Inhibition of Human Papillomavirus DNA Replication by Small Molecule Antagonists of the E1-E2 Protein Interaction

Peter W. White‡, Steve Titolo‡, Karine Brault‡, Louise Thauvette‡, Alex Pelletier‡, Ewald Welchner‡, Lise Bourgon‡, Louise Doyon‡, William W. Ogilvie§, Christiane Yoakim§, Michael G. Cordingley‡ and Jacques Archambault‡¶

From the ‡Department of Biological Sciences and §Department of Chemistry, Boehringer Ingelheim (Canada) Ltd, Laval, Canada, H7S 2G5.

Running title: Inhibitors of HPV E1-E2 interaction

‡To whom correspondence should be addressed: Dept. of Biological Sciences, Boehringer Ingelheim (Canada) Ltd, 2100 Cunard St., Laval, Canada, H7S 2G5. Tel.: (450)-682-4640; Fax: (450)-682-4642;
E-mail: jarchambault@lav.boehringer-ingelheim.com.
SUMMARY

Human papillomavirus (HPV) DNA replication is initiated by recruitment of the E1 helicase by the E2 protein to the viral origin. Screening of our corporate compound collection with an assay measuring the co-operative binding of E1 and E2 to the origin identified a class of small molecule inhibitors of the protein interaction between E1 and E2. Isothermal titration calorimetry and changes in protein fluorescence showed that the inhibitors bind to the transactivation domain (TAD) of E2, the region that interacts with E1. These compounds inhibit E2 of the low-risk HPV types 6 and 11, but not those of high-risk HPV types or of cottontail rabbit papillomavirus (CRPV). Functional evidence that the TAD is the target of inhibition was obtained by swapping this domain between a sensitive (HPV11) and a resistant (CRPV) E2 type and by identifying an amino acid substitution, E100A, that increases inhibition by approximately 10-fold. This class of inhibitors was found to specifically antagonize the E1-E2 interaction in vivo and to inhibit HPV DNA replication in transiently transfected cells. These results highlight the potential of the E1-E2 interaction as a small molecule antiviral target.

INTRODUCTION

Papillomaviruses are a family of small double-stranded DNA viruses that induce benign and malignant hyperproliferative lesions of the differentiating epithelium (reviewed in 1-4). Approximately 25 types of human papillomavirus
(HPV) infect the anogenital region. These HPV types have been classified as low-risk or high-risk types depending on whether they cause benign warts or lesions that can progress to invasive cancer, respectively. Current therapies to remove HPV-induced lesions include a variety of ablative or cytodestructive procedures and the use of immunomodulatory molecules such as imiquimod (5,6) to stimulate a host immune response. Small molecule antivirals for the treatment of HPV infections do not currently exist.

The life cycle of HPV is coupled to the cellular differentiation program that occurs in the epithelium (7). Maintenance of the viral genome in infected cells of the basal layer is essential for the viral life cycle and the ensuing pathology. Maintenance of the HPV episome in primary keratinocyte cultures depends on the function of E1 and E2, a 3'-5' helicase (8), and a sequence-specific DNA binding protein (9), respectively, which are required for replication of the genome (10). HPV DNA replication is initiated by the co-operative binding of E1 and E2 to specific DNA sequences within the viral origin (11-14). Formation of this E1-E2-ori ternary complex is dependent on the interaction of both proteins with DNA but also on a critical interaction between the N-terminal transactivation domain (TAD) of E2 and the C-terminal enzymatic domain of E1 (15-22). Assembly of this initial E1-E2-ori complex serves as a starting point for the recruitment of additional E1 molecules (23, 24) and their assembly into hexamers and double hexamers that have ATPase and helicase activity (25, 26).

Any of the protein-protein and protein-DNA interactions occurring at the origin could in principle be targeted for the development of small molecule
antivirals to treat HPV infections. In reality, however, not all targets are readily amenable to inhibition by small molecules. Protein interactions for example have been considered difficult to inhibit because they often involve large surfaces devoid of small molecule binding pockets (27, 28). In absence of structural information, one often has to rely on screening large compound collections to determine if a protein can be antagonized by small molecules and for the identification of lead inhibitors.

Here we present a class of small molecules that bind reversibly to the E2 transactivation domain and inhibit the E1-E2 protein interaction in vitro and in vivo. The chemical features and synthetic procedures for these molecules will be described elsewhere\textsuperscript{2}. To our knowledge these are the first small molecule inhibitors of HPV DNA replication with cellular activity. As such they highlight the potential of the E1-E2 interaction as an antiviral drug target.

**EXPERIMENTAL PROCEDURES**

**Inhibitors.** The syntheses of inhibitors 1 and 2 will be described elsewhere\textsuperscript{2}. Inhibitor 3 was made by similar procedures using the appropriate indandione, benzaldehyde, and maleimide fragments. 4-fluoro-7-nitrobenzofurazan was purchased from Aldrich.

**Plasmid constructions.** The plasmid used for expression of HPV11 E2 TAD (amino acids 2-201) fused at its N-terminus to a six-histidine tag (amino acid
sequence: MGHHHHHHH) was constructed by inserting a PCR fragment encoding the TAD and the tag in pET28b (Novagen). The plasmid to express CRPV E2 by in vitro transcription/translation was constructed by inserting a PCR fragment corresponding to nucleotides 3107-4287 of the CRPV genome in plasmid pCR3.1 (Invitrogen). The plasmid containing the CRPV origin of replication was constructed by inserting a Pst1 restriction fragment corresponding to nucleotides 192-7111 of CRPV into pBluescript SK(+) (Stratagene). Plasmids pOri-SEAP and pGAL4-SEAP encoding, respectively, the HPV11 origin and five GAL4 binding sites upstream of secreted alkaline phosphatase were constructed by inserting a PCR fragment spanning nucleotides 7884-61 of the HPV11 genome or the five GAL4 binding sites from pG5CAT (Clontech) into pSEAP2-Basic (Clontech). The plasmid encoding E1 fused to the transactivation domain of HSV VP16 (pE1-VP16) was constructed by inserting a PCR fragment encoding E1 into pVP16 (Clontech). HPV/CRPV E2 chimeras were constructed by PCR-mediated ligation as described previously (29) and cloned into pCR3.1. Site directed mutagenesis of E2 was performed with the QuickChange site-directed mutagenesis kit (Stratagene). For the plasmids described above, details of the construction and the sequence of the oligonucleotide primers used for PCR or mutagenesis will be made available upon request.

**Proteins.** His-E2 TAD from HPV11 was expressed from E. coli strain BL21(DE3) pLysS and purified by nickel affinity and size exclusion chromatography (Hi-trap and Superdex 75, Pharmacia) in a buffer composed of
25 mM Tris, 500 mM NaCl, and 5 mM TCEP (pH 8.0 at room temperature). Yield was 11 mg per L of culture and His-TAD was essentially pure as judged by SDS-PAGE or analytical-scale size exclusion chromatography. Preparation of purified SV40 T antigen (30), purified E1 and E1-containing nuclear extracts (31), purified E2 (31), and GST-E2 TAD (20) have been described previously. The TNT coupled reticulocyte lysate system (Promega) was used for in vitro translation of E1 and E2 as described (20).

**E1-E2-ori complex formation and related assays.** E1-E2-ori complex formation was monitored essentially as described previously (31). Unless stated otherwise, all E1-E2-ori complex formation assays were performed with purified E2 and E1-containing nuclear extracts. E1-containing nuclear extracts were titrated in the assay and used at a concentration yielding 70% of maximal activity. Wells without E1 or E2 served as negative controls. In assays performed with in vitro translated E2, 2 µl of translation reaction was used per binding reaction. In this case, wells containing E2 but no E1 were used as negative controls, since the reticulocyte lysate present from the translation reaction decreased background signal.

An assay to detect binding of E2 to the origin in the absence of E1 was carried out in a similar manner using SPA beads linked to anti-mouse IgG and the anti-E2 monoclonal antibody 53.7E8 raised against full-length HPV11 E2.

A similar assay to detect binding of SV40 large T Antigen to its cognate origin was carried out using 100 ng of purified large T antigen and an SV40 ori
probe encompassing nucleotides 5191-31 of the SV40 genome. This probe was generated by PCR using pCH110 (Pharmacia), which contains the SV40 ori, and primers 5'-GCC CCT AAC TCC GCC CAT CCC GC-3' and 5'-ACC AGA CCG CCA CGG CTT ACG GC-3'. Detection was performed using the antibody PAb 101 (32) and protein A SPA beads.

For assays using in vitro translated CRPV E2 proteins or HPV11/CRPV E2 chimeras containing the CRPV DNA binding domain, a radiolabeled probe for the CRPV origin was used. A modified detection procedure was used for the experiment in Figure 6 to compensate for the weaker signal obtained in assays with proteins containing the CRPV DNA binding domain. After the 1 h binding reaction, the mixture was transferred to HVB filter plates (Whatman) to which an SPA bead-E1 antibody suspension had previously been added. After incubating the mixture for one hour with slight shaking, bead-bound protein-DNA complexes were washed by vacuum filtration, which removed the red-colored reticulocyte lysate that slightly quenches detection and also reduced background since unbound probe was washed away. Scintillation fluid was then added (Microscint 20 (Packard), 150 µl per well), the plates were shaken vigorously for several minutes, and counted using a Topcount NXT scintillation detector (Packard).

**Data analysis.** IC$_{50}$ values were determined by a non-linear least-square regression fitting of the inhibition data using the program SAS (software release 6.12, SAS Institute Inc., Cary, NC, USA) or GraFit 5.03 (Erithicus Software Ltd.).
**E1-E2 ELISA.** PBS (Gibco BRL) was used as the buffer for all steps, with additions as noted. Wash buffer contained PBS plus 0.05% Tween-20. 96-well plates (Nunc Immunoplates) were coated overnight at 4°C using the anti-E2 monoclonal antibody 53.7E8 (1 µg in 100 µl /well). Wells were blocked at room temperature with 300 µl PBS plus 3% BSA. Subsequent steps were performed at room temperature on a plate shaker (gentle setting) in a total volume of 100 µl, in PBS supplemented with 1 mM DTT and 0.1 mM EDTA. After blocking, plates were washed and incubated with 25 ng of purified E2 or 4 µl of in vitro translated E2. Negative controls lacked E2 (assays with purified E2) or E1 (assays with in vitro translated E2). After 1.5 h, plates were washed and solutions containing test inhibitors (if used) and 0.5 µl of E1-containing nuclear extract were added. Buffer for this step contained 6% DMSO. After 2 hours, bound E1 was detected by successive incubations with the same anti-E1 serum used in the E1-E2-DNA complex formation assay and then with HRP-linked Goat anti-rabbit antibody (Sigma). Antibody buffers were further supplemented with 0.5% BSA and 0.05% Tween-20. After washing, HRP activity was detected using o-phenylenediamine substrate (Sigma) according to the manufacturer’s instructions.

**Fluorescence quenching experiments.** Fluorescence spectra were acquired using an SLM Aminco 8100 spectrometer with a neutral density filter in place and excitation and emission slits set to 4. Samples of proteins at 400 nM were excited at 280 nm and the emission intensity was recorded over the range from 300-380 nm. For each protein/inhibitor combination, spectra were acquired
from three individually prepared 0.5 ml samples. Signals from the three replicates were averaged, and for each wavelength, the displayed signal is the average from a 3 nm range.

**Isothermal titration calorimetry.** The calorimetry experiment was carried out using the VP-ITC microcalorimeter (Microcal Inc.) at a temperature of 25°C. Purified His-tagged HPV11 TAD was exchanged into an aqueous buffer consisting of 20 mM Tris, 100 mM NaCl, and 0.5 mM TCEP, plus 2% DMSO. Inhibitor 2 was diluted from a DMSO stock solution to give a solution at 200 µM in the same buffer/DMSO mixture. Both solutions were degassed, then the protein solution was passed through a 0.22 µM syringe filter and the inhibitor solution was spun in a microfuge for 5 min at maximum speed, to remove any particulate matter. The concentration of the protein solution after filtration was determined to be 10.0 µM by absorbance at 280 nm (based on a predicted molar extinction coefficient of 4.9 x 10⁴). An initial 4 µl injection was followed by a series of 8 µl injections at a spacing of 240 s. Data were fit using the software Origin (version 5.0, Microcal Inc.), to a one-binding site model after subtraction of the heat of dilution determined by the average value at late injections.

**Reversibility of inhibition.** Reversibility of inhibition was monitored using a modified version of the E1-E2-ori complex formation assay described above. E2 (23 ng/µl) was mixed with inhibitor in assay buffer at a concentration equal to or greater than the IC₅₀. The mixture was then diluted 19-fold and combined with
E1 and radiolabeled probe such that the overall dilution of inhibitor was 32-fold. Controls without inhibitor were run in parallel and used as the reference for calculation of inhibition. The final concentration of E2 was five-fold higher in preincubation assays (10 nM vs. 2 nM) to compensate for the significant decrease in signal observed on preincubation of E2.

**Transient HPV DNA replication assay.** Transient HPV DNA replication was performed as described previously (20, 33, 34). Amount of replicated HPV DNA (pN9) was quantified by exposure on a STORM 860 Phosphorimager (Molecular Dynamics) and normalized to the amplified E1 signal. When applicable, inhibitors were added to the culture medium after transfection in a final concentration of 1% DMSO Each compound concentration was tested in quadruplicate. Cellular DNA replication was measured using the colorimetric Cell Proliferation ELISA kit (Roche).

**Cellular E1-E2 interaction assay.** CHO-K1 cells were transfected using Lipofectamine (Gibco BRL) with three plasmids encoding, respectively, E1-VP16 (2.5 μg), E2 (250 ng) and a SEAP reporter under the control of the minimal origin of DNA replication of HPV11 (62.5 ng). 3 hours post-transfection, cells were treated with trypsin and seeded in 96 well plates (20 X10³ cells/well). When applicable, inhibitors were added to the culture medium after transfection in a final concentration of 1% DMSO. The amount of SEAP in the culture medium was measured by chemiluminescence 48 h post-transfection using the Tropix
Phospha-Light System (Applied Biosystems) according to the instruction supplied by the manufacturer. Each condition was performed in duplicates, which typically varied by less than 10%. As a control for specificity, inhibitors were also tested on cells transfected with two plasmids encoding, respectively, an unrelated SEAP reporter (250 ng of pGAL4-SEAP) and the unrelated transactivator GAL4-VP16 (2.5 µg of pM3-VP16; Clontech) comprised of the GAL4 DNA-binding domain fused to the activation domain of VP16.

RESULTS

Identification of a class of small molecules that inhibit the cooperative binding of HPV11 E1 and E2 to the origin. Co-operative binding of E1 and E2 to the viral origin is an essential step in the initiation of HPV DNA replication. To identify inhibitors of this process, a high-throughput screening assay was developed that measures the binding of E1 and E2 to ³²P-radiolabeled origin DNA. Binding of E1 to the origin probe was detected by immuno-capture of E1-DNA complexes using an anti-E1 antibody coupled to scintillation proximity assay (SPA, 34) beads (Figure 1A). Binding reactions were performed in the presence of an excess of unlabeled non-specific competitor DNA to ensure that binding of E1 to the origin probe was dependent on its association with E2. Figure 1B shows that a strong signal was detected only when E1 and E2 were added to the reaction. This signal was dependent on the association of E1 with E2 since it was reduced to background levels by the E39A amino acid
substitution in E2 that abrogates E1 binding (36-38). In contrast the I73A substitution, which affects primarily the transactivation function of E2 and not its ability to bind to E1 (37, 38), had little effect.

Screening of our corporate compound collection with this assay yielded inhibitor 1 shown in Figure 2. Modification of this inhibitor led to more potent compounds\(^2\) such as 2 and 3 (Figure 2). These three compounds were inactive or only weakly active in a mechanistically related SPA that measures binding of SV40 large T antigen to its cognate origin (Figure 2), demonstrating that they do not interfere with the scintillation proximity detection procedure or bind promiscuously to DNA or protein. Inhibitors 1-3 were also active against E1 and E2 from HPV6 (Figure 2), the most prevalent low-risk anogenital type, albeit at a 10-20 fold lower potency than against the HPV11 proteins.

**The E1-E2 protein interaction is the target of inhibition.** Co-operative binding of E1 and E2 to DNA involves the interaction of both proteins with each other and with DNA. The inhibitors were found to be inactive or only weakly active in assays that measure the binding of E2 to DNA (Figure 2). Compound 1 was also inactive at inhibiting the helicase activity of E1 (IC\(_{50}\) > 100 \(\mu\)M). Hence this class of compounds does not affect the binding of either E1 or E2 to DNA. These results suggested that the E1-E2 protein interaction might be the target of inhibition. This hypothesis was tested using an ELISA. The E2 protein used in this assay was either purified from baculovirus-infected insect cells or obtained by in vitro translation; both sources yielding similar results. Binding of
recombinant E1 to immobilized E2 was detected using an anti-E1 antiserum followed by a secondary antibody coupled to horseradish peroxidase. As seen in Figure 3A, the signal was dependent on the interaction of E1 with E2 since it was reduced by the E39A substitution in E2. As anticipated, the I73A substitution had little effect. As can be seen in Figures 3B and 3C, all three compounds inhibited binding of E1 to E2 with potency comparable to that measured in the E1-E2-ori complex formation assay. The compounds inhibited the E1-E2 interaction regardless of whether the assay was performed with hexameric E1 purified from baculovirus infected insect cells or with monomeric E1 made by in vitro translation (data not shown). Thus the molecular target of these inhibitors is the E1-E2 protein interaction.

_Inhibitors bind to the transactivation domain of E2._ Having found that inhibitor 1 and its derivatives antagonize the E1-E2 protein interaction, we then wished to determine to which protein these compounds were binding. First, we investigated whether addition of compound 2 had any effect on the spectroscopic behavior of either E1 or E2. Fluorescent amino acids such as tryptophan are often found in or close to binding pockets, and the binding of ligands to such sites has been shown to perturb protein fluorescence in some cases (39). Compound 2 was titrated into solutions of 400 nM E1 or E2 (Figure 4A and 4B). While a slight effect was observed on the fluorescence of E1, a much greater level of quenching was observed for E2, consistent with this compound binding to E2. The slight effect observed on E1 and the lack of saturation for E2 at high
compound concentrations could be explained by weak nonspecific binding of compound 2 to other protein sites, reminiscent of the low-level of activity observed in the SV40 T antigen DNA binding assay (Figure 2).

E2 can be divided into an N-terminal transactivation domain (TAD, approximately amino acids 1-200) and a C-terminal DNA binding/dimerization domain (DBD, the last 100 amino acids), separated by a 50-70 amino acid “hinge” with little predicted secondary structure (9). Since for HPV11 it is known that the TAD, but not the DBD, interacts with E1 (16, 40), we repeated the titration with a GST fusion protein encoding the E2 TAD, as well as with GST itself as a negative control (Figure 4C and 4D). The results clearly indicated saturable binding of 2 to the GST-TAD but not to GST, indicating that the compound binds to the TAD. Similar results were also obtained with a polyhistidine-tagged version of the E2 TAD (data not shown).

Next, we quantified the binding of inhibitor 2 to a polyhistidine-tagged TAD using isothermal titration calorimetry (Figure 5). A dissociation constant of 230 nM was obtained by nonlinear regression, in good agreement with the IC_{50} obtained in the E1-E2-ori complex formation assay. The observed 2:1 stoichiometry is consistent with the formation of a 1:1 inhibitor:protein complex, since compound 2 is racemic, and it has been observed for other compounds in this series that only one of the two enantiomers is active (see below, and unpublished data). Collectively, the results presented above provide strong evidence that this class of inhibitor antagonizes the E1-E2 protein interaction by binding to the E2 TAD.
Functional evidence that the E2 TAD is the target of inhibition. During the course of this study we found that in vitro translated CRPV E2 can bind cooperatively with purified HPV11 E1 to either the HPV11 or CRPV origins, albeit weakly. CRPV E2, however, was found to be resistant to inhibition by compound 2 (Figure 6), perhaps not surprisingly given the low level of amino acid identity between the TAD of HPV11 and that of CRPV. Similarly, we found that the inhibitors are inactive against the E2 protein of the high-risk HPV types 16, 18 and 31 (data not shown), which at the amino acid level are only 41-50% identical to HPV11 E2. We made use of the observation that some E2 types are resistant to inhibition to substantiate the finding that the TAD is indeed the target of the inhibitors. Specifically, we constructed chimeric E2 proteins encoding the HPV11 TAD with the hinge and DNA-binding domain from CRPV, or the opposite combination. These chimeras were expressed in vitro and tested in the E1-E2-ori complex formation assay using HPV11 E1. Both chimeras could bind cooperatively with E1 on the origin, but only the one containing the TAD from HPV11 was sensitive to inhibition by compound 2 (Figure 6). This result provides functional evidence that the TAD is indeed the target of inhibition.

Inhibition is reversible. It is generally expected to be difficult to identify inhibitors of protein interactions that act by reversible binding rather than by reacting with the target protein or by some other irreversible mechanism (27). To determine if inhibition of E1-E2-ori complex formation was reversible, we
performed the pre-incubation experiment shown in Figure 7. In this experiment, E2, the target protein, was incubated with a high concentration of inhibitor and then diluted into the E1-E2-ori complex formation assay to a final compound concentration ten-fold below the IC$_{50}$. We reasoned that if the inhibitor is acting by a reversible mechanism, the activity of E2 should be recovered upon dilution of the inhibitor. However, if the mechanism of inhibition is irreversible then significant inhibition should be observed even after dilution. As a control, we used the known reactive electrophile, 4-fluoro-7-nitro-[2,1,3]-benzoxadiazole. Pre-incubation with this compound resulted in significant inhibition even after dilution to a concentration well below the IC$_{50}$. In contrast, pre-incubation of E2 with compound 2 did not lead to any significant inhibition upon dilution (compare third bar to first and second bars from the left in Figure 7). Thus compound 2 acts by a reversible mechanism.

**Inhibition of HPV11 DNA replication in transiently transfected cells.** Next we wished to determine if the most potent inhibitor, compound 3, would be able to inhibit HPV DNA replication in transiently transfected cells. In this assay, plasmids expressing HPV11 E1 and E2 were transfected into CHO cells along with a plasmid carrying the HPV11 minimal origin of DNA replication. Transfected cells were incubated with increasing concentrations of compound 3 ranging from 0.195 to 25 µM and the amount of replicated DNA (Dpn1 resistant) was quantified by PCR 48 hrs post transfection. As a control for specificity, we tested in parallel the effect of the compound on cellular DNA replication as
measured by BrdU incorporation. Figure 8 shows that compound 3 was able to inhibit HPV DNA replication in a dose dependent manner with a 50% inhibitory concentration of approximately 1 µM. In contrast cellular DNA replication (BrdU incorporation) was largely unaffected at a concentration of inhibitor of 12.5 µM. The inhibition of BrdU incorporation seen at the two highest inhibitor concentrations is likely due to cytotoxicity of the compound rather than specific inhibition of cellular DNA replication.

**Inhibition of the E1-E2 protein interaction in vivo.** To ascertain the mechanism of action of the inhibitors in vivo, we established a cellular assay that measures the interaction of HPV11 E1 and E2. Specifically, this assay measures the expression of a secreted alkaline phosphatase (SEAP) reporter gene, placed under the control of the HPV11 minimal origin of replication, upon co-transfection of two plasmids encoding, respectively, E2 and the E1 protein fused to the strong transactivation domain of HSV VP16 (Figure 9A). In this assay, neither E2 nor E1-VP16 could by itself activate expression of the reporter gene (Figure 9B). The lack of transactivation by E2 is consistent with the previous observation that E2 of the low risk HPV types are weak transactivators (41). Maximal expression of the reporter gene was dependent on the expression of both E1-VP16 and E2 and on their interaction, since it was greatly reduced by the E39A substitution in E2 which affects E1-binding (Figure 9B). As expected, the I73A substitution in E2 had no negative effect.
Having confirmed that expression of the reporter gene was dependent on the interaction between E1 and E2, we then tested the effect of compounds 2 and 3 in this assay and in a similar one based on E1 and E2 from HPV6. Compounds 2 and 3 inhibited SEAP synthesis induced by the HPV11 proteins with IC50 values of 14 \( \mu \)M and 2 \( \mu \)M, respectively (Figure 10). The similar potency of compound 3 in the reporter and transient HPV DNA replication assays suggests that its inhibitory activity on viral DNA replication is indeed due to abrogation of the E1-E2 protein interaction. Inhibitors 2 and 3 were also found to be active against the HPV6 proteins, albeit with reduced potency, with IC50 values of 22 \( \mu \)M and 6 \( \mu \)M, respectively. Interestingly the difference in potency between HPV11 and HPV6 protein was about 4-7 fold in this cellular assay compared to 25-30 fold in vitro (Figure 2). The exact reason for the smaller difference in potency against HPV6 and 11 proteins in vivo is currently unknown, but could be related to slight alterations in the conformation of E2 in vivo compared to in vitro (see Discussion). As a control for specificity, we verified that the inhibitors had little effect on the expression of an unrelated SEAP reporter transactivated by a GAL4-VP16 fusion protein (Figure 10). As an additional control for specificity, we showed that an enantiomer of inhibitor 3 that is nearly inactive in vitro (IC50=29 \( \mu \)M in the E1-E2-ori complex formation assay) was also inactive in the cellular assay (data not shown).

To gain additional evidence that the inhibitors were acting in vivo by the same mechanism as in vitro we made use of a mutant E2 protein that has a higher affinity for this class of inhibitors. This mutant protein contains a single
amino substitution in the TAD, E100A, which makes it approximately 10 fold more sensitive to this class of inhibitors than wild type E2, in the in vitro E1-E2-orí complex formation assay. The E100A substitution was discovered during a mutational analysis of the E2 TAD aimed at identifying residues involved in compound binding (to be published elsewhere). Importantly, this amino acid substitution also increased the sensitivity of E2 in the reporter assay (Figure 10). This result provides strong evidence that the mechanism of action of this class of inhibitors is the same in vivo as in vitro.

**DISCUSSION**

*Discovery of inhibitors of the E1-E2 interaction.* In this manuscript we have presented the identification of the first small molecule inhibitors of the E1-E2 protein interaction capable of inhibiting HPV DNA replication in vivo. We have shown by two independent biophysical methods, changes in intrinsic protein fluorescence and isothermal titration calorimetry, that compounds 1-3 bind to the transactivation domain of E2. Functional evidence for binding to the E2 TAD was also obtained by swapping this domain between a sensitive E2 type (HPV11) and a resistant one (CRPV), as well as by identifying an amino acid within the TAD, E100A, that increases inhibition by approximately 10-fold in vitro.

The discovery of inhibitors of the E1-E2 protein interaction from HPV types 6 and 11 highlights the potential of E2 as a drug target for the treatment of anogenital warts. The genome of HPV6 and 11 is present in episomal form in approximately
90% of genital warts (42, 43) and inhibition of the E1-E2 interaction is expected to provide therapeutic benefit (44). In this respect, it is worth noting that E1 and E2 are likely to be expressed at much lower levels in natural infections than in transfected cells, a fact that could lead us to underestimate the true potency of these inhibitors. Determining the real antiviral activity of these inhibitors awaits the development of a straightforward and quantitative HPV viral replication assay.

**Specificity of inhibition.** The inhibitors presented in this study are active against the E2 protein of the two most prevalent low-risk HPV types, 6 and 11, but are nevertheless more potent against the HPV11 protein by approximately 10-40 fold in vitro. This difference in potency was observed both with purified proteins and with proteins produced by in vitro translation. Since we have found no evidence that HPV6 E1 and E2 interacts more strongly than the HPV11 proteins (data not shown), we surmise that the inhibitors must bind less tightly to HPV6 E2. The HPV6 and 11 TAD are 84% identical at the primary amino acid sequence level and any of the amino acid differences or combination thereof could affect binding of the inhibitor. Surprisingly, we found that the difference in potency observed in cellular assays is less pronounced. Indeed, the difference in IC$_{50}$ values is only approximately 3-fold in the transient HPV DNA replication assay (unpublished results). This reduced difference between type 6 and 11 was also observed in the E1-E2 interaction reporter gene assay suggesting that this class of compound does indeed inhibit the E1-E2 interaction of both HPV types.
with comparable potency in cells. It is possible that a conformational change necessary for inhibitor binding has a more similar energetic cost for both HPV types in vivo than in vitro, perhaps because the conformation of E2 in vivo is slightly different as a result of interaction with cellular proteins. Further studies will be required to address this possibility.

**Amino acids in E2 involved in inhibition.** Prior to this report a 15 amino acid peptide derived from HPV16 E2 and spanning residue E39 was shown to be capable of inhibiting the E1-E2 interaction in vitro (45). Several studies have suggested an essential role for E39 in interaction with E1. Specifically, changing this residue to alanine was shown to abrogate the ability of E2 to bind to E1 (36-38), as confirmed in this study, while having little effect on its transcriptional activity. These findings suggested that E39 might form part of the E1-binding surface on E2. Two lines of evidence suggest that our inhibitors may bind near E39 to antagonize the E1-E2 interaction. Firstly, the E100 residue, which when changed to alanine increases the sensitivity of HPV11 E2 to our inhibitors, is located on the same surface as E39 in the crystal structure of the HPV16 E2 TAD (46). Secondly, we found that our inhibitors bind with reduced affinity to the E39A mutant E2 TAD in fluorescence studies similar to those presented in Figure 4 (data not shown). The simplest interpretation of these results is that the surface of the E2 TAD onto which E39 and E100 are located is involved in binding both E1 and our inhibitors. Because these inhibitors are inactive against HPV16 E2 we did not attempt to model them into the published structure of the HPV16 E2
However, further studies are in progress to localize the inhibitor-binding pocket on the HPV11 E2 TAD.

**Protein interactions as drug targets.** The compounds presented here belong to a relatively small group of small molecules which effectively inhibit protein interactions. With only a few exceptions most protein interaction inhibitors have been identified for cases in which the binding surface for one protein is known to be a small contiguous region, such as for receptor-ligand interactions (47, 48). Many of the small molecules reported to inhibit protein interactions are peptides identified either by screening procedures such as phage display, or from panels of overlapping peptides based on the protein sequence, or selected based on results of structural or mutational studies (49). Most of these peptides are relatively weak inhibitors, and while in principle these can serve as lead compounds for drug discovery, in practice transformation of such molecules into potent non-peptidic inhibitors is quite challenging.

A variety of reasons has been proposed for the relatively low number of small molecule inhibitors of protein interactions. One is that small molecules would have difficulty competing against the large surface area typically involved in protein-protein interface. However, it has been shown in a number of cases (e.g. 50) that substitution of a single critical residue at an interface, which is conceptually analogous to tight binding of a small molecule, is sufficient to greatly weaken an interaction. An example of this may be the E39A substitution in E2, which as argued above may abrogate E1-binding because it is located at the E1-
E2 interface. A second reason why protein interactions are thought to be difficult targets for small molecules is because their interfaces can be fairly flat and devoid of concave pockets analogous to enzyme active sites or receptor binding sites (51). Although this is true for some complexes, others have been found to be “bumpy” such that small cavities are often present (52, 53). Perhaps relatively few protein interaction inhibitors have been identified to date only because more effort has been devoted to traditional targets, such as enzymes and receptors. We have in fact succeeded in identifying potent small molecule inhibitors of the E1-E2 interaction by high-throughput screening. This strongly suggests that other investigations will also yield novel small molecule inhibitors of protein interactions as drug candidates.

Acknowledgements. We thank Drs. Craig Fenwick and Steven Mason for critical reading of the manuscript.

REFERENCES


**FOOTNOTES**

1 The abbreviations used are: HPV, human papillomavirus; TAD, transactivation domain; CRPV, cottontail rabbit papillomavirus; HSV, herpes simplex virus; PCR, polymerase chain reaction; SV40, simian virus 40; GST, glutathione S-transferase; SPA, scintillation proximity assay; HRP, horseradish peroxidase; SEAP, secreted alkaline phosphatase; ELISA, enzyme-linked immunosorbent
assay; DBD, DNA-binding domain; TCEP, tris(2-carboxyethyl)phosphine; BrdU, 5-bromo-2'-deoxyuridine; WT, wild type; IC$_{50}$, 50% inhibitory concentration.

2Yoakim, C., Ogilvie, W. W., Goudreau, N., Naud, J., Haché, B., O’Meara, J. A., Cordingley, M. G., Archambault, J., and White, P. W., manuscript submitted for publication

FIGURE LEGENDS

Fig. 1. Assay measuring the co-operative binding of E1 and E2 to the origin. A, principle of the assay. Diagram illustrating the co-operative binding of E1 and E2 to a $^{33}$P-radiolabeled DNA fragment containing the minimal HPV11 origin of DNA replication. E2-dependent binding of E1 to the probe is detected by immunocapture of E1-containing protein-DNA complexes using an anti-E1 antibody ($\alpha$-E1 Ab) coupled to scintillation proximity assay (SPA) beads. Bound radiolabeled DNA excites the scintillant contained within these beads. B, validation of the assay. Bar graph representing the signal measured in counts per minute (cpm) obtained in presence (black bars) or absence (white bars) of recombinant HPV11 E1, and in presence or absence (mock) of wild type (WT) HPV11 E2, or of similar E2 proteins bearing the E39A or I73 amino acid substitutions.
Fig. 2. **Inhibitors identified in this study.** The figure shows the chemical structure of three related inhibitors of different potencies. Compounds 1 and 2 are racemic mixtures, whereas 3 is a single enantiomer with the absolute configuration as shown. The activities of the inhibitors in the various biochemical assays described in the text are indicated below each structure.

Fig. 3. **ELISA demonstrating inhibition of the E1-E2 protein interaction by inhibitors 1, 2 and 3.** A, validation of the ELISA with mutated E2 proteins produced by in vitro translation. The bar graph indicates the signal obtained in presence (black bars) or absence (white bars) of recombinant E1 and in presence or absence of wild type (WT) E2, or of a similar E2 protein bearing the E39A or I73 amino acid substitution. B, IC₅₀ curves. ELISA performed as in A except that purified WT HPV11 E2 (60 ng/well) was used in place of in vitro translated protein. Inhibitory activity was tested for a series of two-fold dilutions of compounds 1 (circles), 2 (squares) and 3 (triangles). C, IC₅₀ values determined by nonlinear regression from the data in panel B.

Fig. 4. **Effect of compound 2 on protein fluorescence.** Fluorescence emission spectra for purified polyhistidine-tagged HPV11 E1 (A), HPV11 E2 (B), GST-HPV11 E2 TAD (C), and GST (D); obtained after addition of 0 µM (diamonds), 0.4 µM (squares), 0.8 µM (triangles), 1.6 µM (circles), or 3.2 µM (crosses) of inhibitor 2. Fluorescence intensity is shown relative to a rhodamine B standard, used to compensate for variation in lamp intensity. The insets show
the proportional decrease in fluorescence as a function of compound concentration, calculated as the average of intensity values recorded between 330 and 340 nm.

Fig. 5. **Determination of the affinity of inhibitor 2 for the E2-TAD by calorimetry.** Top: raw data obtained at 25°C for injections of inhibitor 2 (200 µM) into the sample cell containing His-tagged HPV11 E2 TAD (10 µM). Bottom: calculated enthalpies (squares) after subtraction of the heat of dilution as determined by the later injections. Data were fit to a simple one binding site model to give \( K_a = 4.3 \pm 0.7 \times 10^6 \text{M}^{-1} \), \( \Delta H = 4.68 \pm 0.07 \text{kcal/mol} \) and a stoichiometry of \( 2.03 \pm 0.02 \) inhibitors per protein. This stoichiometry is consistent with a 1:1 TAD:inhibitor binding ratio for the active enantiomer of the compound 2 racemic mixture.

Fig. 6. **Activity of inhibitor 2 against HPV-CRPV E2 chimeric proteins.**

A, tripartite domain structure of E2. The amino acid boundaries of the transactivation domain (TAD), the hinge domain (H) and the DNA-binding domain (DBD) are indicated for HPV11 and CRPV E2. B, diagram of the four in vitro translated E2 proteins tested for inhibition by compound 2. The regions of each protein originating from HPV11 are colored in black while those from CRPV E2 are shown in white. The effect of 2 µM compound 2 is shown as the % inhibition of the signal obtained in the absence of compound, after subtraction of reaction blanks. Signal:background ratios were 17-18 for proteins with the HPV11 DBD
and 3-4 for assays with the CRPV DBD. Each value is the average of twelve measurements with the standard error indicated.

Fig. 7. **Effect of compound preincubation on E1-E2-ori complex formation.** E2 was mixed with the indicated concentration of inhibitor 2 or with 4-fluoro-7-nitrobenzofurazan as a positive control. After 1 hr at room temperature, the mixture was diluted into the assay such that the final concentration of compound was 32-fold lower than during preincubation. In parallel, assays were run without preincubation (standard assay procedure) with either the high or low concentration of compound. Error bars indicate the standard error based on 12-24 reactions in the absence and 4-8 reactions in the presence of each compound.

Fig. 8. **Inhibition of HPV11 DNA replication by compound 3.** The amount of replicated origin DNA was measured by PCR from Dpn1-digested genomic DNA isolated 48 hrs post-transfection from cells transected with three plasmids encoding, respectively, E1, E2 and the origin. A portion of the E1-expressing plasmid devoid of Dpn1 site was amplified as an internal control. A, E1 and origin (ori) PCR products obtained from transfected cells incubated with the indicated amount of inhibitor 3 were analyzed on a 1% agarose gel and stained with the fluorescent dye SYBRGreen I (Molecular Probes). Each inhibitor concentration was tested in quadruplicate. As a negative control, four transfections were performed in absence of the E2 expression plasmid (No E2).
B, graph indicating the amount of cellular DNA replication measured by BrdU incorporation (white circles) and of HPV DNA replication (black circles) obtained from the data in panel A at different concentrations of inhibitor 3. The intensity of the HPV origin fragments shown A was quantified using a Storm Phosphorimager (Molecular Dynamics). Each value is the average of four replicates with the standard deviation indicated. BrdU incorporation and HPV DNA replication values are presented as a percentage of the control activity obtained in absence of inhibitors, which was set arbitrarily at 100 %. Data were fit by non-linear regression to determine that compound 3 inhibits HPV DNA replication with an IC$_{50}$ value of approximately 1 $\mu$M.

Fig. 9. **Cellular assay to monitor the E1-E2 interaction at the origin in vivo.** A, principle of the assay. The diagram shows the SEAP reporter under the transcriptional control of the HPV11 minimal origin of DNA replication. The three E2 binding sites in the origin are shown as black boxes. In this assay, transient expression of E2 (white ellipse) and E1 (black ellipse) fused to the transcriptional activation (TA) domain of HSV VP16 (dotted ellipse) results in transactivation of the reporter gene and expression of SEAP. B, validation of the assay. Bar graph indicating that the amount of SEAP is maximal when both E1-VP16 and E2 are co-expressed. This high level of SEAP is reduced by the E39A substitution in E2, but not by the I73A substitution, suggesting that SEAP expression is dependent on the interaction of E1 with E2. Little SEAP activity is obtained if E1 is not fused to the VP16 activation domain (data not shown).
Fig. 10. **Effect of inhibitors 2 and 3 on the E1-E2 interaction in vivo.**

The graph shows the amount of SEAP activity measured in presence of increasing amounts of inhibitor 2 (A) or inhibitor 3 (B). Levels of SEAP activity are reported as a percentage of the control activity measured in absence of inhibitor, which is set arbitrarily at 100%. Levels of SEAP activity expressed from the ori-SEAP reporter upon expression of HPV11 E2 and HPV11 E1-VP16 are shown as black circles if using wild type E2 and as white squares if using the E100A E2 mutant that is hypersensitive to the inhibitors. Levels of SEAP expressed from the same reporter but upon expression of HPV6 E2 and HPV6 E1-VP16 are shown as white triangles. As a control for specificity, inhibitors were tested against an unrelated SEAP reporter gene (black triangles) that is transactivated by a fusion protein comprised of the yeast GAL4 protein DNA-binding domain fused to the VP16 transactivation domain. Lines represent best fits of the data to a logistic, as determined by nonlinear regression. C, IC$_{50}$ values determined by nonlinear regression from the data in A and B.
- Figure 1 -

A

\[ \alpha\text{-E1 Ab} \]

\[ \text{E1} \]

\[ \text{E2} \]

\[ ^{33}\text{P} \text{ - Origin DNA} \]

B

<table>
<thead>
<tr>
<th></th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>mock</td>
<td>0</td>
</tr>
<tr>
<td>WT</td>
<td>12000</td>
</tr>
<tr>
<td>E39A</td>
<td>3000</td>
</tr>
<tr>
<td>I73A</td>
<td>6000</td>
</tr>
</tbody>
</table>

HPV11 E2

- E1

+ E1
Figure 2

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC_{50} (µM)</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV11 E1-E2-ori</td>
<td>7.8</td>
<td>0.35</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>HPV6 E1-E2-ori</td>
<td>40</td>
<td>8.8</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>SV40 T Ag-DNA</td>
<td>56</td>
<td>60</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>HPV11 E2-ori</td>
<td>&gt; 75</td>
<td>&gt; 40</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>
- Figure 3 -

**A**

![Histogram](chart1.png)

- **A(450)**
- None: □ - E1, ■ + E1
- WT: □ - E1, ■ + E1
- E39A: □ - E1, ■ + E1
- I73A: □ - E1, ■ + E1

HPV11 E2

**B**

![Graph](chart2.png)

- % control activity vs. Inhibitor (µM)
- Inhibitor IC50 (µM)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>0.09</td>
</tr>
<tr>
<td>3</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**C**
- Figure 5 -

![Graph showing Compound:E2 ratio vs. Kcal/mole of injectant](image)
### - Figure 6 -

<table>
<thead>
<tr>
<th></th>
<th>HPV11</th>
<th>CRPV</th>
<th>% Inhibition (2 µM inhibitor 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAD</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>209</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBD</td>
<td>280</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>367</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>208</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>303</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>390</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **HPV/HPV**  : 91 ± 1
- **CRPV/CRPV**: 7 ± 10
- **HPV/CRPV** : 93 ± 2
- **CRPV/HPV** : -20 ± 14
- Figure 7 -

% control activity

<table>
<thead>
<tr>
<th>Assay conc. (µM)</th>
<th>Preincubation</th>
<th>Inhibitor 2</th>
<th>4-Fluoro-7-nitrobenzofurazan</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03</td>
<td>no</td>
<td>1.0 µM</td>
<td>no</td>
</tr>
<tr>
<td>1.0</td>
<td>no</td>
<td>0.03</td>
<td>no</td>
</tr>
<tr>
<td>1.5</td>
<td>no</td>
<td>48</td>
<td>1.5</td>
</tr>
<tr>
<td>48</td>
<td>no</td>
<td>1.5</td>
<td>no</td>
</tr>
</tbody>
</table>

Downloaded from http://www.jbc.org/ by guest on October 1, 2017
Figure 9

A

[Diagram showing HPV11 ori and SEAP with E1 and E2 sites labeled]

B

[Bar chart showing SEAP activity (x 10^5) for none, WT, E39A, and I73A HPV11 E2 with - E1 and + E1 conditions]

SEAP activity (x 10^5)

none  WT  E39A  I73A
HPV11 E2
- Figure 10 -

A

![Graph A](image)

B

![Graph B](image)

C

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC\textsubscript{50} (\textmu{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV11 E1 + E2</td>
<td>14</td>
</tr>
<tr>
<td>HPV6 E1 + E2</td>
<td>22</td>
</tr>
<tr>
<td>HPV11 E1 + E2 E100A</td>
<td>3</td>
</tr>
<tr>
<td>GAL4 reporter</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>
Inhibition of human papillomavirus DNA replication by small molecule antagonists of the E1-E2 protein interaction

Peter W. White, Steve Titolo, Karine Brault, Louise Thauvette, Alex Pelletier, Ewald Welchner, Lise Bourgon, Louise Doyon, William W. Ogilvie, Christiane Yoakim, Michael G. Cordingley and Jacques Archambault

J. Biol. Chem. published online April 30, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303608200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2003/04/30/jbc.M303608200.citation.full.html#ref-list-1