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Identification of Inhibitors to Papillomavirus Type 16 E6 Protein Based on Three-dimensional Structures of Interacting Proteins

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Abstract

Human papillomaviruses (HPV) cause cutaneous and genital warts. A subset of HPV types is associated with a high risk for progression to malignancy. The E6 protein from the high-risk HPV types represents an attractive target for intervention because of its roles in viral propagation and cellular transformation. E6 functions in part by interaction with human cellular proteins, several of which possess a helical E6-binding motif. The role for each amino acid in this motif for binding E6 has been tested through structure determination and site-directed mutagenesis. These structural and molecular biological approaches defined the spatial geometry of functional groups necessary for binding to E6. This E6-binding information (the E6-binding pharmacophore) was transferred into a three-dimensional query format suitable for computational screening of large chemical databases. Compounds were identified and tested using *in vitro* and cell culture-based assays. Several compounds selectively inhibited E6 interaction with the E6-binding protein E6AP and interfered with the ability of E6 to promote p53 degradation. Such compounds provide leads for the development of new pharmacologic agents to treat papillomavirus infections and their associated cancers.

Keywords

Papillomavirus; HPV-16; E6; in silico screening; antiviral; protein-protein interaction

1. Introduction

Papillomaviruses are small double-stranded DNA viruses that infect epithelial tissues and cause cutaneous, mucosal, and anogenital warts. Genital human papillomavirus (HPV) DNA is detected in 5 – 20% of persons between the ages of 14 and 50 and in 10 – 40% of sexually active women between the ages of 16 and 25 in the USA (Phelps et al., 1998). Genital warts are highly transmissible and affect all races and socioeconomic groups. Although not a reportable condition, the Division of STD/HIV Prevention of the Centers for Disease Control estimates that there are 750,000 new cases of genital warts each year and 1.5 million persons

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under treatment. New cases represent only 10% of the ~7 million individuals in whom HPV causes clinically detectable warts.

There are more than 100 genotypes of HPV, a subset of which are associated with the development of malignant lesions and classified as “high-risk” for their ability to promote cancer. DNA from high-risk HPV has been found in over 95% of cervical cancer cases (Munoz et al., 2003). Approximately 50% of all cervical cancers contain HPV-16. Other viral genomes, HPV-18, HPV-31, and HPV-45, together comprise another 20% of cervical cancers (Munoz et al., 2003). The low-risk viruses, such as HPV-6 and HPV-11, are found in genital warts but are rarely associated with cervical cancer.

Papillomavirus infection is thought to begin with invasion of the basal epithelium. In undifferentiated basal cells, the viral genome is maintained extra-chromosomally at low copy number. Since the viral protein coding capacity is small, the virus hijacks cellular factors in order to replicate. As daughter cells begin to differentiate and become non-permissive for DNA synthesis, the virus induces the G1 to S-phase transition to initiate synthesis of viral DNA and expresses early viral genes to prevent cellular stress responses such as p53 activation (McMurray et al., 2001). Unscheduled cellular proliferation such as that caused by viral infection is a signal for cell death via apoptosis. The pro-apoptotic protein p53 is central to this cellular defense mechanism by up-regulating expression of apoptotic proteins in response to cellular stress.

The high-risk papillomaviruses have evolved a mechanism to block the p53 response. Papillomaviruses encode eight major proteins with additional products resulting from alternatively spliced mRNAs. The HPV-16 E6 protein complexes with the cellular factor E6AP (E6-associated protein) and forms an ubiquitin ligase that specifically binds to and targets p53 for ubiquitin-mediated degradation (Huibregtse et al., 1991; Scheffner et al., 1994; Scheffner et al., 1993). E6AP does not bind p53 in the absence of E6. HPV genomes encoding E6 mutants that are unable to degrade p53 cannot replicate in skin keratinocytes (Park and Androphy, 2002; Thomas et al., 1999).

In addition to targeting p53 for degradation, the E6 protein plays other roles in promoting viral replication (Underwood et al., 2000). E6 also disrupts cell cycle checkpoints to promote cellular proliferation (Kaufmann et al., 1997; Malanchi et al., 2002; Thompson et al., 1997). Cells expressing E6 have increased telomerase activity that delays cellular senescence (Klingelutz et al., 1996; Stoppler et al., 1997). E6-induced transcription of the catalytic component of telomerase (hTERT) appears to involve E6AP, which may induce degradation of a repressive factor at its promoter (Gewin et al., 2004; Liu et al., 2005). The HPV E6 proteins also alter the transcriptional pattern of a variety of cellular and viral promoters, which seems to be in large part mediated by its interaction with E6AP (Kelley et al., 2005). E6 thus represents an excellent target for development of antiviral agents.

HPV E6 proteins contain about 150 amino acid residues and two “zinc finger” subdomains (Figure 1) and function through interaction with cellular factors in addition to E6AP including E6BP/ERC-55, E6TP1, ADA3, IRF-3, Bak, MCM-7, Blk, paxillin, CBP/p300, hDlg and other PDZ domain containing proteins (Fehrmann and Laimins, 2003; Scheffner and Whitaker, 2003; Thomas and Chiang, 2005). For many of the factors, the essential core of the binding region has been delineated and contains a consensus sequence of **LxxφLsh**, where **L** is leucine, **s** is a small amino acid (glycine or alanine), **φ** is a hydrophobic residue (usually leucine), and **h** is usually aspartate, asparagine, glutamate, or glutamine and **xx** is a dipeptide where one of the residues is Asp, Glu, Asn, or Gln (Figure 1A). The structure of several peptides containing this “charged leucine” E6-binding motif have been determined in the absence of E6 (Be et al., 2001; Chen et al., 1998). This domain forms an alpha helix, with the leucines forming a

hydrophobic surface on one face of the helix and the charged amino acids on the opposite face (Figure 1B). Replacement of any leucine in the binding motif by alanine disrupts binding to E6, as evidenced by the inability of GST-E6 to pull-down binding proteins (Be et al., 2001; Bohl et al., 2000; Chen et al., 1998). Polar residues that reside on the helix opposite the hydrophobic surface contribute to binding, as mutations in the related E6BP or paxillin proteins show loss in binding (Bohl et al., 2000; Chen et al., 1998).

Although the three-dimensional features of E6-binding motif containing peptides and proteins are known, the binding determinants of E6 are not well characterized (Nomine et al., 2006). Some investigators have found that an N-terminal portion of HPV-16 E6 is sufficient for binding, while others have clearly shown single-point mutants in the C-terminal half can disrupt binding to α -helical partners (Lagrange et al., 2005; Liu et al., 1999b; Nguyen et al., 2002). Several point mutants in E6 directly disrupt binding to E6AP (Liu et al., 1999a; Zimmermann et al., 1999). L37S, L50G, and Y54D are in the top of the first zinc finger, while Q107R, L110Q, H118D, F125V, I128T, G130V, W132R, G134V are in the second zinc finger. A model of the structure of E6 predicts that all of these residues are buried, suggesting that each mutation disrupts E6 structure instead of modifying the surface properties of the protein (Nomine et al., 2006). Because there is evidence that each zinc finger of E6 represents a separately folded domain (Nomine et al., 2003) and since the functional site of many proteins typically lie at the interface of two domains, the α -helical partner E6AP protein may bind into a pocket formed by both zinc fingers of E6. Such a model is supported by the E6AP-binding mutations in E6 that occur in both zinc fingers, but not in the N or C-termini of the protein or in the region connecting the zinc fingers (Figure 1B). Peptides containing the charged leucine helical motif inhibit the interaction between E6 and both E6AP and E6BP, as well as the ability of E6 to promote the degradation of p53 (Bohl et al., 2000; Butz et al., 2000; Elston et al., 1998; Huibregtse et al., 1993; Liu et al., 2004; Sterlinko Grm and Banks, 2004). We hypothesize that non-peptidic compounds can be selected that resemble the structure and functional features of inhibitory peptides to compete with E6AP for binding to HPV-16 E6. Such low molecular weight inhibitors would serve as leads for antiviral agents and help understand the biology of E6.

There are no specific medical treatments targeting papillomavirus-induced diseases. Therapies for cutaneous and genital warts and advanced cases of cervical dysplasia involve destruction or removal of the infected tissue by cytotoxic agents or by surgery (Beutner and Ferenczy, 1997). Prophylactic HPV vaccines have shown promising results in clinical trials (Koutsky et al., 2002; Villa et al., 2005), although there are major challenges to widespread use of a vaccine (Schiller and Davies, 2004). Moreover, a prophylactic HPV vaccine would offer no benefit for the millions of people already infected. Therapeutic nucleic acids that target viral reading frames are also being developed (Alam et al., 2005; DiPaolo and Alvarez-Salas, 2004; Storey et al., 1991). An effective antiviral agent could be used therapeutically to treat papillomavirus infection and would decrease the likelihood of progression to invasive cervical cancer and the spread of virus. In this project, we have discovered a series of lead inhibitors of papillomavirus E6 protein using structure-based approaches.

2. Materials and Methods

Calculation of location spheres for pharmacophore points

The pharmacophore model for binding the high-risk papillomavirus E6 protein comprises three lipophilic points, two hydrogen-bonding points, and a space that excludes the presence of atoms (an exclusion sphere). The locations of these points were derived from two peptide structures that bind E6. The analysis used the best structure of the E6AP peptide and the best structure of the E6BP peptide determined by NMR methods in the absence of E6 protein and the known mutagenesis data (Be et al., 2001; Chen et al., 1998). The sphere of steric exclusion was

centered on a position close to the trajectory between the two H α atoms 3.4 Å (E6AP) and 4.3 Å (E6BP) from the C α position of the conserved glycine. A 120 picosecond quenched MD (molecular dynamics) simulation of the peptide structures at 800K with backbones fixed was performed to obtain a range of structures that cover conformations both free and bound to E6 protein using CDISCOVER 97 (Accelrys Inc., San Diego, CA) and a stepsize of 1 femtosecond. A frame was saved every picosecond and subsequently minimized and the radii of the location spheres were determined by measuring the location distribution of the side chains of the superimposed MD trajectory frames.

Construction and query of a CATALYST database

CATALYST (version 4.0) (Molecular Simulations, Inc. (Accelrys, Inc.), San Diego, CA) versions of the NCI open database and the 1999 edition of the Sigma-Aldrich catalog were built using CATALYST default parameters for large databases following the method published previously for a CHEM-X 3D (Oxford Molecular (Accelrys, Inc., San Diego, CA) version of the NCI database (Milne et al., 1994). Options included selection of the FAST conformation generation, a maximum of 100 conformations stored with a maximum energy threshold of 20 kcal/mol. 97% (240,000 compounds) of the NCI database compounds and 97% (97,000 compounds) of the Sigma-Aldrich entries with structures could be processed. Structures with errors in connection tables or certain substructures that cannot be handled by CATALYST were not used further. Queries used the points of the pharmacophore derived from the E6AP coordinates and the E6BP coordinates and varied the nature of the hydrophilic pharmacophore points ($-\text{CO}_2\text{R}$, where R was hydrogen, NH_2 , or a carbon fragment). Queries took 1 to 60 minutes for completion.

p53 degradation assays in vitro and in cell culture

In vitro degradation assays with ^{35}S -labeled E6 and p53 proteins translated in reticulocyte cell lysate were performed at 25°C as described previously (Sherman et al., 1997). As a control, DMSO was added to a final concentration of 2% for degradation assays in the presence of E6 and p53 to determine p53 levels after uninhibited degradation (0% inhibition). An experiment without added E6 in 2% DMSO was used to assess basal p53 levels in the absence of degradation (100% inhibition). Using these values as standards, the inhibitory potential for each compound was determined at a series of concentrations ranging from 5 to 500 μM . All degradations were performed in duplicate and replicated in at least three separate experiments. The plot of normalized intensities (I) versus inhibitor concentration (C) was fit with the equation $I = 1 / (1 + 10^{(\log C - \log \text{IC}_{50})})$ to determine the concentration at which binding was reduced by 50% (IC_{50}).

Assays in cell culture

Compound 2, which is brightly colored, was added to the culture medium of NIKS cells at 100 and 500 μM , respectively. NIKS cells contain wild-type (WT) p53 and exhibit many characteristics of early-passage keratinocytes, including the ability to stratify, to differentiate, and to maintain the HPV life cycle (Flores et al., 1999). Twenty-four and forty-eight hours later, cells were examined under Nikon Eclipse TE200 inverted microscope.

To assess the ability of the compound to inhibit E6-mediated p53 degradation in cultured cells, NIKS cells were infected with amphotropic retroviruses containing the pBabe Puro vector, HPV-16 E6, and E6 mutants F2V and Y54D that are defective for p53 degradation, and respectively (Allen-Hoffmann et al., 2000; Liu et al., 1999a). After puromycin selection, populations of infected cells were pooled and used within 10 passages. E6 expression was confirmed by real-time PCR and E6 mutations were confirmed by sequencing of the PCR products. To analyze the inhibitory effects of E6 inhibitors, cells were treated with 8 μM mitomycin C (MMC) for 18 hours and then compounds for 24 hours. Cells were harvested and

p53 levels were determined by immunoblot analysis (Alfandari et al., 1999). Filters were cut into strips and reacted with the p53 DO-1 monoclonal antibody (Mab) (SC-126) from Santa Cruz Biotechnology (Santa Cruz, CA); and mouse monoclonal antibody against β -tubulin (T4026, Sigma). Proteins were visualized by enhanced chemiluminescence (SuperSignal, Pierce, Arlington Heights, IL) using peroxidase-conjugated rabbit anti-mouse IgG (A9044, Sigma) according to the manufacturer's protocol. Protein amounts were determined by densitometric scanning (Image Gauge, Fuji, Japan).

In vitro E6 binding assays

An assay was developed to replace the gel-based system used to examine inhibitory molecules (Liu et al., 2004) with a non-radioactive 96-well plate format and will be described in detail elsewhere. In brief, the E6 binding motif region from E6AP was fused in-frame onto the N-terminus of bacterial alkaline phosphatase (BAP) (Yamabhai and Kay, 2001). The BAP fusion was expressed in *E. coli* and purified using the FLAG epitope tag. GST-E6 protein bound to glutathione beads was captured on 96-well filter plates and the E6AP-BAP fusion dispensed into the wells in the presence of inhibitors. Binding efficiency, measured as relative light units (RLU) on a luminometer using the Immunostar-AP substrate (BioRad), was determined as the level of BAP activity remaining in each well after washing. A plot of the data as a function of RLU versus the concentration of the inhibitor, C , was fit assuming single binding site competition with the equation $RLU = RLU_0 / (1 + 10^{(\log C - \log IC_{50})})$ where X was the concentration of inhibitor and RLU_0 is the RLU in the absence of inhibitor. For specificity, anti-FLAG M2 antibody resin (A220 Sigma) was used to measure the ability of compounds to compete with the FLAG-tagged E6AP-BAP fusion for binding to the antibody. A second assay measured the interaction between p107 (a homolog of pRb) and HPV-16 E7 protein using a gel-based method (Liu et al., 2004).

Assessment of compound properties

The LogP values for compounds were calculated using the LogP DB v. 3.5 program (ACD Labs), to indicate the differential solubility or partition coefficient between water and octanol that provides a measure of the hydrophobicity of the compounds. Known drugs typically follow the Lipinski rules and have logP values less than 5, molecular weights less than 500, fewer than 5 hydrogen bond donors and fewer than 10 hydrogen bond acceptors (Lipinski et al., 2001).

3. Results

3.1 Definition of the E6-binding pharmacophore

Conversion of the 3D NMR structures of E6 inhibitory peptides to 3D queries was needed for database searches that could uncover small, nonpeptidic molecules that bind to and inhibit the E6 protein. The points in space assumed necessary for activity were derived by considering the surface of the E6-binding partners available for contact with E6 is primarily composed of side-chain atoms of the polypeptide. The three-dimensional structure determination of E6AP and E6BP peptides provided a rendering of the surface (Be et al., 2001; Chen et al., 1998). Coupling this structural information with our understanding of the role for each amino acid side-chain in contact with the viral E6 protein defined the pharmacophore for binding to E6. Mutagenesis and conservation of residues have shown the critical importance of leucine residues. For example, in E6AP, the loss of the isopropyl group of leucine 9, 12, or 13 in the motif through replacement by alanine correlates with reduced binding (Be et al., 2001). These moieties are located in the three-dimensional structures of the E6AP peptide roughly at the points of an equilateral triangle 7.5 Å apart (Figure 2). Likewise, the replacement by alanine of the side-chain of hydrophilic amino acid residues (Gln 10 and Glu 15 in E6AP, and Glu 22 and Asp 27 in E6BP) defined points in space for hydrogen bonds located approximately 9.5 Å

above the plane of the hydrophobic triangle. The conservation of the small residue glycine (residue 14 in E6AP, residue 26 in E6BP) near the C-terminus of the motif was used to define a region of steric exclusion (Figure 2). The two static pharmacophores for binding the high-risk papillomavirus E6 each comprise three lipophilic points, two hydrogen-bonding points, and one exclusion sphere.

The pharmacophores are based on single representative structures (Be et al., 2001; Chen et al., 1998). To accommodate the ensemble of structures in solution that presumably include the bound conformation, we performed quenched molecular dynamics simulations (MD simulation at a high temperature of 800 K followed by minimization of frames). The resulting space occupied by the side-chains was estimated as spheres 2 to 3 Å in diameter (Figure 2). The inclusion of a range of conformations thus expands the point pharmacophores to more realistic dynamic pharmacophores reflecting the diversity of structures that bind the E6 protein.

The pharmacophores were made more general by consideration of equivalent chemical groups for the amino acid side-chains. For example, the lipophilic phenylalanine side-chain in the E6-binding motif of E6BP (residue 24) is homologous to lipophilic leucine in E6AP (residue 12). The atoms in the hydrophobic group cluster were therefore considered equivalent to aliphatic, alkene, or aromatic atom types. Likewise for the hydrophilic centers, both oxygen and nitrogen atoms were considered equivalent for testing. The final pharmacophore model thus comprised three general hydrophobic centers, two hydrophilic centers, and one exclusion sphere.

3.2 Chemical Database Query

Each pharmacophore, representing a three-dimensional format, was used to query the National Cancer Institute (NCI) open chemical database comprising approximately 240,000 compounds and the Sigma-Aldrich Library of Rare Chemicals of 97,000 compounds. Because the compounds in the database are represented as static structures, a conformational version of the databases was generated using CATALYST 4.0. With both hydrophilic centers presented as carboxylic acids, 144 compounds met the criteria set of the pharmacophores and were selected for testing. In E6AP, replacement of the carboxylic acid with a carboxamide at Gln10 reduced the number of hits to zero; in E6BP, replacement at Glu22 reduced the number from 64 to 2. Replacement of the proton of the carboxylic acid with a carbon fragment did not greatly affect the number of hits, but resulted in additional compounds. In total, 260 compounds were selected—208 compounds from the NCI database and 52 from the Sigma-Aldrich database. A filter was then applied to remove compounds with MW >1000 (because large molecules are unsuitable as drug candidates), heavy metal atoms (because of their potential for cytotoxicity), and acyclic compounds (because of unsuitability as drug candidates due to the many degrees of freedom associated with the flexibility of non-ring structures). The remaining compounds were manually screened to remove porphyrin rings because the CATALYST program did not present them properly (and because of their propensity to bind proteins non-specifically). In addition, scaffolds containing long alkyl chains were excluded because their unsuitability as inhibitors or drug candidates due to the many degrees of freedom associated with the flexible chain. 119 compounds were ordered from the NCI database, of which 48 entries had physical samples that were available for testing. 25 compounds were from Sigma-Aldrich.

3.3 Assay of Pharmacophore-selected Compounds

The 73 available compounds that met the criteria established by the pharmacophore were initially assayed at 500 µM using an *in vitro* p53 degradation assay. Compounds that scored greater than 50% inhibition were subsequently assayed at 500 µM for their ability to inhibit the interaction between E6 and E6AP using an gel-based *in vitro* E6-E6AP binding assay. Compounds that scored greater than 50% inhibition in the both assays were identified as potential E6 inhibitors. 10 compounds, four from the NCI group (1–4, Figure 3) and six from

the Sigma-Aldrich group (**5–10**, Figure 4) showed inhibitory activity in both assays. The large percentage of inactive compounds is not surprising given that the pharmacophore is based on an incomplete understanding of the E6-E6AP molecular interface, and is consistent with observations made in similar systems (Galatin and Abraham, 2004; Lang et al., 2005).

All NCI compounds were dicarboxylic acids, with a central core comprising several aromatic groups. Possibly reflecting the rather larger dimensions of the pharmacophore, the molecular weights were rather high for small molecule leads, ranging from 581 to 911. The calculated logP values ranged from 4 to 8.7 with an average of 6.4, and these compounds were therefore not particularly drug-like or resemble chemical leads (Lipinski et al., 2001). The Sigma-Aldrich compounds showed more diversity, had generally lower molecular weight (ranging from 433 to 691) and lower logP values (ranging from 2.8 to 8.1, with an average of 4.8). Most of the Sigma-Aldrich compounds were fulfilled the Lipinski rules and represent chemical leads (Lipinski et al., 2001).

The compounds were then assayed for inhibition of the E6-E6AP interaction over a range of concentrations and showed a dose response (Figure 5). Each of the 10 compounds tested displayed an IC₅₀ value in the mid μ M range, with the weakest inhibition observed for compound **5** at 52 μ M and the most potent observed for compounds **4** and **10** at about 10 μ M (Table 1). As a positive control, an 18-mer oligopeptide corresponding to the charged leucine domain of E6AP was used to inhibit the interaction between E6 and E6AP. In this assay, the E6AP 18-mer had an IC₅₀ of 7 $\pm$ 4 μ M, close to the 4 $\pm$ 1 μ M affinity (K_d) calculated using surface plasmon resonance (Zanier et al., 2005). To confirm that these inhibitors were functionally active, they were tested over a range of concentrations in the in vitro, gel-based p53 degradation assay. Again all compounds inhibited p53 degradation with IC₅₀ values $\leq$ 130 μ M.

The compounds were also examined for specificity in two assays. The compounds were first tested for their ability to inhibit the interaction of anti-FLAG antibody to the FLAG epitope present on the fusion protein containing E6AP. In this assay, compounds **3**, **4**, **5**, and **8** had IC₅₀ values less than 500 μ M suggesting potential non-specific inhibition. As a chemiluminescent-phosphatase based assay, the E6AP-BAP binding assay may be inhibited by phosphatase inhibitors or colored compounds that block light transmission and may be susceptible to false negatives. Therefore, as a second control for specificity, the interaction between p107 (a homolog of pRb) and HPV-16 E7 protein was measured using a gel-based method (Liu et al., 2004). Eight of the ten showed no inhibition (extrapolated IC₅₀ values were $>$ 5 mM), while two compounds, **8** and **10**, caused some inhibition of the interaction between HPV-16 E7 and p107 at a concentration of 500 μ M.

Compound **8** showed significant inhibitory activities in both specificity control assays indicating that it is not specific. Compounds **3**, **4**, **5**, and **10** showed significant inhibitory activities in one of the specificity control assays suggesting possible non-specificity. Taken together, compounds **1**, **2**, **6**, **7**, and **9** show the best combination of inhibitory activity and selectivity. Note that Compounds **2**, **4**, and **5** have similar core structure scaffolds, but from this limited dataset no meaningful structure-activity relationship is obvious, especially given that the purity of the compounds has not been verified. Compound **9** appears to represent an excellent starting point for synthesis of a series of homologs by condensing aromatic carboxylic acids with N-alkyl anilines to improve specific affinity against E6.

We wished to determine to what extent the compounds are cell permeable. Compound **2** is brightly colored and could be directly monitored for cellular uptake. It showed little cellular uptake in NIKS cells at 100 μ M, but stained cells well at 500 μ M (data not shown). We then proceeded to develop a cellular assay to score E6 inhibition. NIKS cells were pre-treated with MMC before addition of the compound to increase p53 protein levels (Figure 6). We then

examined the effects of E6 inhibitors and controls for their ability to protect p53 from E6-mediated degradation. Compound **2** at a concentration of 100 μM did not consistently increase the steady-state level of p53 (data not shown), probably due to low level of cellular uptake. Significantly, compound **2** reduced E6-mediated p53 degradation at 500 μM by more than 4-fold. In contrast, this compound showed less increase in p53 levels in cells containing vector or E6 mutants (data not shown). The fact that the compound increased p53 level more efficiently in wild-type E6 expressing cells suggests that it can block the E6-E6AP interaction. On the other hand, the ability for this compound to induce p53 in the absence of wild-type E6 suggests that it can also induce p53 in an E6-independent manner, possibly caused by mechanisms including induction of cellular stress.

3.4 Assay of Randomly-selected Compounds

To evaluate whether the procedure for selecting inhibitors selected from the E6-binding pharmacophore model was superior to inhibitors chosen randomly, one hundred compounds were chosen at random from the NCI open chemical database. 59 compounds remained after retaining those compounds that were physically available and excluding compounds with MW >1000, heavy metal atoms, and acyclics. Four of the random set showed >50% inhibition of E6-mediated p53 degradation at 500 μM concentration and were then tested for inhibition of E6AP binding with E6. One compound had no activity, whereas three compounds had IC_{50} values of 48 ± 10 , 312 ± 148 , and 114 ± 27 μM (NCI compounds 9583, 124929, 134042, respectively). Interestingly, these three all shared a naphthoquinone moiety. In the anti-FLAG specificity assay, the compound with the best inhibition of E6, NCI 9583, showed anti-FLAG inhibition of 891 ± 533 μM , whereas the other compounds showed no anti-FLAG inhibition. The 3 best randomly-selected compounds had IC_{50} values that were less potent than the 10 compounds selected using the structure-based pharmacophore method with an average IC_{50} value that was nearly a log less potent than the pharmacophore-selected compounds (158 μM vs. 23 μM , respectively).

4. Discussion

The papillomavirus E6 protein is required for viral infection and progression to cancer and therefore is a logical target for antiviral therapy. In this paper, the three-dimensional structures of the E6-binding sequences were coupled with knowledge of the important groups for interaction with E6 to create a pharmacophore for binding to E6. Compounds selected on the basis of resembling the pharmacophore inhibited the activity of E6. Several of these compounds appear to represent excellent starting points for the selection or synthesis of closely related compounds to establish structure-activity relationship for each scaffold.

Our knowledge of the pharmacophore is incomplete. The current pharmacophore is characterized by six centers based on structural data of unbound E6 ligands and with mutagenesis and conservation data that sample only the amino acid side-chains. As a result, the success rate of finding inhibitors was only 10 out of 73 (14%), although higher than the hit rate from the compounds chosen randomly from the NCI database (3 out of 62, or 5%). If the success rate is applied more stringently to be defined as more than 50% inhibition in p53 degradation and an IC_{50} value of less than 50 μM for inhibiting the E6-E6AP interaction, 9 compounds are scored as hits in the pharmacophore-based approach, and only one from the randomly-selected group. Our hypothesis is that a more detailed and accurate pharmacophore, such as that from structures bound to E6, would increase the rate of finding additional active compounds with different scaffolds that have IC_{50} values in the nanomolar range.

Low molecular weight compounds that target the papillomavirus E6 protein represent a significant advance in finding inhibitors of protein-protein interactions (Arkin and Wells, 2004). One class of compounds directed against E6 promote release of the structural zinc

(Beerheide et al., 1999; Beerheide et al., 2000). The activity of high affinity peptides that block E6 supports the validity of our strategy to identify inhibitory low molecular weight compounds (Butz et al., 2000; Sterlinko Grm et al., 2004). The eventual outcome from this project is a chemically suitable compound with an IC50 in the nanomolar range that can be entered into a drug development program. The final product is expected to effectively treat existing benign and pre-malignant HPV-induced diseases and prevent malignant progression. An effective HPV inhibitor would reduce the incidence of cervical and anal dysplasia and the associated carcinomas that afflict millions of women and men each year.

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A

E6AP	LT L Q E LL G EER
E6BP	V S LQ E FL G DYR
Paxillin (LD1)	DD L Q E LL A DLD
Tuberin (1)	Q T LQ E IL G D P G
Tuberin (2)	L H AQ E LV G RGI
IRF3	D I LQ E LL G NMG
MCM7	M S KQ E LL G DKG
Peptide display	xx φ x E LL G xxx
Consensus	xx L xx φ L s hxxx

B **Figure 1.**

E6-binding structures in sequence and three-dimensional space. (A) Representative amino acid sequences shown to be necessary and sufficient for binding to HPV-16 E6. Sequence comparison among several of the E6-interacting proteins including E6AP, reveals a consensus sequence, **LxxφLsh**, where **L** indicates leucine residues, **φ** is a hydrophobic residue, **h** is an amino acid residue with a side-chain capable of accepting hydrogen bonds (Asp, Glu, Asn, or Gln), **s** represents a small amino acid residue (Gly or Ala) and **xx** is a dipeptide where one of the residues is Asp, Glu, Asn, or Gln. (B) Representative structure of the E6-binding motif (Be et al., 2001) shown interacting with a schematic representing the two zinc fingers of E6.

Side-chain positions of the amino acid residues essential in the motif for binding E6 are indicated by spheres. Published single-amino acid mutations of E6 that disrupt binding are in square boxes whereas mutations that retain binding are circled (Liu et al., 1999a; Nomine et al., 2006; Zimmermann et al., 1999). The E6-binding motif appears to bind to a structure formed between the two zinc finger-like structures of E6.

Figure 2. Pharmacophore description. The pharmacophores for binding the high-risk papillomavirus E6 protein comprises 3 lipophilic points (lip.), 2 hydrogen-bonding points (CO₂H), and one exclusion sphere. The locations of these points were derived from two peptide structures that bind E6 (A, E6AP; and B, E6BP). The radii of the location spheres are calculated from a molecular dynamics simulation of the peptide structures.

Figure 3.

Hits from searching the NCI database using the E6-binding pharmacophore. The compounds are: **1**, NSC83143 (CAS 23870-53-3); **2**, NSC117907 (CAS 55811-23-9); **3**, NSC135098; and **4**, NSC216029.

Figure 4.

Hits from searching the Sigma-Aldrich database using the E6-binding pharmacophore. The compounds, all from Aldrich, are: **5**, s327301; **6**, 207721 (CAS 4680-78-8); **7**, r218634; **8**, r225975; **9**, r278319; and **10**, s204102.

Figure 5. Representative binding curves for (A) Compound **2**, and (B) Compound **9**. Experimental data for binding to E6 are depicted by open circles and by closed squares for anti-FLAG. The best fits of the data are indicated by the lines through the data points.

A **B** **Figure 6.**

Inhibition by Compound 2 of E6-mediated degradation of p53 in cultured cells. NIKS-E6 cells were treated with MMC and then the compound at 500 μ M. Total cell extracts were prepared. (A) p53 levels were determined by Western blot analysis. Tubulin was used as a loading control. (B) Quantitation of p53 levels in NIKS-E6 cells after treatment with Compound 2. The p53 levels in DMSO-treated cells were normalized to 1. Data represent the mean and standard deviation of seven experiments.

Table 1

Compound Inhibition^a

Name ^b	E6AP ^c binding IC ₅₀	p53 Degrad. IC ₅₀	Control 1 Anti-FLAG IC ₅₀	Control 2 p107-E7 IC ₅₀
1 NCI 83143	29±6	63±34	>5000	>5000
2 NCI 117907	29±6	81±41	4100±1200	>5000
3 NCI 135098	22±3	69±44	140±20	>5000
4 NCI 216029	12±2	89±74	170±40	5100±600
5 A s327301	52±7	43±42	380±50	>5000
6 A 207721	21±7	31±16	>5000	>5000
7 A t218634	27±5	68±51	800±500	>5000
8 A t225975	12±2	21±17	77±19	460±205
9 A t278319	17±2	15±13	>5000	>5000
10 A s204102	11±1	118±51	>5000	190±250

^a IC₅₀ values are given in micromolar. n.d. = not determined.

^b “NCI” refers to the chemical repository at the National Cancer Institute. “A” refers to the Sigma-Aldrich library of rare chemicals.