Flavonol and imidazole derivatives block HPV16 E6 activities and reactivate apoptotic pathways in HPV\textsuperscript{+} cells

C-H Yuan\textsuperscript{1}, M Filippova\textsuperscript{1}, JL Krstenansky\textsuperscript{2} and PJ Duerksen-Hughes\textsuperscript{*,1}

High-risk human papillomaviruses (HR-HPVs) cause nearly all cases of cervical cancer, as well as approximately 30% of head and neck cancers. HPV 16 E6, one of two major viral oncoproteins, protects cells from apoptosis by binding to and accelerating the degradation of several proteins important in apoptotic signaling, including caspase 8 and p53. We proposed that blocking the interactions between HPV E6 and its partners using small molecules had the potential to re-sensitize HPV\textsuperscript{+} cells to apoptosis. To test this idea, we screened libraries of small molecules for candidates that could block E6/caspase 8 binding and identified several candidates from different chemical classes. We tested hits for dose-dependency and specificity in vitro and for toxicity in a cell-based assay and then used this information to select the two best candidates for further testing: myricetin, a flavonol, and spinacine, an imidazole amino-acid derivative of histidine. Both compounds clearly inhibited the ability of E6 to bind in vitro to both caspase 8 and E6AP, the protein that mediates p53 degradation. In addition, both compounds were able to increase the level of caspase 8 and p53 in SiHa cervical cancer cells, resulting in an increase of caspase 3/7 activity. Finally, both myricetin and spinacine sensitized HPV\textsuperscript{+} cervical and oral cancer cells, but not HPV\textsuperscript{−} cervical and oral cancer cells, to apoptosis induced by the cancer-specific ligand TRAIL, as well as the chemotherapeutic agents doxorubicin and cisplatin. New therapies based on this work may improve treatment for HPV\textsuperscript{+} cancer patients.

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High-risk types of human papillomavirus (HPV), especially types 16 and 18, are the causative agents of nearly all cases of human cervical cancer, in addition to up to 70% of head and neck cancers (HNC).\textsuperscript{1} Although the overall incidence of HNC has stabilized during the past decade, the incidence of HPV-associated cases, especially of oropharyngeal squamous cell carcinoma, has dramatically increased.\textsuperscript{2} HPVs are small, double-stranded DNA viruses that infect epithelial tissues. The HPV-encoded oncoproteins E6 and E7 are responsible for cellular immortalization and transformation and, consequently, for the development of HPV-associated cancer. Although E7 is best known for the inactivation of Rb, E6 accelerates the degradation of several molecules involved in apoptosis.

Two HPV vaccines, Gardasil (MSD, Merck, Kenilworth, NJ, USA) and Cervarix (GSK, Glaxo SmithKline, London, UK), have been approved and are currently in use for the prevention of HPV infection. However, they offer no benefit to an individual who has already been infected and only protect against 2 of the 15 types of high-risk viruses, HPV-16 and -18. Surgical and ablative techniques are used to remove developed tumors; however, these approaches are invasive and cytodestructive, and lesions frequently recur following treatment. Chemotherapy, utilizing agents such as cisplatin and doxorubicin, has also been used to treat cervical cancer but with mixed results.\textsuperscript{3–8} As researchers and clinicians have worked to move beyond these relatively non-specific and toxic agents, reagents that activate the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated, extrinsic apoptotic pathway have garnered considerable interest owing to their promise in the treatment of several types of tumors.\textsuperscript{9–17} Unfortunately, therapies that function by activating apoptosis, including those based on TRAIL, cisplatin and doxorubicin, are handicapped in their ability to effectively treat HPV-associated malignancies, because high-risk E6 proteins subvert both the extrinsic and intrinsic apoptotic pathways. E6 proteins from high-risk types of HPV are well known for their ability to mediate the rapid degradation of p53,\textsuperscript{18–21} an important mediator of intrinsic apoptotic pathways, thereby increasing the growth and survival of transformed cells.\textsuperscript{22,23} E6 also interacts with other partner proteins, a number of which participate in extrinsic, receptor-mediated apoptosis. For example, our laboratory found that HPV 16 E6 binds to and inactivates several molecules involved in these pathways, including TNF R1,\textsuperscript{24} Fas-associated protein with death domain (FADD),\textsuperscript{25} and caspase 8.\textsuperscript{26,27} As a result, engagement of either the extrinsic or the intrinsic apoptotic pathways fails to result in the transduction of the intended death signal because the mediator molecules – p53 in the case of the intrinsic pathway, and FADD and caspase 8 in the case of the extrinsic pathway – are missing. Therefore, if any of these
apoptosis-inducing signaling pathways are to be used as effective tools for the elimination of HPV-associated malignancies, it will be necessary to restore the missing signaling molecules.

In our previous work, we identified myricetin as a compound that can inhibit the E6/caspase 8 interaction in vitro. Unfortunately, myricetin is known to also inhibit a number of cellular proteins, including several tyrosine kinases, and its structure makes modification for drug development difficult. The identification of additional, more tractable inhibitors of the E6/procaspase 8 interaction was therefore pursued. Screening an ActiProbe 2 K library enabled us to identify several compounds of interest, and we further examined the ability of one particular compound, spinacine (5), to inhibit specific interactions between E6 and its partner proteins. We found that both myricetin and spinacine can re-sensitize HPV+ cells to apoptosis triggered by apoptotic inducers such as TRAIL, cisplatin, and doxorubicin by increasing caspase 3/7 activity and restoring the level of apoptotic proteins in HPV+ cells. These findings identify a novel small molecule that can inhibit E6 functions and may serve as the basis for further discovery and development of effective and novel therapeutic approaches for the treatment of HPV-mediated cancers.

Results

Imidazole derivatives specifically inhibit the interaction of HPV E6 with caspase 8. We previously reported that HPV E6 binds to caspase 8 and that myricetin can block the E6/caspase 8 interaction in vitro. In that study, we utilized a bead-based assay based on AlphaScreen Technology (Perkin-Elmer, Waltham, MA, USA) to identify inhibitors of the E6/caspase 8 interaction. In the current work, we extended our search by screening the ActiProbe 2 K (2000 compounds) library using the same approach. In our primary screen, 118 (5.9%) compounds demonstrated an ability to inhibit the E6/caspase 8 interaction. Seventy-nine of these 118 compounds presented EC50 values <10 μM and were therefore chosen for further analysis. Twenty-three of these 79 compounds also demonstrated specific inhibition of the E6/caspase 8 interaction, in that they were unable to block formation of the caspase 8/caspase 8 homodimer (counter-screen). These 23 compounds represented three different chemical classes, of which the imidazoles and the related benzimidazoles showed the best inhibition of E6/caspase 8 binding. Analysis of the structures and physical/chemical characteristics of those molecules led to the selection of five additional compounds: 3-(1H-indol-1-yl)propan-1-amine methanesulfonate (1), benzimidazole (2), 1H-benzimidazole-1-methanol (3), 2-(methoxymethyl)-1H-benzimidazole (4), and spinacine (5) for further study (Figure 1).

To determine the dose-responsiveness of these five compounds, variable concentrations of those small molecules were first tested for their ability to inhibit E6/caspase 8 binding. The results showed that only compounds 1, 2, and 5 demonstrated dose-dependent inhibition of E6/caspase 8 binding (Figure 2a). We then asked whether these molecules, which had been selected for their ability to interfere with E6/caspase 8 binding, could also affect other interactions of E6, such as its binding to E6-associated protein (E6AP). The E6/E6AP interaction is required for the E6-mediated acceleration of p53 degradation. We therefore tested the ability of these five compounds to inhibit E6/E6AP binding and found that compounds 1, 2, and 5, the same agents that had inhibited E6/caspase 8 binding, could also inhibit the E6/E6AP interaction and could do so nearly as well as they had inhibited the E6/caspase 8 interaction (Figures 2a and b).

To ask whether this inhibition was specific, we employed a counter-screening assay in which we assessed the ability of candidates to inhibit the binding of GST-caspase 8 to His-caspase 8; those that did were to be eliminated. In this counter-screening assay, the materials in each well were the same as in the primary screen, with the exception that GST-caspase 8 replaced GST-E6. Compounds 1 and 2 were able to inhibit the caspase 8/caspase 8 interaction about as well as they inhibited the E6/caspase 8 interaction, indicating...
that their actions were non-specific. However, spinacine (5) did not significantly inhibit the binding of caspase 8 to itself, providing evidence of its specificity for E6 (Figure 2c). These results are summarized in Table 1, which shows that, of the compounds tested, spinacine displayed the lowest EC50 value for the inhibition of E6/caspase 8 and E6/E6AP interactions, while not inhibiting caspase 8/caspase 8 binding.

Myricetin and spinacine sensitize SiHa cells to TRAIL-induced apoptosis. We next asked which of the molecules that block E6/caspase 8 binding in vitro could also act in the context of a cell. SiHa cells are an HPV+ cell line, derived from a cervical carcinoma, which serves as a commonly used model for HPV-associated malignancies. To determine whether HPV+ SiHa cells are resistant to TRAIL-induced apoptosis, SiHa cells were treated with TRAIL and cell viability was assessed. TRAIL-sensitive, HPV− U2OS cells served as a positive control. The results (Figure 3a) demonstrate that, in comparison to U2OS cells, SiHa cells are relatively resistant to treatment with TRAIL, as predicted. Furthermore, both myricetin and spinacine displayed low toxicity to SiHa cells in the absence of TRAIL (Figure 3b). We next asked whether myricetin and/or spinacine could sensitize these HPV+ cells to TRAIL-induced apoptosis. The indicated concentrations (0–125 μM) of myricetin, (3) (negative control), and spinacine (5) were added to SiHa cells in the presence of TRAIL, and Figure 3c shows that, in the presence of TRAIL, myricetin and spinacine were both able to reduce the viability of SiHa cells to 30–40% at a dose of 125 μM, in contrast to the negative control, 3. Table 2 lists and compares the concentrations of the three tested compounds required to induce death in 50% of SiHa cells

Table 1  EC50 (μM) values of tested small molecules for the indicated protein–protein interactions

|                  | (1) [μM] | (2) [μM] | (3) [μM] | (4) [μM] | Spinacine (5) [μM] |
|------------------|----------|----------|----------|----------|-------------------|
| GST-E6/His-caspase 8 | 0.63     | 1.038    | No Inhibition | No inhibition | 0.017             |
| GST-E6/His-E6AP  | 0.67     | 1.481    | No Inhibition | No inhibition | 0.02              |
| GST-caspase 8/His-caspase 8 | 0.83     | 1.051    | No Inhibition | No inhibition | No Inhibition     |

Figure 2  3-(1H-indol-1yl)propan-1-amine (1), three benzimidazole derivatives (2–4), and spinacine (5) inhibit protein–protein interactions. Five compounds at the indicated concentrations (1.4 μM–3.2 mM) were tested using our bead-based screening assay for their ability to inhibit three different protein/protein interactions: (a) GST-E6/His-caspase 8; (b) GST-E6/His-E6AP; and (c) GST-caspase 8/His-caspase 8. Binding in the presence of 1.4 μM of the test compound was set at 100%. Experiments were performed in triplicate, and error bars indicate the S.D.
Figure 3  (a) HPV+ SiHa cells are resistant to TRAIL-induced apoptosis as compared with the human osteosarcoma cell line U2OS. Cells (2 × 10^4 cells per well) were seeded into a 96-well plate, allowed to incubate overnight, and then treated with the indicated concentration of TRAIL in the presence of cycloheximide (5 μg/ml). The viability of cells was measured after overnight incubation using the MTT assay; the viability of cells untreated with TRAIL was set at 100% for each group. (b) The indicated concentrations of three small molecules were added to SiHa cells, and an MTT assay was performed after overnight incubation. The viability of cells untreated with small molecules was set at 100%. (c) The indicated concentrations of myricetin or spinacine were added 4 h prior to TRAIL (100 ng/ml) in the presence of cycloheximide (5 μg/ml) and incubated at 37 °C overnight. The viability of cells untreated with small molecules was set at 100%. (d and e) SiHa cells (2 × 10^4 cells per well) were seeded into a 96-well plate and allowed to incubate overnight. In all, 100 μM of myricetin and 50 μM of spinacine were added 4 h prior to either (d) doxorubicin or (e) cisplatin. Cell viability was measured after overnight incubation using the MTT assay, and the viability of cells untreated with either myricetin or spinacine was set at 100%. Experiments were performed in triplicate, and error bars indicate the S.D.
in the presence or absence of TRAIL and demonstrates that both myricetin and spinacine induced more cell death (an increase of 3.5-fold for myricetin and of 8.6-fold for spinacine) in the presence than in the absence of TRAIL (Table 2). These results provide strong evidence that inhibiting the E6/caspase 8 interaction can restore the sensitivity of HPV+ cells to apoptotic signals.

Myricetin and spinacine increase the sensitivity of SiHa cells to doxorubicin and cisplatin. As noted earlier, both myricetin and spinacine can also inhibit the binding of E6 to E6AP, an E3 ligase involved in the degradation of p53, in vitro. Based on this observation, we next asked whether myricetin and/ or spinacine would increase the sensitivity of SiHa cells to two drugs, doxorubicin and cisplatin, that are thought to act by inducing intrinsic apoptosis through the activation of pathways that involve p53. We found that, as compared with the negative control, both myricetin and spinacine increased the sensitivity of SiHa cells to doxorubicin and cisplatin (Figures 3d and e). These results suggest that the ability of both myricetin and spinacine to inhibit the interaction between E6 and E6AP, resulting in a predicted increase in p53, results in the sensitization of HPV+ cells to inducers of p53-mediated apoptosis.

Myricetin and spinacine increase caspase 3/7 activity. Activation of caspase 3/7 is an essential marker for both extrinsic and intrinsic apoptosis and can be used to determine whether cell death occurs through the apoptotic pathway. Our previous data (Figure 2) demonstrates that both myricetin and spinacine can inhibit the E6/caspase 8 and E6/E6AP interactions in vitro, resulting in increased cell death. If this cell death occurred through apoptosis, one prediction is that pretreatment of cells with either myricetin or spinacine should increase the level of TRAIL-, cisplatin-, and doxorubicin-induced activation of caspase 3/7. To test this prediction, caspase 3/7 activity was measured in SiHa cells following treatment with myricetin or spinacine. As shown in Figure 4a, we found that the caspase 3/7 activity increased when myricetin or spinacine were combined with TRAIL as compared with controls. Similar results were obtained following treatment with cisplatin and doxorubicin (Figures 4b and c). These data demonstrate that the increased cell death induced by the combination of apoptotic inducers and myricetin/spinacine occurs through the apoptotic pathway.

Myricetin and spinacine re-sensitize HPV+ cells to TRAIL-induced apoptosis by blocking the binding of HPV E6 to caspase 8. To determine whether myricetin and spinacine re-sensitize SiHa cells to TRAIL-induced apoptosis by specifically blocking the binding of E6 to caspase 8, we compared responses in the HPV+ SiHa cells with those from the HPV+ human cervical carcinoma cell, C33A, as these cells do not express E6. We found that, although the HPV+ SiHa cells displayed resistance to TRAIL at concentrations up to 100 ng/ml, their sensitivity increased dramatically in the presence of 100 μM myricetin. In contrast, the HPV+ C33A cells remained resistant to TRAIL-mediated apoptosis even in the presence of myricetin (Figure 5a). Similar results were also found following pretreatment with 50 μM spinacine (Figure 5b). Together, these results suggest that myricetin and spinacine can re-sensitize HPV+ cells to TRAIL-mediated apoptosis by specifically blocking the binding of HPV E6 to apoptotic proteins, such as caspase 8.

Myricetin and spinacine increase the levels of caspase 8 and p53 in SiHa cells. The data described above demonstrate that myricetin and spinacine are able to increase caspase 3/7 activity (Figure 4) and to sensitize SiHa cells to TRAIL (Figures 3a and c and 5) and p53 (Figures 3d and e) induced apoptosis. If this ability is due to inhibition of E6 functions, then an increase in the levels of caspase 8 and p53 should be observable in SiHa cells following treatment with myricetin and spinacine. To test this prediction, we treated SiHa cells with 50, 100, and 200 μM of myricetin or spinacine for 24 h and then measured the level of caspase 8 by immunoblot. The results (Figure 6a) demonstrate that treatment with myricetin caused an increase in caspase 8 levels at doses of 100 and 200 μM, while spinacine caused an increase in caspase 8 at doses from 50 to 200 μM. To ask how p53 levels were affected, we employed an ELISA assay. For comparison, C33A cells, which lack HPV E6 and express mutant p53, were also treated with the same concentrations of spinacine and myricetin. Mitomycin C was included in the assay to cause DNA damage and therefore increase p53 levels. The results from this experiment (Figure 6b) demonstrate that treatment of SiHa cells with myricetin caused a 4.4-fold increase in p53 at a dose of 200 μM and that treatment with spinacine caused a 3.4-fold increase in p53 at a dose of 100 μM, as compared with untreated cells. Furthermore, there was no significant change in p53 levels in C33A cells, confirming our earlier conclusion that myricetin and spinacine impact apoptotic pathways only in the presence of E6. Together, these results suggest that these small molecules are able to increase the sensitivity of SiHa cells to both TRAIL- and p53-induced apoptosis by blocking the ability of E6 to bind to its cellular partners, thereby reducing the degradation of caspase 8 and p53 in HPV+ SiHa cells.

Myricetin and spinacine sensitize HPV+ head and neck squamous cell carcinoma (HNSCC) cells to TRAIL-induced cell death. As shown above, myricetin and spinacine sensitize SiHa cells to TRAIL and chemotherapy drugs. These data provide proof-of-principle that small-molecule inhibitors can block HPV E6 functions and trigger HPV+ cervical cancer cell lines to undergo apoptosis. We next examined whether other types of HPV+ cancer cells, and in particular, HNSCC cells can also be sensitized in this
Myricetin and spinacine (5) increase caspase 3/7 activity in SiHa cells following treatment with TRAIL and chemotherapy drugs. SiHa cells (2 × 10^4 cells per well) were seeded into a 96-well plate and allowed to incubate overnight and then pretreated with 100 μM of myricetin or 50 μM of spinacine for 4 h. (a) TRAIL (100 ng/ml), along with cycloheximide (5 μg/ml), (b) cisplatin (50 μM), or (c) doxorubicin (2 μM) were added respectively. Caspase 3/7 activity was measured after 0, 1.5, 3, and 6 h using the CellTiter-Glo assay. Activity at 0 h of treatment was set at 100% for each group. Experiments were performed in triplicate, and error bars indicate the S.D.

Myricetin and spinacine (5) can re-sensitize HPV+, but not HPV−, cells to treatment with TRAIL. SiHa (2 × 10^4 cells per well) and C33A cells (1 × 10^4 cells per well) were seeded into a 96-well plate and allowed to incubate overnight, and then cells were pretreated in the presence or absence of (a) myricetin (100 μM) or (b) spinacine (50 μM) for 4 h. The indicated concentration of TRAIL along with cycloheximide (5 μg/ml) was added, and the cells were allowed to incubate overnight. Cell viability was measured by MTT assay, and the viability of cells untreated with TRAIL was set at 100%. Experiments were performed in triplicate, and error bars indicate the S.D.
Small molecules block E6 and trigger apoptosis in HPV+ cells

CH Yuan et al

Discussion

Currently, no small molecules targeting any of the HPV proteins are available for clinical use, although a few groups are examining the possibility of developing these sorts of small, inhibitory molecules to expand and enhance the limited therapeutic options currently available for HPV-associated malignancies. E6 and E7 oncoproteins are expressed at relatively high levels during cancer development and therefore have the potential to serve as useful targets. For example, inhibition of E6 is predicted to lead to increased cell death, as E6 normally functions to block both intrinsic and extrinsic apoptotic pathways.

One approach to blocking the interactions between E6 and its cellular partners is to use peptides, and previous work from our laboratory and others has identified peptide inhibitors that can specifically inhibit the interactions between E6 and caspase 8 or FADD or between E6 and E6AP. As compared with peptide inhibitors, however, small molecules possess numerous advantages because they are more stable, penetrate target cells more easily, and can more readily be modified and optimized by organic chemists during drug development. Some progress has been made in this area, as published studies have identified several compounds (some with EC50 values between 17 and 29 μM) that could inhibit the interaction between E6 and E6AP. However, no structure–activity relationship (SAR) has been carried out for those compounds because of the limited data set, and none of these findings have yet led to clinically useful interventions.

Our laboratory previously reported that the flavonol myricetin can inhibit interactions between E6 and caspase 8 in vitro, and preliminary results (data not shown) indicate that myricetin binds to E6 at a ratio of 2:1. Unfortunately, myricetin is known to inhibit a number of cellular proteins (including several kinases), indicating a lack of sufficient specificity. In addition, the high EC50 value observed in the context of a cell-based model system (Table 2) suggests that its ability to negatively affect tumors may be limited, perhaps because its polar nature limits its ability to enter cells. As polar groups are an important feature in assessing SARs, optimization of this series of compounds would be difficult without resorting to a pro-drug approach. Therefore, we engaged in additional screening efforts with libraries containing more 'lead-like' members that are expected to provide better platforms for optimization.

Of the five tested compounds (Figure 1), spinacine (5) was best able to specifically inhibit the interactions of E6 with both caspase 8 and E6AP (Figures 2a and b), demonstrating the
lowest EC_{50} values for both E6/Caspase 8 and E6/E6AP binding. However, it did not inhibit caspase 8/caspase 8 binding, thus demonstrating the desired specificity (Table 1). In the experiments that involved treating SiHa cells with TRAIL, the EC_{50} values demonstrate that, as compared with myricetin, spinacine may be better able to penetrate into cells and thus re-sensitize cells more efficiently to TRAIL-induced apoptosis (Table 2).

**Figure 7** Myricetin and spinacine can re-sensitize HPV^+, but not HPV^−, head and neck cancer cell lines to treatment with TRAIL. (a) Both HPV^− and HPV^+ HN cancer cell lines display resistance to TRAIL treatment. (b) HPV^− (UMSCC 29 and SCC 84) and (c) HPV^+ (UMSCC 47, UPCI-SCC90-UP-Clone 35, and UM-SCC47-TC-Clone 3) head and neck cancer cell lines (2 × 10^4 cells per well) were seeded into 96-well plates and allowed to incubate overnight, and then cells were pretreated with myricetin (0–200 μM) or spinacine (0–100 μM) for 4 h. In all, 50 μM of TRAIL along with cycloheximide (5 μg/ml) was added, and cells were allowed to incubate overnight. Cell viability was measured by the MTT assay, and the viability of cells untreated with small molecules was set at 100%. Experiments were performed in triplicate, and error bars indicate the S.D.
Therapies based on TRAIL-mediated apoptosis, alone or in combination with other agents, are attractive possibilities for the treatment of many different types of cancer. However, these treatments are unlikely to be helpful in the context of HPV on their own, owing to the ability of E6 to compromise apoptotic pathways. Our study found that the combination of TRAIL with small molecules can re-sensitize E6-expressing cells to TRAIL-induced apoptosis, potentially filling this gap. In addition to sensitizing cells to TRAIL, we found that both myricetin and spinacine sensitized cells to intrinsic apoptosis triggered by chemotherapy drugs, such as doxorubicin and cisplatin. This may be clinically relevant, because cervical cancer tends to be relatively resistant to chemotherapeutic treatments, such as cisplatin. We note that myricetin required a higher concentration (100 μM) than did spinacine (50 μM) to inhibit binding and to sensitize cells, suggesting that spinacine may be a more efficient agent. Taken together, these data suggest that myricetin and spinacine can block the...
Myricetin and spinacine (5) can re-sensitize HPV+ but not HPV− HNSCC cell lines to treatment with doxorubicin. Two HPV− (UMMSCC 29 and SCC 84) and three HPV+ (UMSCC 47, UPCI-SCC90-UP-Clone 35, and UM-SCC47-TC Clone 3) head and neck cancer cell lines (2 × 10⁴ cells per well) were seeded into 96-well plates and allowed to incubate overnight, and then cells were pretreated with myricetin or spinacine (0–12.5 μM) for 4 h. In all, 2 μM of doxorubicin was added, and the cells were allowed to incubate overnight. Cell viability was measured by MTT assay, and the viability of cells untreated with small molecules was set at 100%. Experiments were performed in triplicate, and error bars indicate the S.D.

**Figure 8** Myricetin and spinacine (5) can re-sensitize HPV+, but not HPV−, HNSCC cell lines to treatment with doxorubicin. Two HPV− (UMMSCC 29 and SCC 84) and three HPV+ (UMSCC 47, UPCI-SCC90-UP-Clone 35, and UM-SCC47-TC Clone 3) head and neck cancer cell lines (2 × 10⁴ cells per well) were seeded into 96-well plates and allowed to incubate overnight, and then cells were pretreated with myricetin or spinacine (0–12.5 μM) for 4 h. In all, 2 μM of doxorubicin was added, and the cells were allowed to incubate overnight. Cell viability was measured by MTT assay, and the viability of cells untreated with small molecules was set at 100%. Experiments were performed in triplicate, and error bars indicate the S.D.
binding of E6 to multiple apoptotic proteins, including caspase 8 and E6AP/p53, thereby reactivating the E6-compromised apoptotic pathways and rendering HPV+ cells sensitive to both intrinsic and extrinsic inducers of apoptosis.

The ability of myricetin and spinacine to sensitize HPV+ SiHa cells to apoptosis induced by TRAIL and chemotherapy agents led us to ask whether the same effects could be seen in HPV+ HNC cells. We found that the result from our HNC cells closely mirrored those from the cervical cells, indicating that our results should have broad applicability to HPV-associated malignancies regardless of the site of origin.

Our data suggest a mode of action in which the small molecule interacts directly with E6, either destabilizing the virus protein and changing its conformation, or blocking the interactions by direct interference. If the latter, one possibility is that myricetin/spinacine binds to a region on E6 required by both E6AP and caspase 8. Further work is necessary to develop a detailed model for the interactions between E6 and E6AP/caspase 8, which can then be used to design one or more highly specific inhibitors.

Overall, these results regarding the combination of small molecules with TRAIL/chemotherapeutic agents suggest a promising and novel therapeutic approach for the effective and selective killing of HPV+ cancer cells and may provide the basis for developing an effective therapeutic strategy to treat HPV-mediated cancers.

Materials and Methods
Protein purification. The construction of the pGEX-E6, pTrEx-E6AP, and pTrEx-Caspase-8 DED plasmids has been reported. Expression and purification of GSTE6, His-E6AP, and His-Caspase-8 DED were carried out as previously described. GST-tagged and His-tagged proteins were diluted into GST dilution buffer (PBS pH 8.0, 5% glycerol, 2 mM DTT) and His dilution buffer (20 mM Heps pH 7.4, 150 mM NaCl, 2 mM MKCI, 5% glycerol, 2 mM DTT), respectively. Protein concentration was measured using Coomassie Plus – The Better Bradford Assay Reagent (Thermo Scientific, Waltham, MA, USA). Purity of the isolated proteins was estimated following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining.

Small-molecule library and acquisition of additional compounds. The 2000-compound small-molecule library (ActiProbe 2K) was acquired from TimTec, LLC (Newark, DE, USA) and was chosen because it encompasses a highly diverse selection of lead-like compounds. Five additional derivatives were purchased from Sigma (St. Louis, MO, USA) (benzimidazole, 2-(methylcarboxyethyl)-1H-benzimidazole, and 3-(1H-indol-1-yl)propan-1-amine methanesulfonate) and TimTec (1H-benzimidazole-1-methanol and 4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid). (Note: The material provided by TimTec as 6,7-dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid whose com- plex used as monoclonal capture antibodies. This antibody has a broad specificity, covering a diverse selection of lead-like compounds. Five additional derivatives were purchased from Sigma (St. Louis, MO, USA) (benzimidazole, 2-(methylcarboxyethyl)-1H-benzimidazole, and 3-(1H-indol-1-yl)propan-1-amine methanesulfonate) and TimTec (1H-benzimidazole-1-methanol and 4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid). (Note: The material provided by TimTec as 6,7-dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid whose com- plex used as monoclonal capture antibodies. This antibody has a broad specificity, covering a diverse selection of lead-like compounds. Five additional derivatives were purchased from Sigma (St. Louis, MO, USA) (benzimidazole, 2-(methylcarboxyethyl)-1H-benzimidazole, and 3-(1H-indol-1-yl)propan-1-amine methanesulfonate) and TimTec (1H-benzimidazole-1-methanol and 4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid). (Note: The material provided by TimTec as 6,7-dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid whose complex used as monoclonal capture antibodies. This antibody has a broad specificity, covering a diverse selection of lead-like compounds. Five additional derivatives were purchased from Sigma (St. Louis, MO, USA) (benzimidazole, 2-(methylcarboxyethyl)-1H-benzimidazole, and 3-(1H-indol-1-yl)propan-1-amine methanesulfonate) and TimTec (1H-benzimidazole-1-methanol and 4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid).)
forms of the protein. These antibodies were absorbed (50 μl per well, 4 μg/ml) onto the surfaces of a Nunc-immuno plate, MaxiSorp Surface (NalgeneNunc International, Rochester, NY, USA) by incubation overnight at 4 °C. The plates were then washed on an Auto Strip Washer, ELx50 (Bio-Tek Instruments, Inc.) using PBST (PBS plus 0.1% Tween 20) six times. Nonspecific binding sites were blocked by incubation with 200 μl per well of PBS, including 10% calf serum (Invitrogen) (blocking buffer) for 2 h at room temperature, followed by washing as described above. In all, 100 μl of each cell lysate to be tested were then added to the coated wells and allowed to incubate overnight at 4 °C. After washing, 100 μl of a solution containing 4 μg/ml biotinylated anti-p53 antibodies (polyclonal, produced in sheep, Roche) diluted into blocking buffer was added to each well and allowed to incubate for 45 min at room temperature. The plates were washed, and then the avidin–peroxidase conjugate (Sigma; 100 μl/well, 2.5 μg/ml diluted into blocking buffer) was added and allowed to incubate for 30 min at room temperature. After washing, 100 μl of the substrate (0.3 mg/ml ABTS (2,2′-azino-di-(3-ethylbenzthiazolin sulfonate) dissolved into 0.1 M citric acid, pH 4.5, with 1 μl 30% H2O2 added just before use) was added to each well and allowed to incubate for approximately 30 min. The absorbance at 405 nm was read with a microplate reader (Dynex Technologies; MRX Revelation software, Chantilly, VA, USA). The protein concentration of each lysate was also measured using the BCA method (Pierce, Chantilly, VA, USA) and used to normalize the measured p53 values for possible variations in the number of cells per well. Each p53 value (obtained from the ELISA assay) was divided by the protein concentration to obtain a normalized p53 value (ng p53 per mg total protein). The average and S.D. of the replicates (a minimum of three) were calculated, normalized to the control, and used to prepare the graph.

Conflict of Interest

The authors declare no conflict of interest.