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ARTICLE

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Amiloride derivatives block ion channel activity and enhancement of virus-like particle budding caused by HIV-1 protein Vpu

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Abstract The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels and enhances the process of virion budding and release. Mutagenesis studies have shown that the N-terminal transmembrane domain primarily controls both of these activities. Here we report that the Vpu ion channel is inhibited by the amiloride derivatives 5-(*N*,*N*-hexamethylene)amiloride and 5-(*N*,*N*-dimethyl)amiloride but not by amiloride itself, nor by amantadine. Hexamethyleneamiloride also inhibits budding of virus-like particles from HeLa cells expressing HIV-1 Gag and Vpu proteins. These results confirm the link between Vpu ion channel activity and the budding process and also suggest that amiloride derivatives might have useful anti-HIV-1 properties.

Keywords Human immunodeficiency virus · Vpu protein · Amiloride derivatives · Ion channels

Introduction

Some enveloped viruses, including influenza viruses (A and B) and the immunodeficiency virus (HIV-1), encode small proteins with hydrophobic segments that form ion channels (Duff et al. 1992; Ewart et al. 1996; Piller et al. 1996; Pinto et al. 1992; Sunstrom et al. 1996). The influenza A virus contains mRNA for a small protein, M2, that forms a proton channel in the virus envelope and in the membrane of the trans-Golgi network in infected cells; these channels are essential for normal replication of the virus (reviewed in Lamb and Pinto 1997). The channel formed by M2 can be blocked with amantadine, a drug that also depresses replication of the virus. The influenza B virus is relatively unaffected by amantadine. It encodes a small protein, NB, that also forms ion channels (Sunstrom et al. 1996) but they are resistant to the effects of amantadine.

HIV encodes several small "auxiliary" proteins, among them Vpr and Vpu, both of which form ion channels (Ewart et al. 1996; Piller et al. 1996). Vpu is an integral membrane protein encoded by HIV-1. It associates with the Golgi and endoplasmic reticulum membranes but has not been detected in the viral envelope nor in the plasma membrane of cells except when artificially overexpressed (Jabbar 1995; Maldarelli et al. 1993; Schubert et al. 1996a). Vpu contains about 82 amino acids (depending on the viral isolate) and has an N-terminal transmembrane anchor and a hydrophilic phosphorylated cytoplasmic C-terminal domain (Schubert et al. 1992). Vpu forms homo-oligomers in membranes (Maldarelli et al. 1993).

Vpu is multifunctional in infected cells. It is involved in the budding and release of virus particles (Klimkait et al. 1990), degradation of HIV-1 receptor CD4 in the endoplasmic reticulum (Willey et al. 1992) and processing of other membrane proteins, including MHC class 1 (Kerkau et al. 1997). These functions are mediated by distinct structural domains of Vpu (Schubert et al. 1996a). Mutations in the transmembrane anchor domain prevent enhancement of virus release by Vpu but do not affect the CD4 degradation activity (Tiganos et al. 1998). In contrast, mutations to the phosphorylated serine residues at positions 52 and 57 in the cytoplasmic tail abolish Vpu-dependant CD4 degradation but do not affect the virus release function. Experiments with mutants and synthetic peptides demonstrate that the ion channel activity of Vpu resides in the N-terminal transmembrane domain (Schubert et al. 1996b). Further, mutations that knock out the ion channel function also ablate Vpu enhancement of virus release (Schubert et al. 1996b), establishing a link between the two activities.

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We have identified chemical compounds that depress ion channel activity of Vpu and show that they also antagonize the enhancement of budding and release of virus-like particles (VLPs) induced by Vpu.

Materials and methods

Bacterial expression and purification of recombinant Vpu protein

Purified Vpu protein, for reconstitution into planar lipid bilayers, was prepared after expression in *Escherichia coli* as described previously (Ewart et al. 1996). Briefly, cells containing the plasmid p2GEXVpu – which expresses HIV-1 Vpu (isolate HXB2) with an N-terminal glutathione *S*-transferase (GST) tag – were grown to approximately 250 Klett units at 30 °C, then induced with IPTG (0.01 mM) for a further 4 h before harvesting. Combined cell debris and membrane fractions were prepared after osmotic disruption and centrifugation. The pellet was resuspended and GST-Vpu was solubilized by stirring on ice for 1 h in the presence of CHAPS detergent (2% wt/vol) and glycerol (20% wt/vol) in MTPBS-DTT (16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl, 1 mM dithiothreitol, pH 7.3). Insoluble material was removed by centrifugation at 400,000×*g* for 1 h. The supernatant was applied to a glutathione-agarose chromatography column, and after thorough washing, the resin was treated with human thrombin (100 U/mL; 37 °C for 1 h) to elute the Vpu portion of the fusion protein. This Vpu fraction could be further purified to homogeneity by anion exchange and immunoaffinity chromatography (Ewart et al. 1996).

For planar lipid bilayer experiments, purified Vpu was reconstituted into phospholipid vesicles by dialysis as follows. A mixture of palmitoyl-oleoyl-phoshatidylethanolamine (PE), palmitoyl-oleoyl-phoshatidylcholine (PC) and palmitoyl-oleoyl-phoshatidyl-serine (PS) (7:2:1, 5 mg total lipid) in chloroform was evaporated to dryness under a stream of N_2 and resuspended in 100 µL of CHAPS solution (400 mg/mL). Vpu (1 µg) was added to the lipid suspension in 400 µL of buffer containing CHAPS (0.5%), glycerol (5%), DTT (1 mM) and Tris (50 mM), pH 7.5. After sonication in a bath sonicator for 10 min, the mixture was injected into a 1 mL ''Slide-a-lyser'' (Pierce) and dialysed overnight against two 500 mL volumes of MES (10 mM)/NaCl (200 mM) (pH 7.2). The resultant suspension of proteoliposomes was used the next day in bilayer experiments or stored for up to two days at 4 °C.

Lipid bilayer techniques

Bilayers were painted across a small circular hole (approximately 100 µm diameter) in a Delrin plastic wall separating aqueous solutions in the CIS and TRANS chambers. A lipid mixture of 7:2:1 PE:PC:PS in *n*-decane was used and both chambers initially contained 10 mM MES (morpholine-ethanesulfonic acid), 50 mM NaCl, 10 mM $CaCl_2$, pH 7.2. Aliquots of Vpu proteoliposomes representing 10–50 ng of Vpu were added to the CIS chamber and stirred for 2 min to assist collision of proteoliposomes with the planar lipid bilayer. Then the concentration of NaCl in the CIS chamber was adjusted to 500 mM and stirring was continued until Vpu channel activity was detected. The CIS chamber was earthed and the TRANS chamber was held at various holding potentials ranging between +100 and –100 mV. The standard electrophysiological bilayer techniques used are described fully elsewhere (Miller 1986). Currents were recorded with an Axopatch 200 amplifier and recorded directly to videotape using a Vetter PCM recorder at a sampling rate of 1 kHz. For further analysis, the raw data was digitized at 0.5 kHz. Average currents were calculated for continuous segments (5–80 s duration) of channel activity. Average conductance was calculated by dividing average current by holding potential minus the reversal potential of the currents. The reversal potential in the presence of a 500/50 mM NaCl concentration gradient was 31 ± 1.2 mV, as reported previously (Ewart et al. 1996).

Synthetic Vpu N-terminal peptide

An oligopeptide (Vpu-N) corresponding to the N-terminal 32 amino acids of Vpu was synthesized at the BioMolecular Resource Facility (JCSMR ANU) with an Applied Biosystems model 477a machine. Its sequence is MQPIPIVAIVALVVAIIIAIVVWSIVIIEYRK (MW 3544). The peptide was further purified by reverse-phase HPLC using an RP304 column (BioRad). Initially, the peptide was dissolved in 95% acetonitrile/0.1% trifluoroacetic acid (TFA) and the rate of solubilization was assisted by bath sonication. For loading onto the column, the solution was diluted with 10% acetonitrile/0.1% TFA to 52.5% acetonitrile and eluted with a 52.5–100% gradient of acetonitrile in 0.1% TFA. The full-length peptide, which began to elute at approximately 75% acetonitrile, was collected and dried under vacuum. It was stored at –20 °C. Mass spectroscopy (matrix assisted laser desorption time of flight; MALDI-TOF) of purified Vpu-N revealed a single molecular ion of molecular mass 3543.5, in good agreement with that expected for the full-length peptide. For bilayer experiments, a stock solution of purified Vpu-N of 10 mg/mL was prepared in trifluoroethanol and diluted, as required, before addition of 1 µL aliquots to the CIS chamber with stirring. The final concentration of hydrophobic peptide in the CIS chamber was thereby approximately 2.8 µM.

Chemicals

Amiloride, 5-(*N*,*N*-hexamethylene)amiloride (HMA), 5-(*N*,*N*-dimethyl)amiloride (DMA) and amantadine-HCl were obtained from Sigma. Aqueous stock solutions (10 mM) were prepared by conversion to the HCl salt form and stored in small aliquots at –20 °C in the dark. Phospholipids were obtained from Avanti Polar Lipids (Alabaster, Ala., USA).

Mammalian expression vectors

Vectors for the expression of Gag (pAN1343) and Vpu (pAN1300) in HeLa cells were constructed as follows. The Gag gene from HIV isolate SF2 was amplified as a 1508 bp product by PCR from a plasmid containing the *gag* cDNA (pUC19ARV; obtained from Dr. Scott Thomson, John Curtin School of Medical Research, Australian National University) and ligated, after digestion with *Bam*H1 and *Eco*R1, into the vector pcDNA3.1+ (Invitrogen). The PCR reaction employed *Pfu*-thermostable DNA polymerase (0.5 units), 200 µM dNTPs and 20 pmol each of the 5′ oligonucleotide (GAGGGATCC**ATG**GGTGCGAGAGCGTCGGTATTAAG; *Bam*H1 site underlined; Gag start codon in boldface), and the 3′ antisense oligonucleotide (CCCGAATTC**TTA**TTGTGACGAGGG; *Eco*R1 site underlined; Gag stop codon in boldface). The thermocycler was programmed for 30 cycles of 94 °C for 1 min; 50 °C for 1 min; and 72 °C for 4 min.

To construct pAN1300, a 268 bp Vpu-encoding fragment was amplified and cloned into *Bam*H1/*Eco*R1 digested pcDNA3.1+ as described previously (Ewart et al. 1996).

Virus-like particle production in HeLa cells driven by HIV-1 Gag and Vpu

The budding of virus-like particles (VLPs) from HeLa cells was induced by the expression of the Gag protein and further enhancement of VLP budding was induced by the co-expression of Vpu. T7 RNA polymerase expressed from the recombinant vaccinia virus vTF7-3 (Fuerst et al. 1987) was used to drive the expression of Gag and Vpu via T7 promoters in the vectors, essentially as described elsewhere (Paul et al. 1998). Except during experimental manipulation, HeLa cell cultures were maintained at 37 °C under 5% CO_2. For VLP production, approximately 5×10^5 HeLa cells at 60–70% confluence were infected for 1 h with vTF7-3 at a 10-fold multiplicity of infection. The cells were then transfected, either with pAN1343 alone or in combination with pAN1300, in the presence of lipofectin (Gibco BRL) for 3.5–5 h,

according to the manufacturers specifications. Subsequently, the medium was replaced and the cultures were incubated for various times before being processed for assessment of VLP budding either by electron microscopy or metabolic radioactive labelling, followed by immunoprecipitation and fluorography. To test for an effect on VLP budding, HMA was added to culture media immediately after transfection with the expression plasmids.

Observation of VLPs by electron microscopy

Cultures were grown and processed on glass coverslips which, at 16 h post-infection, were immersed in primary fixative (2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 2 h. After washing, they were immersed in secondary fixative (1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4) for 1.5 h followed by staining in 2% uranyl acetate and dehydration by soaking in an increasing series of ethanol solutions, up to 100%. The coverslips were then equilibrated in Spurr's resin, which was subsequently polymerized at 70 °C overnight. Thin sections of 80 nm were cut and stained with lead citrate for 8 min. Sections were viewed in a Hitachi H7000 electron microscope at 75 kV (Electron Microscopy Facility, JCSMR, ANU).

Metabolic labelling of VLPs

The culture medium was replaced with methionine-free RPMI (GIBCO-BRL) for 1 h before incubation in the presence of 250 μCi of Tran^{35}S Cys/Met (ICN) for 30 min. After labelling, the medium was replaced with fresh F15 supplemented with 10% fetal calf serum. At 16 h post-infection, the culture medium was removed centrifuged and filtered through 0.45 μM Acrodiscs (Gelman) to remove contaminating cells. The filtrate was then layered over a solution of 20% sucrose in phosphate buffered saline and centrifuged for 1.5 h at 32,000 rpm in an SW55 rotor to pellet the VLPs, which were then resuspended in a small volume of RIPA buffer [150 mM NaCl, 1% IGEPAL CA 630 (Sigma), 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5]. For immunoprecipitation of the Gag protein, the solution was pre-cleared with Protein A agarose for 30 min at 4 °C before addition of monoclonal antibodies recognizing the p24 domain (a gift from Dr. Alistair Ramsay, JCSMR, ANU, Australia). Immunoprecipitates were dissociated in SDS-containing sample buffer for 20 min at 90 °C before electrophoresis through polyacrylamide gels. After electrophoresis, the gels were equilibrated in Amplify (Amersham) before drying and exposure to X-ray film.

Results

HMA inhibits ion channels formed by Vpu

When purified recombinant Vpu was reconstituted into a planar lipid bilayer by vesicle fusion, as outlined in Materials and methods, ion channel activity was observed as reported previously (Ewart et al. 1996). Bilayers were observed for several minutes to ensure a stable baseline and absence of channel activity in the bilayer before Vpu was added to the CIS chamber. Typical currents observed after incorporation of Vpu are shown for one experiment at three different holding potentials in Fig. 1A. In the same experiment, channel activity disappeared following addition – with stirring – of HMA (50 μM) to the aqueous solutions in the CIS and TRANS chambers (Fig. 1B). Similar inhibition of Vpu ion channel activity was seen in 28 experiments with HMA concentrations from 25 to 125 μM and at holding potentials between +100 and –100 mV. The average channel conductances before and after HMA addition were calculated to be 14.1 ± 1.6 pS and 0.9 ± 0.4 pS, respectively (mean ± SEM, $n = 28$). Taking into account all sources of random variation introduced during reconstitution of different preparations of the protein into different planar bilayers, as well as variation due to the effects of holding potential and drug concentration, there was an extremely significant inhibitory effect of HMA on Vpu channel activity ($P_{\text{single factor ANOVA}} = 10^{-9}$).

At lower concentrations of HMA (25 and 50 μM), a trend towards less complete inhibition at more negative holding potentials was observed (not shown). Nevertheless, for experiments in which data was collected at –80 mV or –100 mV, comparison of mean conductance ($n = 8$) before (19.7 ± 2.2 pS) and after (2.42 ± 1.6 pS) addition of HMA again revealed an extremely significant inhibition of channel activity in the presence of the drug ($P_{\text{single factor ANOVA}} = 3 \times 10^{-5}$). Some inhibition of Vpu channels was seen with 10 μM HMA, but the results were variable (not shown) and the data were not analysed quantitatively for the purposes of this study.

HMA also blocks channels formed by an N-terminal peptide

Vpu-N, a synthetic peptide corresponding to the 32 N-terminal amino acids of Vpu (Materials and methods), was also found to form ion channels in planar lipid bilayers, consistent with previous experiments with a shorter N-terminal peptide (residues 1–27) (Schubert et al. 1996b). The size of the average currents generated by Vpu-N was dependent on the concentration of peptide in the CIS chamber. Figure 2A shows representative currents (0 mV holding potential) generated at the highest concentration of peptide tested (2.8 μM). At this concentration, the mean current-voltage relationship was linear in the range –20 to +20 mV with an average conductance of 390 pS. When lower concentrations of peptide were used, single discrete "channel" openings, presumably single channels, were observed with a conductance of 15.4 pS (not shown). The higher conductance observed at higher peptide concentrations presumably represents the contribution of many channels in the same bilayer, or possibly the existence of channels formed by a large number of Vpu monomers. As shown in Fig. 2B, addition of 100 μM HMA resulted in complete inhibition of the Vpu-N channel activity.

Effect of other compounds

DMA also inhibited ion channels formed by Vpu, as illustrated in Fig. 3. The concentration of DMA was 125 μM. Again, as seen with HMA, the inhibition was

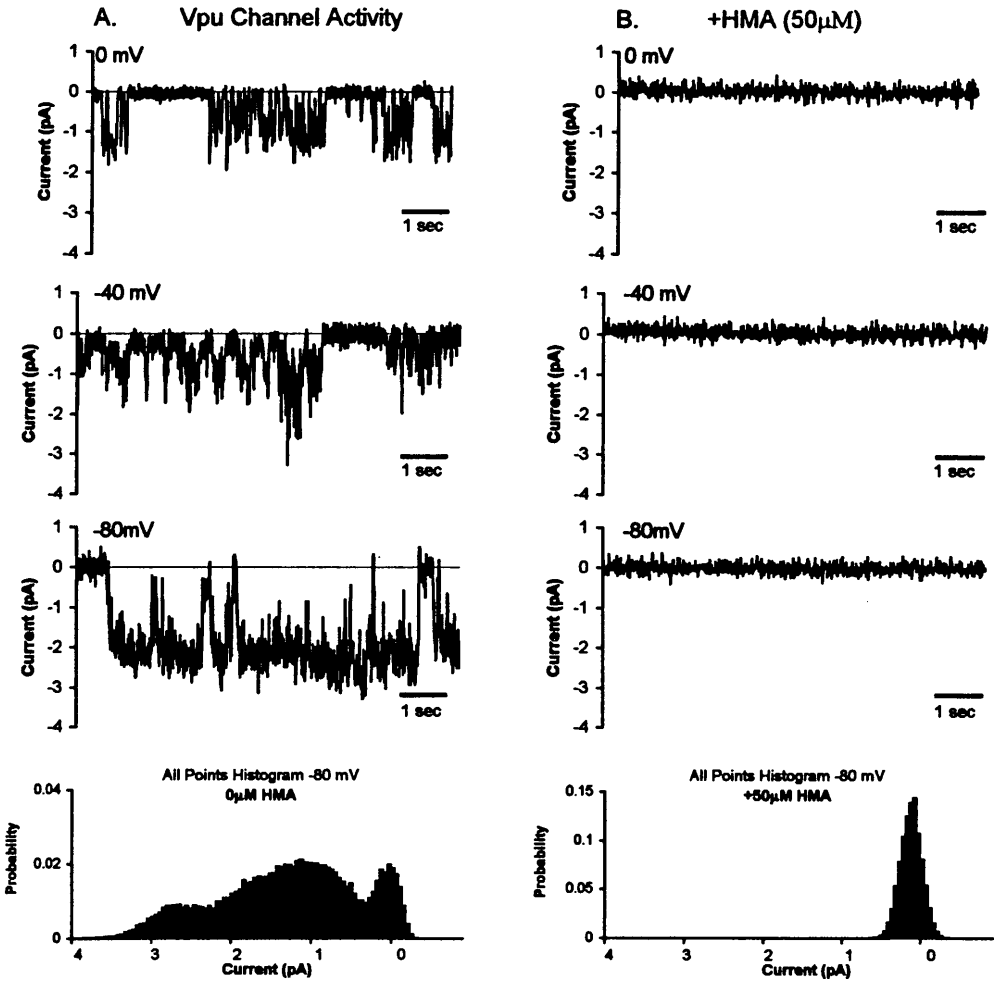


Fig 1A, B HMA inhibits Vpu ion channel activity. Purified recombinant Vpu was incorporated into planar lipid bilayer as described in Materials and methods. **A** Representative current fluctuations at 0, –40 and –80 mV and an all-points histogram generated from a 60-s continuous trace for channel activity observed at –80 mV holding potential. **B** The corresponding current traces and histogram from the same bilayer after addition of 50 μM HMA to the CIS and TRANS chambers, with stirring. For presentation purposes, the data were filtered at 100 Hz

effective at each holding potential and there was a trend towards less complete blocking at more negative potentials (–80 mV). Similar results were obtained in four separate bilayers. The average conductances before and after addition of DMA, calculated from average current traces at 0, –40, –60 and –80 mV, were 16.1 ± 1.5 and 0.5 ± 0.2 pS, respectively ($P_{\text{single factor ANOVA}} = 5\times10^{-5}$). Preliminary observations suggest that DMA is a less potent channel blocker than HMA, since at a lower concentration (50 μM) the degree of inhibition was variable and residual channel activity was present even at holding potentials as low as 0 mV (data not shown).

Amiloride, the parent compound, did not depress Vpu ion channel activity at concentrations up to 375 μM (Fig. 4).

Amantadine, which at 20 μM inhibited ion channels formed by M2 in bilayer experiments (Duff and Ashley 1992), did not inhibit Vpu channels when concentrations up to 1 mM were added to the CIS and TRANS chambers (data not shown).

Purified rabbit antibodies, raised against a peptide corresponding to the C-terminal 20 amino acids of Vpu (Ewart et al. 1996), had no effect on Vpu channel activity (data not shown).

HMA inhibits budding of virus-like particles

Electron microscopic examination revealed that expression of the HIV-1 Gag polyprotein in HeLa cells induced budding and release of VLPs from the plasma membrane of the cells into the culture medium (Fig. 5A, D), as has been described previously (Gelderblom 1991; Haffar et al. 1990). Co-expression of the Vpu protein has also previously been shown to enhance the budding process (Gottlinger et al. 1993; Paul et al. 1998), and in our hands, release of immunoprecipitable Gag into the culture supernatant was increased up to 13-fold (Fig. 5F; compare lanes 1 and 3). In agreement with previous reports, in the presence of Gag alone, many incipient and immature buds were observed on regions of the cell surface (Fig. 5D) and relatively few VLPs were observed surrounding the cell (Fig. 5A). In cultures of cells co-expressing Gag and Vpu, the cell surfaces were typically coated with buds at a later stage of maturation (Fig. 5E) and many cells were observed to be surrounded by an increased number of VLPs (Fig. 5B).

VLP release from cells co-expressing Gag and Vpu was inhibited by HMA. As shown in Fig. 5F (lane 2), 10 μM HMA reduced the amount of Gag immunoprecipitated from culture supernatants by more than 90%.

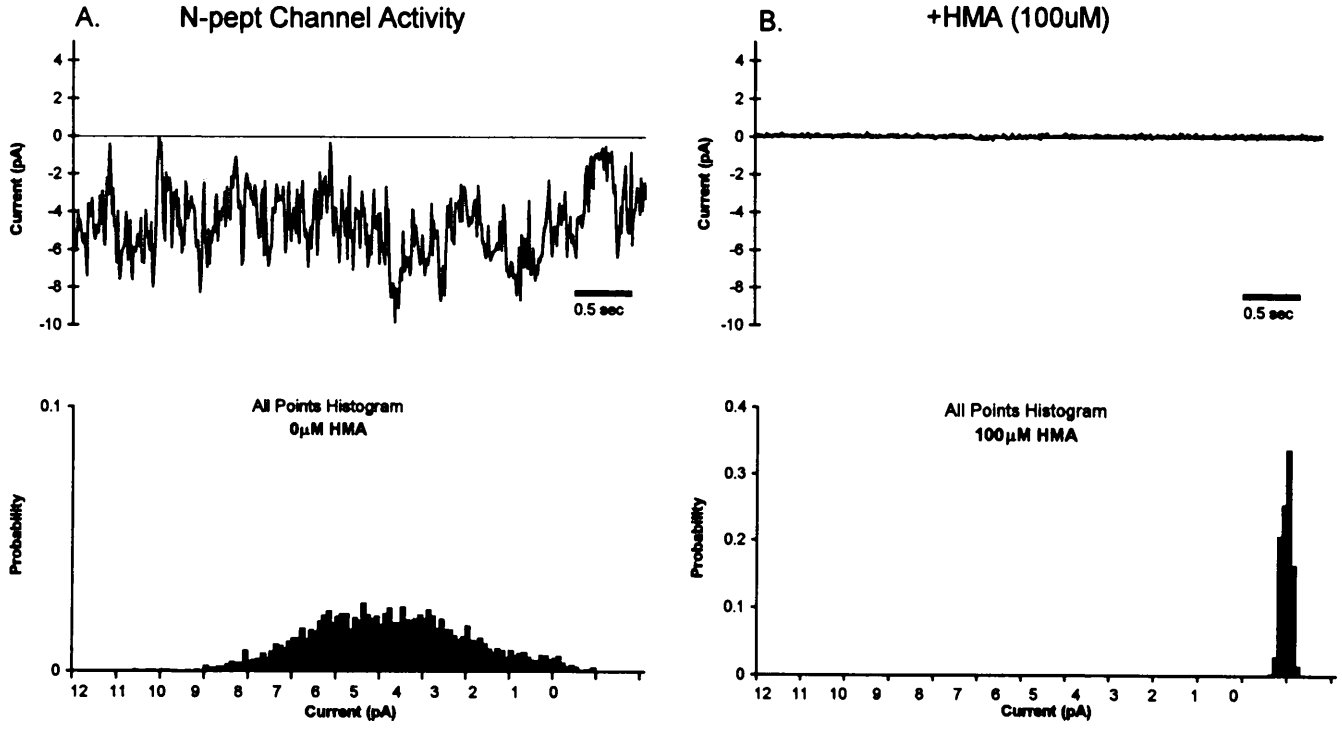
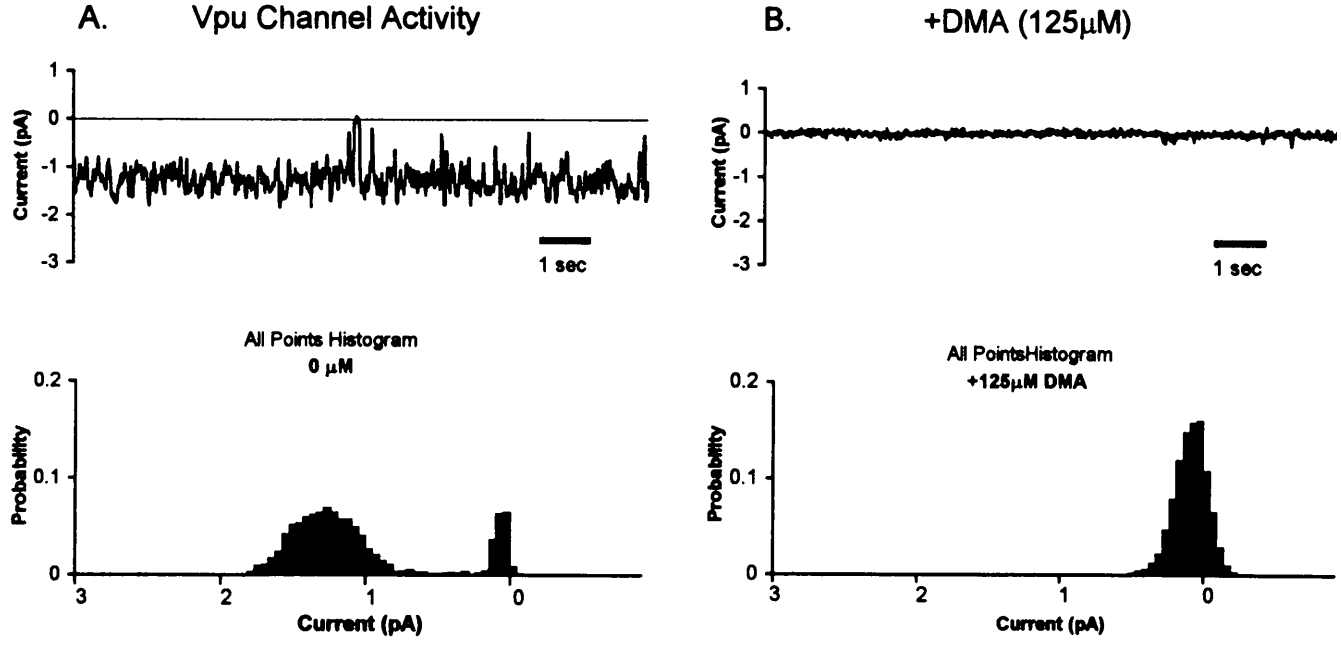


Fig. 2A, B HMA inhibits ion channel activity associated with the synthetic peptide Vpu-N. Vpu-N in 1 μL trifluoroethanol was added to the CIS chamber to a final concentration of 2.8 μM and the solution in the chamber was stirred until channel activity was detected. **A** Representative current fluctuations with the bilayer holding potential set at 0 mV. **B** The same bilayer with the same holding potential after addition of 100 μM HMA to the CIS and TRANS chambers. Corresponding all points histograms of currents generated from 15 s of data are shown underneath each current trace

Correspondingly, electron micrographs of cells expressing both Gag and Vpu showed a greatly reduced number of VLPs in close proximity to the cells when 10 μM HMA was present in the culture medium, as illustrated in Fig. 5C. At this concentration, HMA was not toxic to the cells, as assessed by both visual observation and staining of cells with trypan blue. In fact, by these criteria, cells expressing Gag and Vpu appeared healthier in the presence of the drug than in its absence. Further, analysis of cell lysates revealed that the level of Gag

Fig. 3A, B DMA inhibits Vpu ion channel activity. Representative current traces and all-points histograms for Vpu channel activity observed in the same bilayer held at –40 mV before (**A**) and after (**B**) addition of 125 μM DMA to the CIS and TRANS chambers



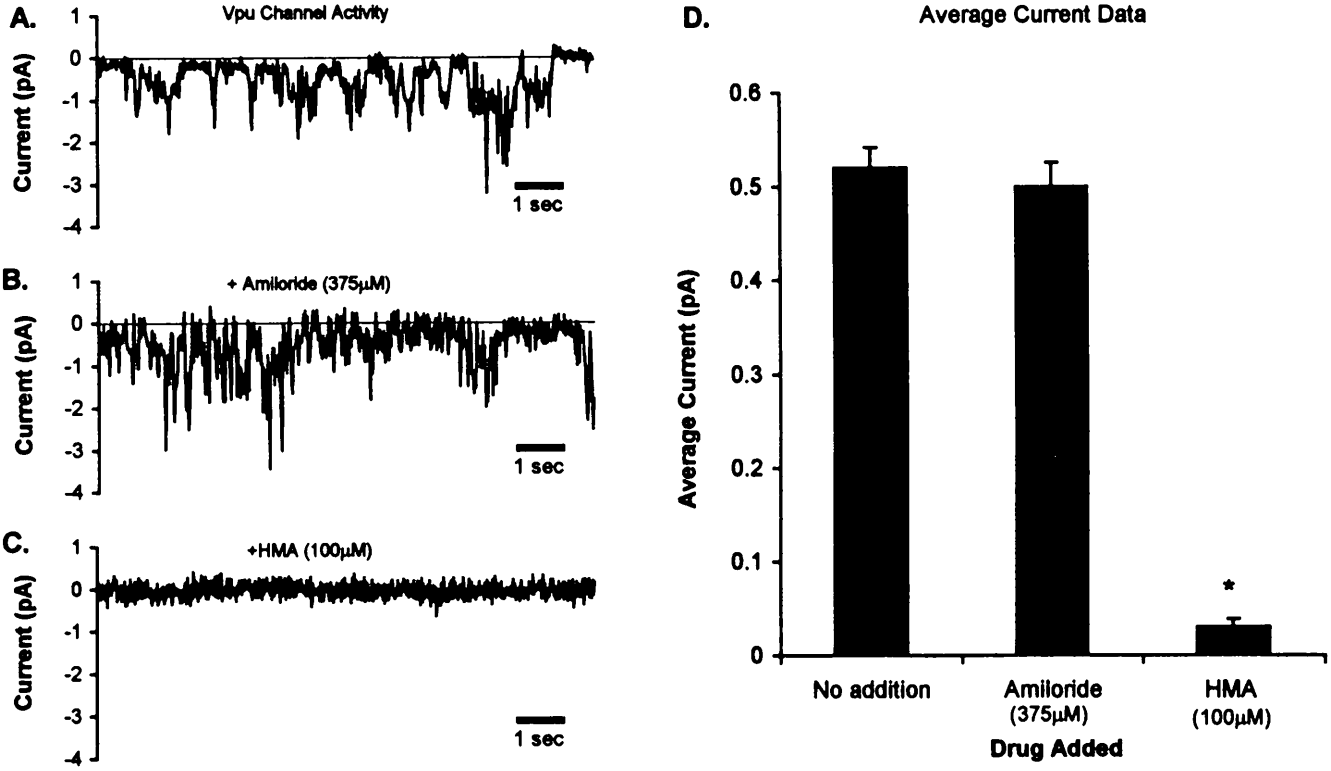


Fig. 4A–D Comparative effects of amiloride and HMA on Vpu ion channel activity. Representative traces of channel activity are shown for the same bilayer held at –40 mV: **A** before addition of any drug; **B** after addition of 375 μM amiloride to both chambers; and **C** after subsequent addition of 100 μM HMA to both chambers. Average currents (±1 SEM) calculated from the data shown in **A**–**C** are plotted in **D**. No significant effect of amiloride was seen on the average current, but the effect of HMA is highly significant ($P \ll 0.001$)

synthesis was relatively unaffected in the presence of HMA (Fig. 5F).

Discussion

This paper reports the first identification of drugs which inhibit the ion channel activity of HIV-1 Vpu. The two effective drugs tested, HMA and DMA, are derivatives of amiloride (see Fig. 6) with aliphatic substituents on the N atom of the amino group at the 5-position of the pyrazine ring. A hydrophobic substituent at this position appears to be important for the inhibition of Vpu channels, as amiloride itself is not inhibitory (see Fig. 4).

Amiloride and a large number of its derivatives are reversible inhibitors of ion transport (reviewed in Kleyman and Cragoe 1988, 1990). Amiloride itself inhibits Na^+ channels in urinary epithelia (ENaC) and is used therapeutically as a potassium-sparing diuretic. As a consequence, much research has been focused on the effects of amiloride on amiloride-sensitive Na^+ channels (reviewed in Alvarez de la Rosa et al. 2000). Structural modelling suggests that amiloride interacts with several parts of ENaC, with the positively charged guanidinium group penetrating into the pore while the pyrazine group interacts with the outer mouth of the channel (Horisberger 1998). In the present study, the inhibition by HMA of the channels formed by the synthetic peptide Vpu-N (Fig. 2) confirms that the drug binding site is in the N-terminal transmembrane domain of Vpu, as would be expected if the drug interacts with the channel outer mouth. Future mutagenesis studies will be focused on identifying the sites of interaction between the drug and the individual amino acid residues of Vpu involved.

As well as blocking ENaC, amiloride and many derivatives also inhibit Na^+/H^+ antiporters and Na^+/Ca^+ exchangers found in the plasma membrane of many eukaryotic cells. Reported IC_{50} values for amiloride at these three sites are 0.34, 83.8 and 1100 μM, respectively (Kleyman and Cragoe 1988). HMA and DMA are both more potent inhibitors of the Na^+/H^+ antiporter than amiloride (by approximately 500- and 20-fold, respectively), but less potent inhibitors of the epithelial Na^+ channel (>30-fold). This order of potency was seen with these drugs in blocking of the Vpu channel. While IC_{50} values have not been determined here, we find that the Vpu channel can be completely inhibited by 50 μM HMA (Fig. 1) or 125 μM DMA (Fig. 3), but is not blocked by amiloride at concentrations as high as 375 μM (Fig. 4). Whether or not this reflects greater structural similarity of Vpu channels to Na^+/H^+ antiporters than to ENaC – or other channel families – may be revealed by future comparative structural modelling.

In our hands, amantadine was not found to inhibit the Vpu ion channel, even at 1 mM. However, it could

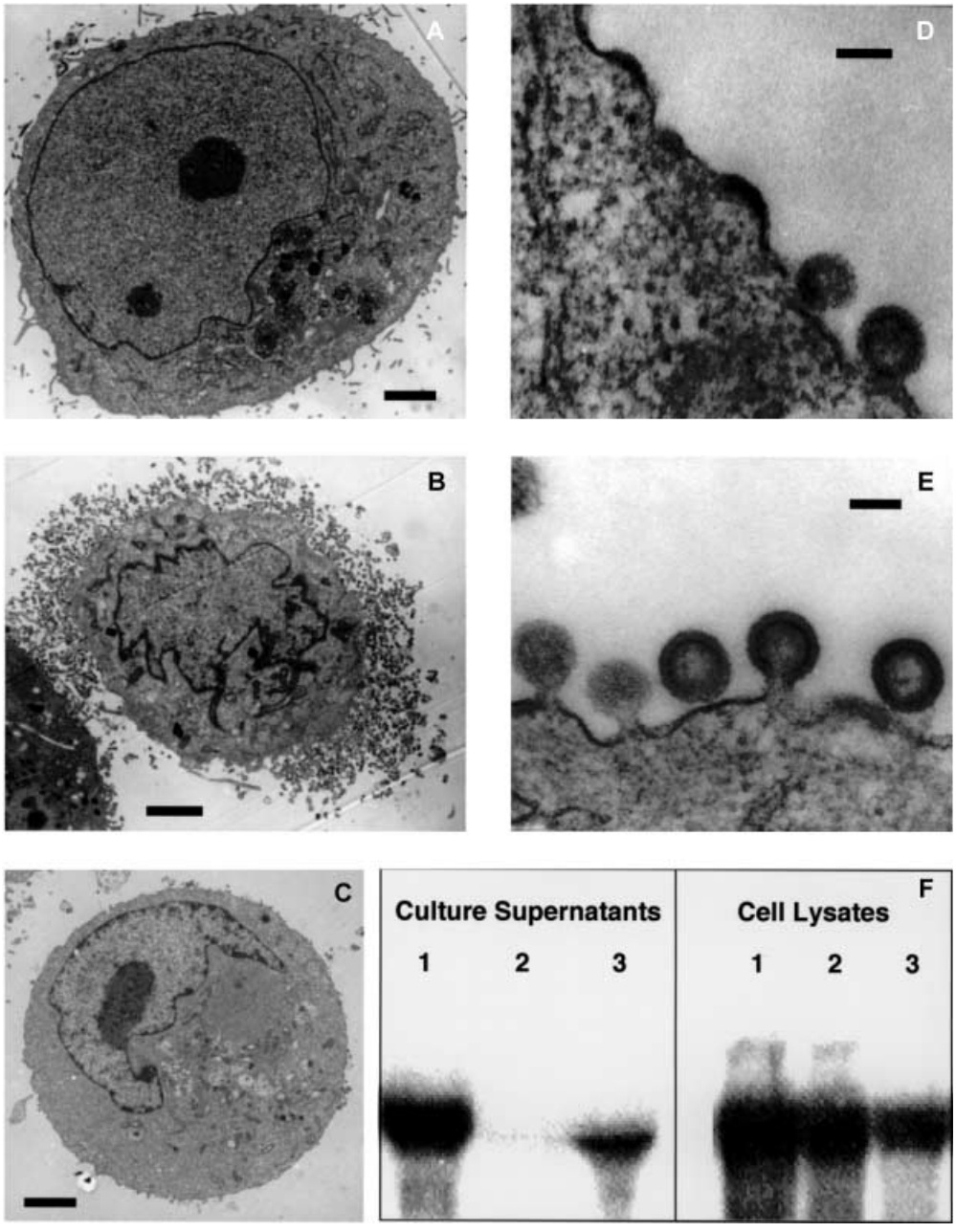


Fig. 5A–F The effect of HMA on budding of virus-like particles from HeLa cells. **A–E** Electron micrographs of fixed and sectioned HeLa cells showing the cell, budded VLPs and incipient buds. The cells were infected with vTF7.3 and subsequently transfected with pAN1343 alone – for the expression of HIV-1 Gag (**A** and **D**) – or with both pAN1342 and pAN1300 – for co-expression of Gag and Vpu (**B**, **C** and **E**). **C** A representative cell co-expressing Gag and Vpu in the presence of 10 μM HMA, showing reduced levels of incipient bud formation and associated VLPs. Scale bars: **A–C**, 2 μm; **D** and **E**, 100 nm. **F** An autoradiograph of PAGE gels showing the relative levels of Gag protein immunoprecipitated from culture supernatants and cell lysates prepared from cultures of cells transfected with Gag and Vpu expression plasmids, in the absence (lanes 1) or presence (lanes 2) of 10 μM HMA. Lanes 3 were loaded with equivalent samples from cultures of cells transfected with the Gag expression plasmid but not the Vpu expression plasmid

be noted that Kolocouris et al. (1996) have identified a number of chemical derivatives of amantadine that have ''borderline anti-HIV-1 activity'' but no effect on HIV-2. In the light of this, it may be worthwhile to test those compounds for their ability to inhibit the Vpu ion channel.

In addition to blocking the Vpu channel, HMA and DMA were also found to inhibit another activity associated with Vpu: enhancement of Gag-driven budding of VLPs from the plasma membrane of a mammalian cell (Fig. 5). This result confirms the previously proposed mechanistic link between the ion channel and the budding process established with mutations of the N-terminal TM segment (Schubert et al. 1996b). In the presence of 10 μM HMA, many fewer VLPs were seen in close proximity to cells in fixed sections (Fig. 5C) than in cells co-expressing Gag and Vpu in the absence of HMA (Fig. 5B). Interestingly, although incipient buds and VLPs were still present (Fig. 5C), the amount of Gag detected in culture supernatants in the presence of HMA was less than the amount recovered in the Gag-only control (Fig. 5F, lanes 2 and 3). While it was expected that the HMA depression of budding would not be complete because Gag alone drives a low level of budding in the absence of Vpu (Paul et al. 1998; Deora et al. 2000), these results indicate that the presence of drug-inhibited Vpu channels interferes with the Gag-driven budding mechanism in some way. HMA does not inhibit cellular synthesis of the Gag protein: analysis of cell lysates shows that the level of Gag (Fig. 5F)

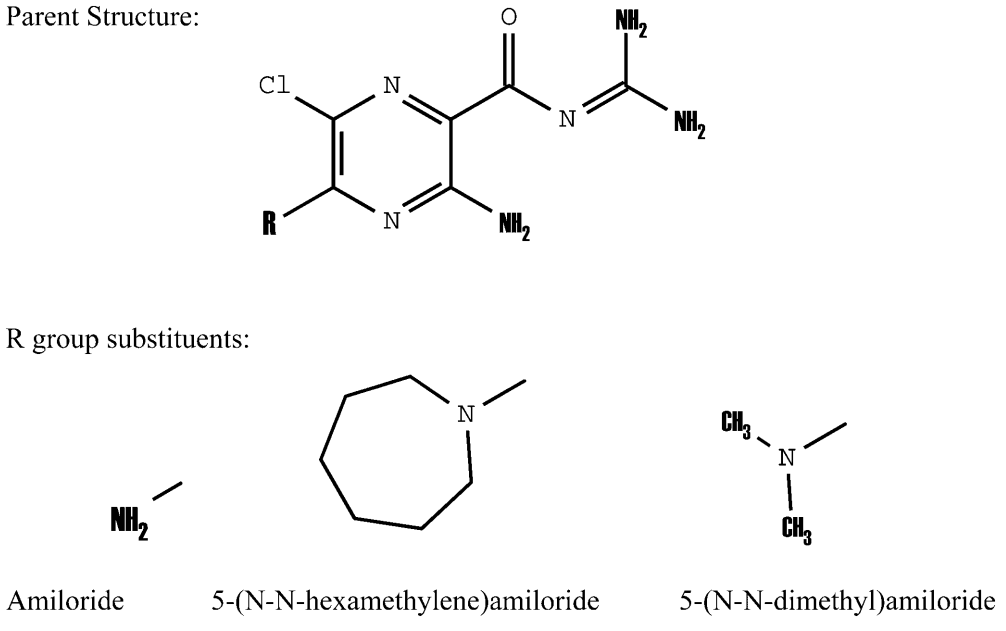


Fig. 6 Amiloride derivatives used in this study

and Vpu (not shown) in the cell is not affected by the drug.

Although 10 μM HMA was found to be effective at inhibiting VLP budding enhancement, this concentration of drug produced variable levels of inhibition of the Vpu channel in bilayer experiments. The reason for this apparent discrepancy is unknown. However, although there may be other factors involved, it is possible that the explanation lies in differences in the structural conformation of the channel arising from the different physical environments in the two experiments. Such conformational differences may alter the exposure and availability of drug binding sites, for example. Interestingly, 20–100 μM amantadine was used to block M2 channels in bilayer experiments (Duff and Ashley 1992; Pinto et al. 1992), while in a HeLa cell-based assay of M2 activity, 5 μM amantadine was used (Sakaguchi et al. 1996).

Virion budding is clearly of fundamental importance in the life cycle of HIV-1 and hence an obvious question raised by the results presented here is whether amiloride analogues might be useful drugs in preventing or delaying the development of AIDS in HIV-1-positive individuals. Unfortunately, a complete understanding of the roles played by Vpu in the course of HIV-1 infection in vivo remains elusive, hindered by the lack of a direct animal model. Certainly, the relevance of Vpu, and in particular its ion channel activity, to the replication of HIV and development of AIDS has been questioned (Lamb and Pinto 1997). Nevertheless, the evidence from a number of investigations is clear: Vpu has been shown to enhance virion release in various cultured human cell types infected with live virus. Interestingly, the enhancement is much more pronounced in macrophages than in peripheral blood mononuclear cells (PBMC) (Schubert et al. 1995). One study reported a decrease in virus release of approximately three orders of magnitude in macrophages when Vpu was ablated. However, in the same study, the release of virus from PBMC was virtually unaffected (Balliet et al. 1994). Hence, the relative effect of Vpu on virion budding is dependent on both host cell type and HIV-1 isolate. The matter is further complicated by a redundancy in the virus release function: the Env protein is able to substitute for Vpu to variable degrees depending on the viral isolates (Schubert et al. 1999). Despite this variation in the relative importance of Vpu in virus replication, it seems likely that drugs that target Vpu and inhibit HIV-1 budding in certain cell types in vivo may be useful in the fight against AIDS. The clear dependence on Vpu of virus release in macrophages is particularly relevant, given that infection by macrophage-trophic isolates of HIV-1 has been proposed to be necessary and sufficient for the development of AIDS (Mosier and Sieburg 1994; see comments). It has also been argued that chronic replication of HIV-1 in macrophages is critical for AIDS pathogenesis (Schuitemaker 1994).

Recently, a highly pathogenic chimeric simian-human immunodeficiency virus ($SHIV_{KU-1}$) was isolated after passage of non-pathogenic SHIV-4 in pig-tailed macaques (McCormick-Davis et al. 1998). Studying the chronology of the genetic changes during acquisition of virulence, it was found that reversion of the *vpu* open reading frame – allowing expression of human Vpu – correlated with the major phase of $CD4^+$ T-cell loss and profound loss of immunocompetance. Interestingly, a subsequent study, introducing a deletion mutation into the *vpu* gene of the pathogenic isolate, showed that Vpu was not essential for T-cell loss or development of neuro-AIDS (McCormick-Davis et al. 2000). It was suggested that expression of Vpu in the original non-pathogenic strain may have conferred a replicative advantage, allowing accumulation of mutations in Env

and/or Nef that were ultimately responsible for pathogenicity.

Drugs that modify the ion channel function of Vpu may well serve as useful tools for probing the role of this activity in the mechanism of virus budding. They may also prove to be beneficial in AIDS therapies. In this regard, both HMA and DMA have already been shown to be stable and well tolerated in mammals: plasma concentrations of up to 40 μM were reported in pigs (Holthe et al. 1992; Lee and Tannock 1996; Meng et al. 1990). In addition, amiloride itself is currently prescribed as a pharmaceutical potassium-sparing diuretic at doses of 10–20 mg per day. Therefore, testing of these or other amiloride derivatives or analogues might be expected to proceed rapidly through initial phases of clinical trials. There is also a need for further fundamental research to define the structure of the Vpu ion channel and to understand its mechanism. Such information, together with structure-activity relationship data for amiloride derivatives and analogues, could then be used to rationally design blocking drugs with maximum affinity and specificity.

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