Small molecule inhibitors of the annexin A2 heterotetramer prevent human papillomavirus type 16 infection

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Objectives: High-risk human papillomavirus (HPV) infection leads to the development of several human cancers that cause significant morbidity and mortality worldwide. HPV type 16 (HPV16) is the most common of the cancer-causing genotypes and gains entry to the basal cells of the epithelium through a non-canonical endocytic pathway that involves the annexin A2/S100A10 heterotetramer (A2t). A2t is composed of two annexin A2 monomers bound to an S100A10 dimer and this interaction is a potential target to block HPV16 infection. Here, recently identified small molecule inhibitors of A2t (A2ti) were investigated for their ability to prevent HPV16 infection in vitro.

Methods: A2ti were added to HeLa cells in increasing concentrations prior to the addition of HPV16. Cytotoxicity was evaluated via trypan blue exclusion. HPV16 pseudovirion infection and fluorescently labelled HPV16 capsid internalization was measured with flow cytometry.

Results: A2ti blocked HPV16 infection by 100% without substantial cellular toxicity or reduction in cell growth. Furthermore, A2ti blocked HPV16 entry into epithelial cells by 65%, indicating that the observed inhibition of HPV16 infection is in part due to a block in entry and that non-infectious entry may occur in the absence of A2t binding.

Conclusions: These results demonstrate that targeting A2t may be an effective strategy to prevent HPV16 infection.

Keywords: HPV16, annexin A2/S100A10 heterotetramer, A2t

Introduction

High-risk human papillomavirus (hr-HPV) infection leads to the development of several infection-related cancers including cervical, anogenital and head and neck cancers that are a significant health burden worldwide.1–3 HPV type 16 (HPV16), the most common of the hr-HPV genotypes, is an obligatory intracellular non-enveloped virus that must gain entry into host basal cells of the epithelium to deliver its double-stranded DNA to the nucleus and the HPV16 capsid proteins play a vital role in these steps.5 We previously reported that the annexin A2/S100A10 heterotetramer (A2t) facilitates infectious entry of HPV16 into epithelial cells through a direct protein–protein interaction (PPI) between the S100A10 subunit of A2t and the HPV16 L2 minor capsid protein.6 PPIs are increasingly being explored for small molecule drug discovery and the identification of A2t as an HPV16 receptor makes it a promising target for inhibition. Annexin A2 (A2) is found cytoplasmically as a monomer or at the cell surface as a heterotetramer consisting of two A2 monomers bridged non-covalently to an S100A10 dimer.7 The dimeric S100A10 structure yields two binding pockets that accommodate the N-terminus of A28 and it is this interaction that preliminary drug discovery studies have targeted.9 Recently, inhibitors of A2t (A2ti) that specifically disrupt the PPI between A2 and S100A10 have been identified,10 but have not been explored in the context of HPV infection. Here, we investigated the ability of A2ti to inhibit HPV16 entry and infection of epithelial cells in vitro.
**Materials and methods**

**Cells, reagents and HPV16 pseudovirions (PsV)**

HeLa cells (ATCC, Manassas, VA, USA) were maintained in IMDM with 10% FBS and PenStrep (Lonza, Walkersville, MD, USA) at 37°C with 5% CO₂. Spontaneously transformed HaCaT keratinocytes (ATCC) were maintained in Keratinocyte Serum-Free Media (Life Technologies, Carlsbad, CA, USA) with manufacturer-provided growth supplement at 37°C with 5% CO₂. A2t inhibitors were purchased from Asinex (Moscow, Russia) and reconstituted in DMSO. The ability of A2ti to disrupt HPV16 infection was verified via isothermal titration calorimetry following standard procedures. HPV16 PsVs were produced by co-transfection of 293TT cells with plasmids encoding codon-optimized HPV16 L1 and L2 following published procedures. HPV16 PsVs were produced by co-transfection of 293TT cells with plasmids encoding codon-optimized HPV16 L1 and L2 following published procedures.

**Toxicity assay**

HeLa cells were incubated at 37°C in 5% CO₂ with increasing concentrations of A2ti-1 or A2ti-2 for 72 h. Cells were then counted and viability was measured by trypan blue exclusion. In control experiments, cells were left untreated or were treated with DMSO at matched concentrations to A2ti delivery.

**HPV16 PsV infection assay with A2ti**

HeLa or HaCaT cells seeded at 2 × 10⁵ cells/well were incubated overnight in 24-well plates at 37°C with increasing concentrations of A2ti-1 or A2ti-2. The following day, cells were incubated with HPV16 PsVs containing a GFP reporter plasmid using an moi of 50. Using flow cytometry, infection was measured 48 h post-HPV16 PsV treatment as the percentage of GFP-positive cells. In control experiments, cells were left untreated or were treated with DMSO.

**HPV16 PsV internalization assay with A2ti**

HPV16 PsVs were labelled with a pH-dependent rhodamine fluorophore (pHrodo, Life Technologies) or carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Life Technologies). HeLa cells seeded at 2 × 10⁵ cells/well were incubated overnight with increasing concentrations of A2ti-1 or A2ti-2. The next day, pHrodo- or CFDA-SE-labelled HPV16 PsVs were added at 1 μg/10⁶ cells and incubated at 37°C for 6 h. The mean fluorescence intensity (MFI) of the cells was analysed by flow cytometry. In control experiments, cells were left untreated or were treated with DMSO.

**Results**

In the current study, we examined the ability of two A2ti to block HPV16 entry and infection in vitro. The first of these inhibitors (A2ti-1) has a reported IC₅₀ of 24 μM and it was hypothesized that A2ti-1 would block HPV16 PsV infection within a reasonable range of this value. The second of the tested inhibitors (A2ti-2) is similar in chemical structure, but the deletion of an ethyl group results in an increased IC₅₀ of 230 μM and was therefore predicted to be less effective in blocking HPV16 infection while having similar, if any, off-target effects. A2ti-1, A2ti-2 and DMSO matched to the concentration of A2ti delivery were found not affect total cells recovered after 72 h (Figure 1a and c). Before performing internalization and infection assays, we confirmed that A2ti targeted A2t with isothermal titration calorimetry and found that A2ti targeted the S100A10 dimer of A2t (Figure S1, available as Supplementary data at JAC Online). Next, we sought to determine the effect of A2ti on HPV16 PsV infection. We found that the higher-affinity A2ti-1 reduced HPV16 PsV infection of HeLa cells in a dose-dependent manner with 100% inhibition of infection observed at 100 μM (Figure 2a) and similar results were observed in HaCaT cells (data not shown). As predicted, lower-affinity A2ti-2 was less effective with <50% reduction in HPV16 PsV infection achieved at 100 μM while DMSO vehicle had no effect. To determine whether the reduction in infection was due to a decrease in capsid entry, we next evaluated the ability of A2ti to block internalization of HPV16 into cells using PsV

**Figure 1.** A2ti do not affect cell growth and are non-toxic to HeLa cells. (a) Chemical structures and IC₅₀ values of A2ti-1 and A2ti-2. HeLa cells were left untreated or treated with A2ti-1, A2ti-2 or DMSO. After 72 h, cells were counted (b) and viability was measured (c) via trypan blue exclusion. The mean ± SD percentage viability is presented (n = 6). The graph is representative of three independent experiments.
Figure 2. A2ti block HPV16 infection and entry into HeLa cells. (a) HeLa cells were treated with increasing concentrations of A2ti-1, A2ti-2 or DMSO control. The following day cells were infected with GFP-plasmid-containing HPV16 PsV. GFP-positive cells were measured after 48 h by flow cytometry. The mean ± SD percentage of infected cells normalized to the PsV-only group is presented. (b) HeLa cells were incubated with increasing concentrations of A2ti-1, A2ti-2 or DMSO control. The next day, pHrodo-labelled HPV16 PsV were added to the cells for 6 h and the MFI of cells was analysed by flow cytometry. The mean ± SD fold change in MFI normalized to the untreated group is presented (n=4). Each graph is representative of three independent experiments (*P<0.05, **P<0.01 and ***P<0.001 as determined by a two-tailed, unpaired t-test compared with PsV only).
Discussion
Our group previously identified A2t as an HPV16 uptake receptor using a combination of cellular, molecular and biochemical techniques including small hairpin RNA knockdown, antibody (Ab) neutralization and electron paramagnetic resonance. Specifically, we demonstrated that the S100A10 subunit of A2t binds to amino acids 108–126 of HPV16 L2; A2t coimmunoprecipitates with HPV16 at the cell surface; A2t mediates HPV16 entry and infection in an L2-dependent manner; and a previously identified natural A2t ligand, secretory leucocyte protease inhibitor, reduced HPV16 PsV infection of epithelial cells. Dzidusko and Ozburn independently confirmed the role of A2t in HPV16 entry and infection by showing that (i) early HPV16 binding results in the translocation of A2t to the extracellular surface, (ii) A2t cointernalizes with HPV16 and mediates intracellular trafficking and (iii) anti-A2 and anti-S100A10 Abs block HPV16 PsV infection at different stages of HPV16 infection both pre- and post-entry. In the current study, we show a significant decrease in HPV16 PsV internalization and a complete reduction in HPV16 infection with A2ti(A2ti-1). Taken together, these results highlight the importance of A2t in HPV16 infection and demonstrate the potential for targeting A2t to block HPV16 infection. Additionally, A2t has been implicated in infection by other viruses including CMV, RSV, enterovirus 71 and HIV infection of macrophages. Consequently, A2t inhibition has broad appeal as an antiviral strategy.

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Transparency declarations
None to declare.

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Supplementary data
Figures S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References

