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Small molecule inhibitors of the HPV16-E6 interaction with caspase 8

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ABSTRACT

High-risk strains of human papillomaviruses (HPVs) cause nearly all cases of cervical cancer as well as a growing number of head and neck cancers. The oncogenicity of these viruses can be attributed to the activities of their two primary oncoproteins, E6 and E7. The E6 protein has among its functions the ability to prevent apoptosis of infected cells through its binding to FADD and caspase 8. A small molecule library was screened for candidates that could inhibit E6 binding to FADD and caspase 8. Flavonols were found to possess this activity with the rank order of myricetin > morin > quercetin > kaempferol = galangin ≫ (apigenin, 7-hydroxyflavonol, rhamnetin, isorhamnetin, geraldol, datiscetin, fisetin, 6-hydroxyflavonol). Counter screening, where the ability of these chosen flavonols to inhibit caspase 8 binding to itself was assessed, demonstrated that myricetin, morin and quercetin inhibited GST-E6 and His-caspase 8 binding in a specific manner. The structure–activity relationships suggested by these data are unique and do not match prior reports on flavonols in the literature for a variety of anticancer assays.

Human papillomavirus (HPVs) are small DNA viruses that infect human epithelial tissue such as that found in the genital tract, hands, and feet. There are more than 200 types of HPVs, which can be classified both on the basis of sequence homology, leading to assignments of species, and on the basis of whether infection with a particular type is likely to lead to cancer (high-risk vs low-risk). Most, though not all, of the high-risk types are located within species 9 (reviewed in Munoz et al., 2006).[1] HPV types 16 (species 9) and 18 (species 7) are high-risk types, and are the causative agents of nearly all cases of human cervical cancer, between 20% and 30% of head and neck cancer cases, and some cases of other cancers.[2–5] The HPV-encoded oncogenes E6 and E7 are responsible for cellular immortalization and transformation.[6–8] While E7 is best known for the inactivation of Rb and thus for the immortalization of differentiated cells, E6 induces degradation of p53, which serves as a critical tumor suppressor. In addition, E6 binds to and inactivates other cellular proteins involved in normal cellular functions.[6–9]

In our previous work, we found that E6 interacts with the extrinsic apoptosis pathway by binding to key signaling molecules such as TNF R1, FADD and caspase 8 and altering their functions, thus allowing HPV-infected cells to avoid clearance through apoptosis.[10–13] Activation of the extrinsic apoptotic pathway normally begins with the binding of ligands such as TNF-α, FasL or TRAIL to their receptors, which then leads to recruitment of adaptor proteins such as FADD, TRADD and TRAF. A Death Inducing Signaling Complex (DISC) is then formed, which includes the initiator caspase, procaspase-8, bound to the adapter protein FADD. Procaspase 8 cleavage releases active cysteine-aspartic proteases, which then enable caspase 8 to cleave and activate effector caspases such as caspase 3 and 7 and to initiate apoptosis. If the HPVE6 oncoprotein is expressed in the cell, however, this sequence cannot occur. E6 binds to FADD and procaspase-8 and blocks the interaction between the two cellular proteins; this binding also increases the degradation of FADD and procaspase-8. Both events prevent the successful completion of apoptosis, and cells become resistant to cell death induced by TNF-, Fas-, and TRAIL.[11–13]

Although two vaccines designed to prevent cervical cancer are now available,[14–16] the development of effective therapeutic drugs directed against HPV infection and disease remains an urgent need. Because the vaccine is prophylactic rather than therapeutic, it will bring no benefit to women and men who are already infected. In fact, it will not have a significant effect on human health for decades, as most women become infected in their late teens/early twenties, while cancer appears in their late forties/early fifties.

Abbreviation: HPV, human papillomavirus; TNF R1, tumor necrosis factor receptor 1; FADD, fas-associated protein with death domain; TRAIL, TNF-related apoptosis-inducing ligand protein; TRADD, tumor necrosis factor receptor type 1-associated death domain protein; TRAF, TNF receptor associated factors; DISC, death-inducing signaling complex; SAR, structure–activity relationship; GST, glutathione s-transferase; DMSO, dimethyl sulfoxide.

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Furthermore, the vaccine targets only two of the high-risk strains (16 and 18), which together account for 70–75% of the cases, leaving 25–30% of the high-risk infections unaffected by an individual's vaccination status. Finally, the high cost and multiple boosters needed (three shots total) make it unlikely that this vaccine will become readily available in the developing world in the near future. For these reasons, the development of novel and effective therapeutic interventions for established cases of cervical cancer remains an urgent need.

Small molecule approaches have been used to inhibit important signal transduction pathways that are involved in breast, colon, pancreatic and lung cancer formation.[17,18] However, such small molecule-based therapeutic agents have not yet been fully developed for HPV-mediated cancers. Our current knowledge of the molecular mechanisms by which HPV 16 E6 prevents apoptosis through the extrinsic pathway[8–12] has now allowed us to propose a novel approach for using small molecules to inhibit the binding between E6 and the key players of extrinsic apoptosis, FADD and procaspase 8. If this binding can be prevented, cells should be resensitized to apoptotic mediators such as TRAIL, and TRAIL-based therapy can then be successfully used to initiate apoptosis of E6-expressing tumor cells. In this paper we report on our results from screening a small molecule library for molecules that can prevent E6/FADD and E6/caspase 8 binding.

In this study, we used AlphaScreen™ technology (Perkin–Elmer) to search for molecules capable of inhibiting E6/FADD and E6/caspase 8 binding. Secondary screening allowed us to eliminate a number of molecules from consideration, and suggested two classes of chemicals for further analysis. The best candidate was the flavonol, myricetin, which served as a potent inhibitor of E6/caspase 8 binding and helped to define the structure–activity relationship (SAR) of the ligands.

The construction of the pGEX-E6 and pTriEx-Caspase-8 DED plasmids has been reported.[8] Expression and purification of GST-E6, His-FADD and His-Caspase-8 DED were carried out as previously described.[8,19] GST-tagged and His-tagged proteins were diluted in GST dilution buffer (PBS pH 8.0, 5% glycerol, 2 mM DTT) and His dilution buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 2 mM KCl, 5% glycerol, 2 mM DTT). Protein concentration was measured using Coomassie Plus—The Better Bradford Assay Reagent (Thermo Scientific). The purity of the isolated proteins was estimated following separation by SDS–PAGE and Coomassie staining (Fig. 1).

We began by using AlphaScreen™ technology to screen a 949 member small-molecule library from TimTec, LLC (Newark, DE) designed for similarity to kinase inhibitors. Members of the library were present at 10 μM in DMSO. Briefly, GST-E6 and His-FADD proteins were purified from *Escherichia coli* lysates after induction of protein expression by IPTG (Fig. 1). 5 μl (1 ng) of GST-E6 and 5 μl (338 ng) of His-FADD were included in each reaction mixture with 5 μl blocking buffer (0.5 mg BSA, 0.5% Tween 20 in PBS) in the absence or presence of 10 μM of each test chemical. After a one-hour incubation of the mixture at room temperature, 5 μl donor beads and 5 μl acceptor beads (Perkin–Elmer) were added to each well according to the manufacturer's protocol. The mixture was incubated in the dark at room temperature overnight, and the emitted signal was detected using the Envision Multilabel plate reader (Perkin–Elmer). In the presence of test chemicals, the binding affinity was calculated as a percentage of the binding in the presence of carrier only (DMSO). Of the 949 chemicals initially screened, 108 chemicals demonstrated some ability to interfere with E6 binding (11.4% of the original set of chemicals). These chemicals were then re-tested in triplicate to confirm activity, and 61 of the 108 showed some inhibitory activity (6.4% of the initial 949 chemicals). The compounds that demonstrated a high level of activity (inhibition of 90% and higher) were tested again in triplicate at 1:10 and 1:100 dilutions (1 μm and 0.1 μm). Finally, those compounds that appeared to show a dose–response relationship were retested at 1:50 and 1:500 dilutions in triplicate.

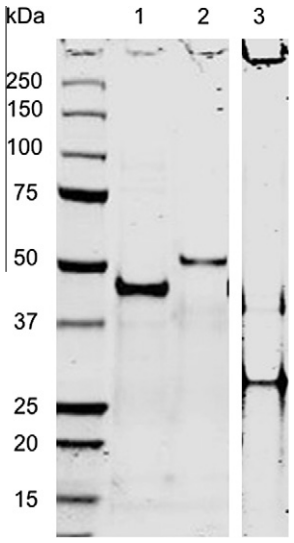

Figure 1. Purity of isolated proteins. Purified proteins (10 μg) were separated by SDS–PAGE. The gel was then stained using the Coomassie reagent. Lane 1: GST-E6, Lane 2: GST-caspase 8, Lane 3: His-caspase 8.

To analyze this screening data, we began with a SD file of the structures and the corresponding well layout provided by TimTec, LLC and imported it into an initial ChemFinder 11.0 database. The database was then exported into a ChemOffice for Excel spreadsheet. The structures were reviewed, and from these structures, a series of physical properties was calculated using the functions available in ChemOffice for Excel. These properties were: (1) *c*log*P*: calculated log octanol/water partition coefficient; (2) number of hydrogen bond donor atoms; (3) number of hydrogen bond acceptor atoms; (4) number of rotating bonds; (5) polar surface area; (6) molar refractivity; (7) number of heavy atoms.

From these data, another column assessed these parameters and the compounds were judged as passing or failing the Lipinski Rule of Five.[20] The structures were also assessed visually for possible reactivity with thiol groups (e.g., Michael acceptors), as HPVE6 has 6 surface Cys thiol residues. Compounds that failed the Lipinski Rule of Five, were not 'lead-like'[21] ($100 < MW < 350$ & $1 < c\log P < 3$) or were deemed potentially thiol-reactive were removed from consideration. After testing and data analysis we were left with 19 compounds from several different structural classes out of the original 949 compounds in the library.

Among the most potent of the 19 were a flavonol, kaempferol, and a flavone, chrysin 7-methyl ether. Notably, flavone and apigenin were in the original library and did not exhibit sufficient potency for selection. These data indicate that this class of compounds exhibits clear SAR at this binding site. Additionally, the literature contained several descriptions of this class of compounds having potential antitumor activity.[22–26]

We had shown previously that the E6 binding motifs on FADD and procaspase 8 proteins have a similar structure, and that the E6 binding to FADD and to procaspase 8 can be blocked by the same blocking peptide in both in vivo and in vitro assays.[19] Consistent with these findings, we were able to verify that kaempferol could indeed inhibit both the His-FADD and His-caspase 8 interaction with GST-E6 in a dose-dependent manner. Therefore, later analyzes were carried out using His-caspase 8 DED rather than His-FADD. Two advantages for the change were: (1) the His-caspase 8 DED protein proved easier to consistently purify than His-FADD as a properly folded protein, therefore giving us greater consistency in