1-43)
VP5(1-22)
VP5(23-43)
VP5(280-301)