6-2013

Small Molecule Inhibitors of HPV16 E6

Chung-Hsiang Yuan

LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Small Molecule Inhibitors of HPV16 E6

by

Chung-Hsiang Yuan

A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Microbiology and Molecular Genetics

June 2013

Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

_____, Chairperson
Penelope J. Duerksen-Hughes, Professor of Biochemistry

_____
Maria Filippova, Assistant Research Professor of Microbiology

_____
Daila S. Gridley, Professor of Microbiology and Radiation Research

_____
John L. Krstenansky, Professor and Director of Medicinal Chemistry

_____
Ubaldo Soto, Assistant Research Professor of Microbiology

_____
Ron E. Swensen, Associate Professor of Obsterics and Gynecology

ACKNOWLEDGEMENTS

I would like to show my gratitude to the Department of Microbiology and Molecular Genetics at Loma Linda University for allowing me to conduct my research and providing any assistance requested. In this program, I have learned a lot not only from the class but also in my research. The success of this study required the help of various individuals. Without them, I might not meet my objectives in doing this study. First, I want to give gratitude to my mentor Dr. Duerksen-Hughes for giving the guidance, support, and knowledge in exploring things. She has supported me throughout my thesis with her patience and knowledge. I truly appreciate her encouragement and effort on my PhD degree and without her this thesis, too, would not have been completed or written.

I would also like to thank Masha, Dr. Maria Filippova, for giving ideas for the improvement of my research; and for her patience, understanding and encouragement. She has provided me the skills to find out the questions, design experiments and a set of molecular biology tools. The most important thing she told me is “to think logically”, and I will never forget it. With her guidance and help, I am able to move forward to be a research scientist. It is my honor to work with all the members of the Dr. Duerksen-Hughes lab. I would like to thank Dr. Valery Filippov, Vonetta Williams, Whitney Evans, and Yan Chen for all of their help and advice in my lab work.

Last but not least, I would like to thank my family and my girlfriend, Chun-Chiao Lee, for supporting me throughout all my studies. With the help of them, I have overcome difficulties and maintained focus on my goal.

CONTENTS

FIGURES

Figure Page

TABLES

Tables Page

ABSTRACT OF THE DISSERTATION

Small Molecule Inhibitors of HPV16 E6

by

Chung-Hsiang Yuan

Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, June 2013
Penelope Duerksen-Hughes, Chairperson

High-risk human papillomaviruses (HR-HPVs) cause nearly all cases of cervical cancer. HPV 16 E6, one of two viral oncogenes, protects cells from apoptosis by binding to and accelerating the degradation of several apoptotic proteins, 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^+ cells to apoptosis. To test this prediction, we screened libraries of small molecules for candidates that could block E6/caspase 8 binding, and identified several candidates from different chemical classes. Testing hits for dose-dependency and specificity *in vitro* and for toxicity in a cell-based assay allowed us to select the two best candidates, myricetin and 6,7-dihydro-1H-imidazo[4,5-c] pyridine-6-carboxylic acid (DIPC), for further testing. Both compounds significantly and specifically inhibited the ability of E6 to bind *in vitro* to both caspase 8 and to E6AP, the protein that mediates p53 degradation. In addition, both compounds were able to increase caspase 3/7 activity and to sensitize HPV^+ SiHa cells, but not HPV^- C33A cells, to apoptosis induced by TRAIL, a cancer-specific ligand, as well as the chemotherapeutic agents doxorubicin and cisplatin. New therapies based on this work may improve treatment for HPV^+ cancer patients.

CHAPTER ONE

INTRODUCTION

Human Papillomavirus Infections and Cancer

Human papillomaviruses (HPV) are small, double stranded DNA viruses that infect epithelial tissues, including those of the anogenital tract. Of the more than 200 different identified types of HPV, about 40 are involved in genital tract infections. Some types of HPV cause warts, while others can increase the risk of malignancy and lead to cancer of the cervix, other anogenital regions, and the head and neck areas [1]. HPV types are classified as either high risk or low risk, with high-risk types being associated with cancer formation while low risk types are not. For example, HPV types 6 and 11 are classified as low risk types, and infection with these types results in the proliferation of epithelial cells and manifests as warts or papillomas on the skin. However, these infections are generally self-limiting and do not lead to malignancy. On the other hand, HPV types 16 and 18 are major high-risk genotypes, which together cause up to 75% of cervical cancer cases as well as a significant number of head and neck squamous cell carcinomas. The association between HPV and human cancer has been studied for more than three decades. HPV-induced cervical cancer is the second most common cancer and the fifth leading cause of cancer-related deaths among women worldwide [2, 3]. Cervical cancer is not the only cancer caused by high-risk types of HPV; in fact, biological and functional studies have demonstrated the role of HPV in the development of several

additional types of cancer, such as cancer of the head and neck, vaginal, vulvar, anal, and penile areas [4, 5].

When high-risk HPV infects young females, most infections cause no or only minor symptoms, and usually disappear without any treatment within a few years. However, in 5% to 10% of infected women, the infection can persist for many years [6]. Persistent infections are at relatively high risk for causing cell abnormalities and developing lesions of the cervix, which can in turn lead to the development of cervical cancer. In 2010, 12,000 women in the United States were diagnosed with HPV-induced cervical cancer, of which 4000 were expected to die. Furthermore, cervical cancer is the second most common cancer in women worldwide. There are approximately 400,000 new cases of this disease diagnosed each year and approximately half of these patients will die. The development of HPV-induced cervical cancer usually requires 15–20 years, providing a window of opportunity for detecting the presence of HPV and/or HPV-induced cell abnormalities.

Unfortunately, current approaches for the prevention and treatment of HPV-associated malignancies are not always effective. Significant progress has been made with regards to prevention, in that two vaccines that target high-risk HPVs, Cervarix and Gardasil, have both demonstrated remarkable efficacy in preventing infection within the vaccinated population [7]. These vaccines have been shown to reduce the infection rate (by at least 90%), as well as the incidence of early neoplasia, caused by the targeted HPV types (HPV type 16 and 18 in the case of Cervarix; HPV types 6, 11, 16, and 18 in the case of Gardasil) [8-10]. However, because these vaccines are prophylactic rather than therapeutic, they are not effective interventions for patients already infected with HPV.

Surgical and ablative techniques are available for these patients, including excision, electro- and cryosurgery, and laser-based treatments. However, these procedures are invasive and cytodestructive, and lesions frequently recur following treatment. Combined chemoradiotherapies have also been assessed, but with mixed results. Topical treatments for warts are available, and include trichloroacetic acid, liquid nitrogen, imiquimod, interferon-α injections and podophyllin resin [11]. However, repeated treatments are again necessary, treatment is not always effective, and imiquimod is not approved for use in the oral cavity. Also, while these treatments may remove warts, they do not cure the HPV infection itself. Viral DNA can be detected before cell abnormalities are observed, and the Papanicolaou test, also called a Pap smear or Pap test, is used to screen and detect abnormal cells, possible precursors for malignancies. However, Pap tests may not always detect the abnormal cells. First, the sample may not contain abnormal cells that may be present on the cervix. Second, the abnormal cells may exist high up in the cervix or deep in the glands of the cervix (adenocarcinoma), and it is difficult to obtain samples from these areas. Due to the limitations of current approaches, therefore, there remains an urgent need for the development of effective and novel therapies that are based on a solid understanding of the biology of the virus.

HPV Virology

HPV Genes and Proteins

The HPV genome is divided into an early region (E), a late region (L) and a non-coding long control region (LCR) (Figure 1A). The E region encodes seven non-structural proteins: E1, E2, E4, E5, E6, E6* and E7, while the L region encodes two

structural proteins: L1 and L2, which code for the major and minor capsid proteins, respectively. Early genes are responsible for modulating epithelial cell function so as to favor virus production. For example, their gene products modulate keratinocyte differentiation, promote viral DNA replication and segregation, and inhibit viral clearance by the immune system. All early HPV proteins perform multiple functions, and act by interacting with a variety of cellular partners [12, 13]. These interactions in turn modify the flow through numerous cellular pathways, including apoptosis. The core proteins of the late genes, L1 and L2, together with HPV DNA, participate in the assembly of virus particles.

HPV Life Cycle

HPV infects cells in the basal layer, below the surface of the epithelium, and carries out a life cycle that is closely tied to the differentiation program of the host cell (Figure 1B). During the early stages of infection, the episomal viral genome is usually present at fewer than 100 copies per cell, and functions as a slowly replicating plasmid. Shortly after infection and uncoating, low-level expression of early genes (E6, E6*, E7, E1 and E2) as directed by the early promoter enables maintenance of the episomal state of the HPV genome [14-18].

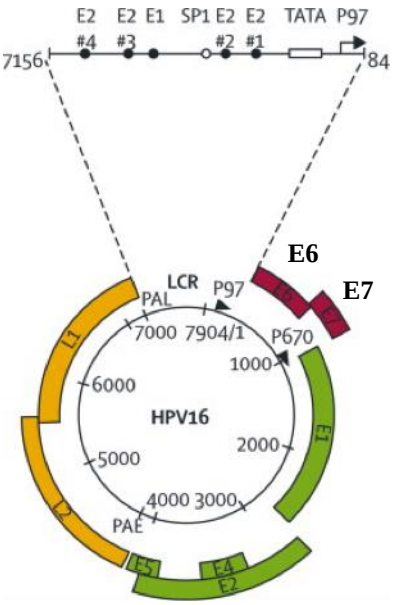
As cells move up through the layers, they begin to proliferate. The ability of E6 and E7 to suppress apoptosis and to alter the function of factors involved in cell-cycle regulation, respectively, facilitates prolongation of the proliferative stage of keratinocyte differentiation [19, 20]. During this proliferative stage, E1 and E2 function to regulate viral DNA replication and transcription such that viral DNA is replicated coordinately

with the host cell chromosome. E2 binds to the HPV upstream regulatory region and recruits the E1 DNA helicase to the viral origin of replication. In addition, E2 acts to bind the necessary factors onto mitotic chromatin and to tether the virus genome to host chromosomes during mitosis [21]. Meanwhile, E1 binds to the viral origin of replication. It then uses ATP to unwind double-stranded DNA, acting like a helicase.

Amplification of viral genomes begins in the proliferative layers, and an increased level of expression of all the early viral gene products, including E4 and E5 [22-24], is necessary for this process. During this stage, an increase in transcription of the genes coding for the E1, E2, and E1-E4 fusion proteins is observed [25, 26]. The sequence of E4 is located entirely within the E2 open reading frame (ORF), and the protein is expressed relatively late during viral infection. E2 is co-expressed with and binds to E1^E4 protein and this interaction results in the stabilization of both proteins [27]. Overexpression of the E1^E4 protein enables reorganization of keratin network during keratinocyte differentiation and viral genome amplification [28].

Both the E4 and E5 proteins participate in the late viral functions, although details of these mechanisms are not yet entirely defined. It has been shown that E5 plays a subtle role during the productive stage of the HPV16 life cycle [23, 29]. When the infected cells reach the point of terminal differentiation, the viral copy number is increased up to a thousand per cell, and the late genes L1 and L2, which encode viral capsid proteins, are expressed. The viral capsid proteins assemble into virus particles and are released and spread to other host cells. Through these mechanisms, HPV is able to enhance the survival of infected cells in order to facilitate its own replication cycle, and thus ensure the production and spread of progeny.

A



B

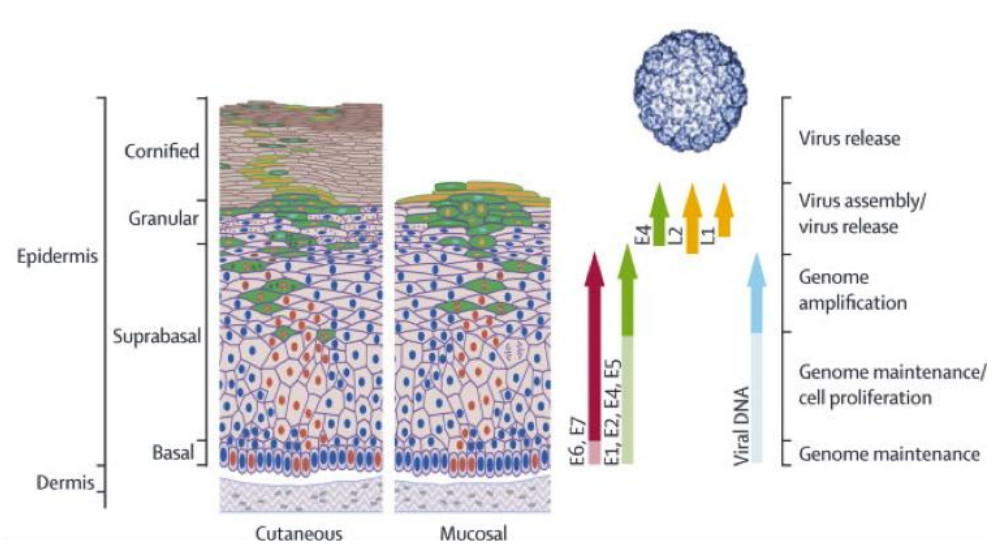


Figure 1 (A) Genomic map of HPV 16 (B) Viral gene expression following HPV infection. Taken from Doorbar et al, 2006 [30].

Integration and Cancer

During the normal viral life cycle, the HPV genome exists in host cells as an episome. In rare cases, however, integration of the viral genome into that of the host can occur. Interestingly, this integration is not required for the HPV life cycle and is, in fact, detrimental to the virus, as integrated viral sequences do not reproduce. However, integration is closely tied to the development of cancer, as most cases of HPV-induced cervical cancer feature an integrated form of the HPV genome. Such integration typically leads to an increase in the expression of HPV early proteins, including the E6 and E7 oncoproteins, and a consequential increase in cellular transformation and the probability of HPV induced carcinogenesis [31]. The activities of E6 are of particular relevance to cancer development, because E6 functions to block both intrinsic and extrinsic apoptosis.

Apoptosis

Apoptosis, also known as programmed cell death, plays an important role in development, cellular homeostasis and the pathophysiology of many diseases [32]. Apoptosis helps to eliminate damaged cells and also contributes to the elimination of virus-infected cells [33]. If inappropriately up- or down-regulated, apoptosis can also contribute to various diseases such as autoimmunity and cancer [34]. Two major classic apoptotic pathways have been identified: the extrinsic pathway, triggered by the engagement of "death receptors", and the intrinsic pathway, which responds to a variety of stress signals (for example, radiation) by triggering the release of intracellular signals that promote cell death [35-37].

Extrinsic Pathway

Apoptosis is triggered through the extrinsic pathway when specific receptors are activated by their corresponding ligands. The majority of these "death" ligands belong to the TNF (tumor necrosis factor) family, and their corresponding cell surface "death" receptors to the TNF receptor family. TNF-alpha binds to TNF R1 or R2 receptors, Fas ligand to Fas, and TRAIL (TNF-related apoptosis-inducing ligand) binds to Death Receptor (DR) 1, DR2, DR3, DR4, and DR5. Other receptors within this family include FADD-like interleukin-1 beta-converting enzyme (FLICE) and TNF-like weak inducer of apoptosis (TWEAK) [38]. Adaptor molecules such as TRADD (Tumor necrosis factor receptor type 1-associated DEATH domain protein) and FADD (Fas-Associated protein with Death Domain) are engaged, and initiator caspases such as procaspase-8 are activated. Interactions of regions such as the death domain (DD) allow TRADD, FADD, and pro-caspase 8 to combine to form the death-inducing signaling complex (DISC) [39]. Formation of the DISC enables cleavage of caspase-8, which then cleaves and activates executioner caspases such as procaspase-3/7. The active caspase-3/7 can cleave many different target substrates, including poly(ADP-ribose) polymerase (PARP), and these cleavages lead to the demise of the cell. PARP is a nuclear enzyme that catalyzes the transfer of the ADP-ribose moiety of NAD+ to a specific subset of nuclear substrates in response to DNA damage. Active caspase-3 cleaves PARP into 24 kDa and 89 kDa segments, thereby eliminating the ability of PARP to function in DNA repair and thus promoting apoptosis. In the case of Fas-mediated apoptosis, two pathways have been shown to operate in a cell type-dependent manner (types I and II) [40]. In the type I cell model, a robust activation of caspase-8 can be observed following DISC formation.

Overexpression of anti-apoptotic proteins such as Bcl-2 or Bcl-XL cannot stop the cleavage of caspase-8 and caspase-3, and mitochondrial activity is not required to trigger apoptosis in type I cells. In contrast, DISC formation in type II cells is weak, and additional signaling from the mitochondria is necessary for apoptosis. Low levels of DISC and capsase-8 are detected in type II cells. In this context, caspase-8 cleaves Bid and triggers mitochondrial depolarization and the release of cytochrome c. Following its release, cytochrome c forms a complex with apoptosis protease activating factor 1, (Apaf-1) and procaspase-9, called the apoptosome. The apoptosome is analogous in function to the DISC, and mediates the activation of caspase-9, which in turn activates caspase-3. The intracellular events of apoptosis then give way to the external characteristics of this form of cell death, which include chromatin condensation, phosphatidlyserine exposure, cytoplasmic shrinkage and membrane blebbing. Anti-apoptotic proteins such as Bcl-2 and Bcl-XL can block the activation of caspase in type II cells and significantly reduce apoptosis [40, 41]. For ligands other than those of the TNF family, initiator caspases include capsase-10 and caspase-2, and the corresponding adaptor proteins are RIP, RAIDD/CRADD, and TRAFS. In most of these cases, activation of the executor caspases 3/7 initiates the apoptotic cascade [42-45].

Intrinsic Pathway

The classic intrinsic apoptotic pathway, also referred to as the mitochondrial pathway, can respond to a variety of external stimuli such as DNA damage, radiation and osmotic stress [46, 47]. Activation of this pathway results in the release of cytochrome c from the mitochondrial intermembrane space to the cytosol. Apoptotic protease activating

factor-1 (Apaf-1), pro-caspase 9 and cytochrome c then form a complex called the apoptosome in the cytosol [48, 49]. The binding of Apaf-1 and cytochrome c to procaspase-9 leads to the auto-cleavage and activation of procaspase-9. The active caspase-9 then cleaves and activates caspase-3, which induces the cleavage of additional substrates, such as PARP gelsolin, and protein kinase C-delta [50-52] (see Figure 2).

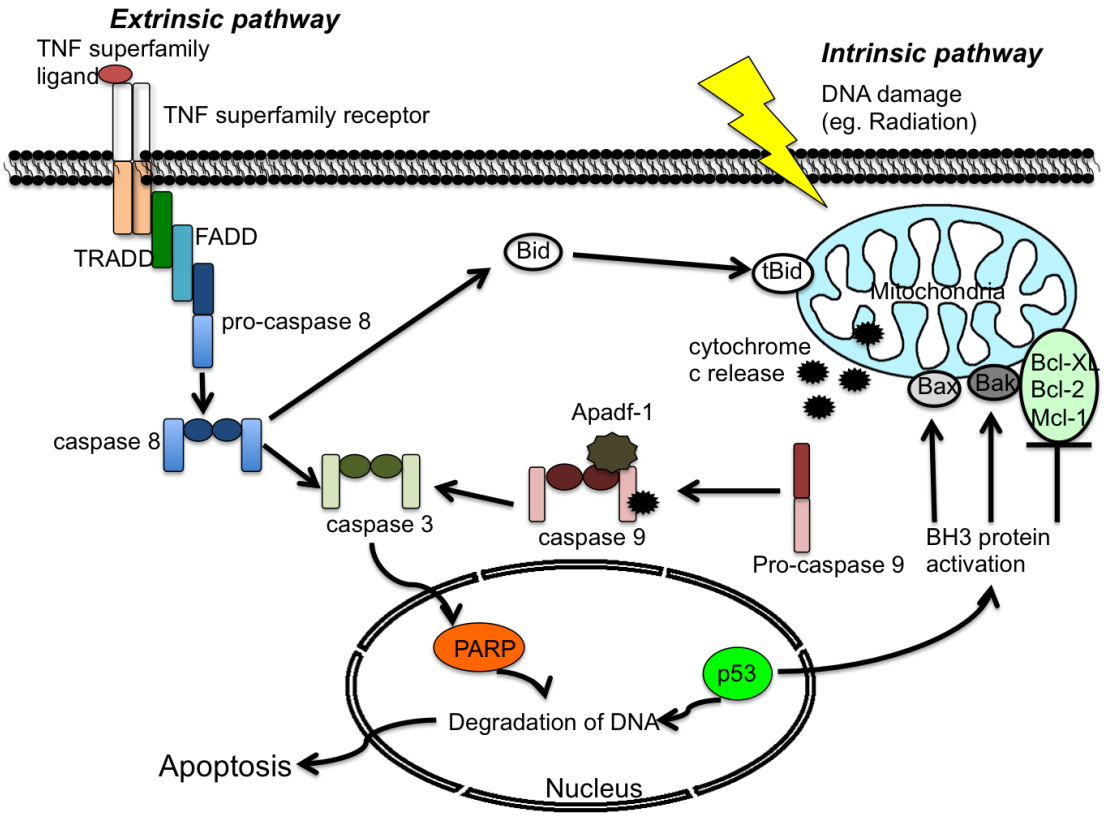


Figure 2. Generalized overview of the extrinsic and intrinsic apoptosis pathways. The extrinsic pathway is initiated by the binding of a tumor necrosis factor (TNF) family ligand to its receptor, followed by activation of downstream signaling proteins. In the intrinsic pathway, cellular stress causes BH3 activation and cytochrome c release. Both pathways activate the effector caspase-3 and promote cell death. Proteins are identified within the text [53].

Virus-Mediated Modulation of Apoptosis

Following infection of host cells, a virus typically employs a number of different mechanisms to avoid the host immune response and to initiate and maintain efficient production and spread of its progeny. In most cases, a host organism will attempt to initiate apoptosis in order to eliminate infected cells. For this reason, many viruses, especially the DNA viruses, encode a variety of viral proteins that can inhibit or delay these protective actions, often by targeting key cellular proteins [37]. For example, human adenovirus employs the E3 14.7 kDa protein to reduce TNF- and Fas-induced apoptosis; these proteins work by inhibiting the TNFR-induced release of arachidonic acid [54]. Poxvirus produces two proteins, CrmB and CrmC, that change the conformation of TNFR in order to block the binding of TNF [55]. In addition, poxvirus and herpesvirus both encode proteins with homology to the DED domain, and thus block the binding of death receptors and the FADD adaptor protein to caspase-8 [56]. CD40 and CD40L form a receptor/ligand pair found on antigen presenting and T cells, respectively, and is required for their activation. Epstein-Barr virus (EBV), a causal agent of epithelial and lymph tumors, can increase the expression of CD40L but down-regulates the expression of CD40. This abnormal interaction between CD40L and CD40 contributes to decreased apoptosis of infected cells and the development of myeloma cells [57]. Furthermore, the Epstein-Barr nuclear antigen 3C (EBNA3C) is known to possess anti-apoptotic properties and to regulate cell proliferation. For example, EBNA3C can bind to p53 and Gemin3 so as to inhibit the binding between p53 and DNA, thus blocking the p53-mediated apoptotic pathway [58]. HPV also expresses several proteins that affect cellular apoptotic pathways, as detailed below.

HPV Proteins That Modulate Apoptosis

Apoptosis is an important mechanism through which the host can eliminate infected cells, and the ability to avoid apoptosis can therefore enhance virus survival. In addition, apoptosis plays an important role during keratinocyte differentiation [59]. For these reasons, it is to the virus' advantage to intervene in apoptotic pathways, and five papillomavirus proteins, E2, E5, E6, E6* and E7, have been shown to do so. E5 and E6 can protect host cells from apoptosis, while E2 and E6* appear to possess pro-apoptotic properties. E7 can act in either a pro-apoptotic or an anti-apoptotic manner, depending on the cell type and circumstances. While it is not entirely clear why pro-apoptotic properties of virus proteins might enhance virus survival and propagation, it is likely that the influence of each HPV protein on host apoptotic pathways is related to specific stages of the virus life cycle. Overexpression of the major oncogenes, E6 and E7, during cell transformation generally results in resistance of transformed cells to apoptosis; this ability to avoid apoptosis is one of the major hallmarks of carcinogenesis [60].

HPV E6

The essential oncoprotein HPV E6 has been widely studied over the last two to three decades. E6 is a small protein, consisting of 151 amino acids and presenting two atypical zinc fingers with motifs that contain two cysteines (Cys-X-X-Cys). The p53 tumor suppressor is the first-described and best-known target of HPV E6 [61]. p53 acts as a transcriptional factor, and can trigger cell cycle arrest or apoptosis in response to cellular stress or DNA damage. Under normal conditions, triggers such as DNA damage cause increases in the level of p53, which then lead to downstream effects such as cell

cycle arrest and/or apoptosis, depending on the intensity or amplitude of the damage or stimulus. The presence of E6 from high-risk types of HPV interferes with this process, because E6 binds to both p53 and the E6-associated protein ligase (E6AP), causing ubiquitinylation and the subsequent degradation of p53. This loss then prevents p53 from inducing either growth arrest or apoptosis of infected cells [62]. During this process, E6 first binds to E6AP, which functions as an E3 ubiquitin protein ligase. Then, the E6/E6AP complex binds to the core domain of p53. E6AP catalyzes the transfer of ubiquitin to p53, thereby enabling its proteasome-mediated degradation. This E6-mediated inhibition of p53 activity can increase the survival of transformed cells [63, 64]. Interestingly, a recent study showed that E6 could also interact with ubiquitin ligases other than E6AP in order to promote p53 degradation [65].

In addition to p53, E6 also interacts with other partner proteins that play a variety of roles in the cell [12, 66]. Following binding to E6, many of these proteins are lost by an E6AP-mediated ubiquitinylation and degradation process similar to that observed for p53. E6 partners include proteins involved in apoptosis such as Bak [67], c-Myc [68], TNF receptor 1 (TNF-R1) [69], FADD, and caspase-8 [12, 70-72]. Bak and Myc were the first apoptosis-related proteins, other than p53, to be identified as targets of E6. Following binding to E6, both Bak and Myc are degraded through the ubiquitin-proteasome pathway [68]. This loss of Bak has biological consequences, as E6 has been shown to inhibit TNF-mediated apoptosis by reducing the expression of Bak in a process independent of the regulation of caspase-3 and caspase-8 expression [73]. Other key proteins involved in cellular apoptosis can also be compromised by E6. For example, E6 can bind to the death effector domains (DEDs) of FADD and procaspase-8 and accelerate

their degradation [70-72, 74]. The resulting lower amounts of FADD and procaspase 8 in E6-expressing cells then hinders formation of the apoptotic Death Inducing Signaling Complex (DISC) that would normally be triggered by members of the TNF superfamily, thereby compromising the ability of TNF, FasL and TRAIL to initiate apoptosis [69, 70, 72, 75]. In addition, E6 exerts effects on other apoptotic and anti-apoptotic proteins at the transcriptional level. For example, E6 can up-regulate the activity of the survivin promoter [76].

In each of these cases, E6 functions to reduce cell death by compromising the integrity of cellular pathways leading to apoptosis. In the context of the virus life cycle, the prevention of apoptosis is critical because it protects infected cells from elimination by the immune system, and therefore enables them to continue serving as hosts. This ability may also prolong the early stages of the life cycle. During carcinogenesis, the expression of E6 is typically increased, resulting in cells that do not undergo apoptosis and that are then susceptible to E6 and E7-mediated transformation. One recent study found that E6-mediated cervical cancer relies on the continuous expression of E7 along with constitutively expressed E6, and that continuous expression of E7 is required for the maintenance of cervical cancer and precancerous lesions in transgenic mice [77].

E6 has been considered a promising target molecule for therapy due to its abilities to block apoptosis and promote cancer development. For this reason, inhibition of E6 is predicted to lead to more cell death. Some progress was reported in 2004 and 2006 regarding the development of inhibitors of E6-E6AP interactions, a necessary step in the loss of p53 [78-80]. However, to the best of our knowledge, no group other than our own

is exploring the possibility of interfering with E6 function by blocking its ability to inhibit apoptotic signaling.

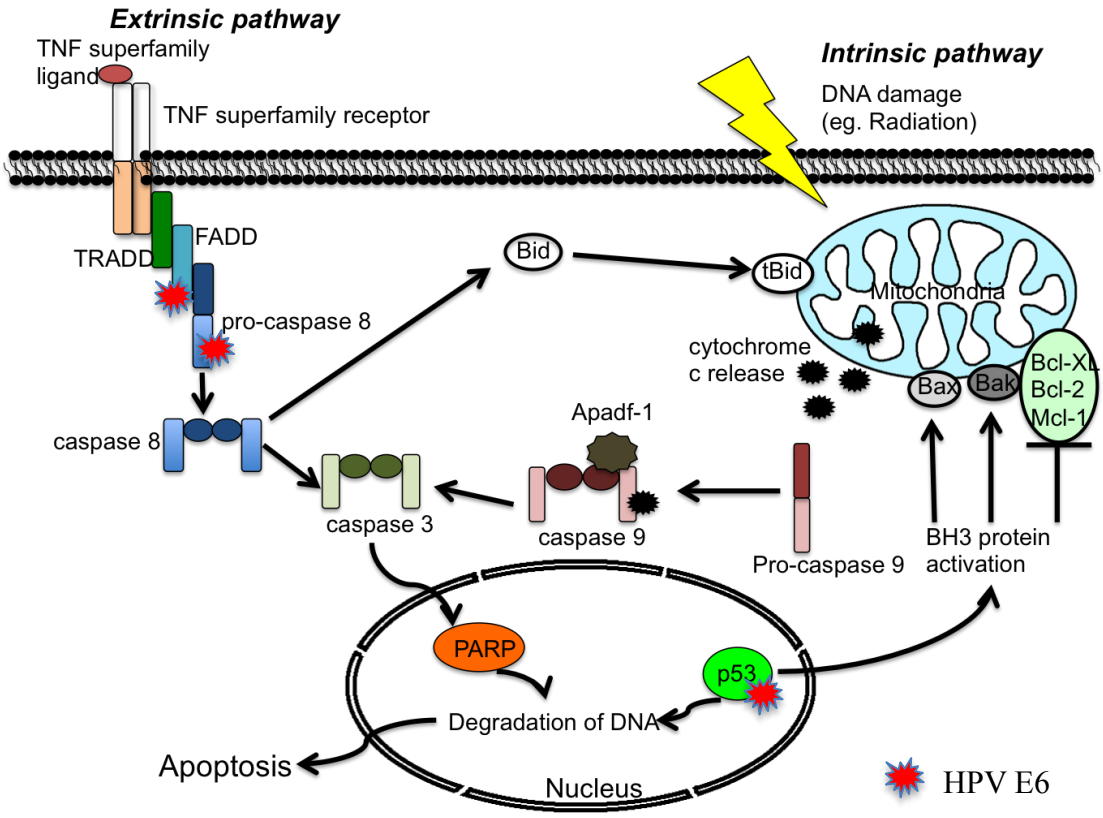


Figure 3. Interactions between E6 and apoptotic proteins. E6 binds to apoptotic proteins that are involved in both extrinsic and intrinsic apoptosis pathways and accelerates their degradation, thereby blocking apoptotic signal transduction.

Re-Engagement of Apoptotic Pathways as a Therapeutic Approach for HPV-Induced Cancers

Currently, therapeutic approaches that rely on activation of either the intrinsic or the extrinsic apoptotic pathways are unlikely to be helpful for HPV-associated diseases due to the ability of high-risk E6 proteins to subvert both extrinsic and intrinsic apoptotic pathways by mediating the rapid degradation of signaling molecules (p53 in the case of the intrinsic pathway and FADD and caspase 8 in the case of the extrinsic pathway). As a result of these E6 activities, therefore, engagement of either the extrinsic or the intrinsic apoptotic pathways does not result in the transduction of the intended death signal because the mediator molecules are missing. Therefore, if any of these apoptosis-inducing signaling pathways are to be used as tools for the elimination of HPV-associated malignancies, it will be necessary to restore the missing signaling molecules.

An approach based on inhibiting the ability of E6 to mediate the rapid degradation of its cellular partners has the potential to re-sensitize HPV^+ cells to inducers of apoptosis, and could therefore make certain existing cancer treatments available to those suffering from HPV-associated malignancies.

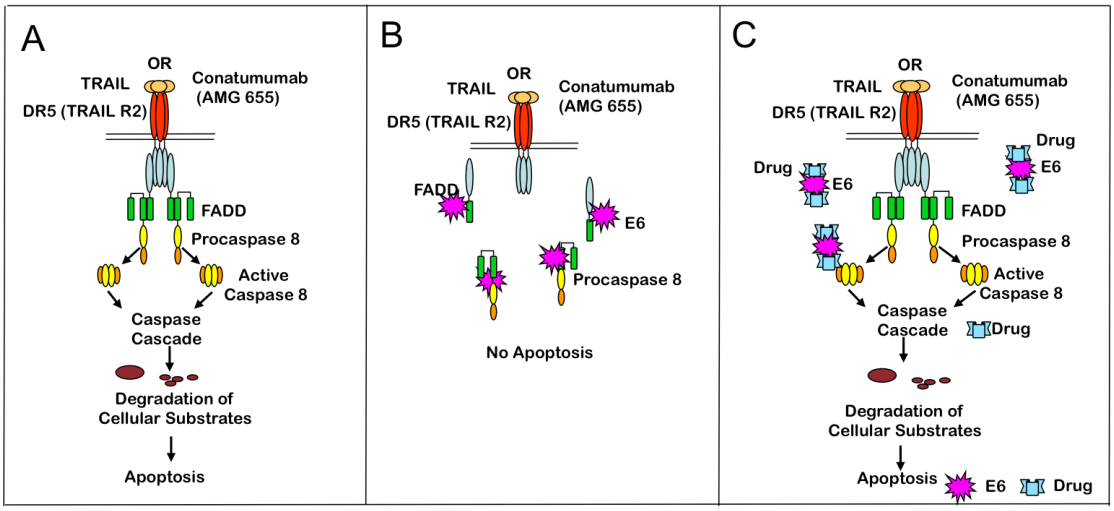


Figure 4. Working model and strategy. (A) TRAIL-mediated apoptotic pathway. The binding of either TRAIL or antibodies directed against the TRAIL R (e. g., AMG 655) to DR5 activates the pathway, leading to complex formation, activation of caspase 8 and initiation of the apoptotic cascade. (B) The HPV E6 proteins binds to p53 (not shown in figure), FADD and (pro)caspase 8, causing their rapid degradation and thus blocking apoptotic signaling. (C) Small, drug-like molecules that interfere with the ability of E6 to interact with caspase 8 and FADD have the potential to rescue the apoptotic pathway and thus re-sensitize cells to apoptosis.

Reactivating Intrinsic Apoptosis

Progress has been made in the search for inhibitors of the E6/E6AP interaction; this approach has the potential to re-activate the intrinsic apoptotic pathway. First, the essential binding motif through which E6 and E6AP interact was identified and verified [24, 81, 82]. Structural information regarding the E6/E6AP binding motif was then transferred to a three-dimensional query format suitable for computational screening of large chemical databases. This 3D format was used to query the National Cancer Institute (NCI) open chemical database comprising approximately 240,000 compounds, as well as the Sigma-Aldrich Library of Rare Chemicals of 97,000 compounds. Selected molecules that were predicted to fit into the E6/E6AP binding motif were then analyzed for their ability to inhibit E6/E6AP interactions and thereby interfere with E6-promoted p53 degradation. Following these *in vitro* and cell-based assays, several compounds with these characteristics were identified. Although these compounds have the potential for development into new therapeutic agents for treatment of HPV-associated cancers [79], no significant progress has been reported for the last several years.

Reactivating Extrinsic Apoptosis

It may also be possible to target the extrinsic apoptotic pathway. For example, activation of the TRAIL-mediated, extrinsic apoptotic pathway shows promise in the treatment of several types of tumors. TRAIL-based therapies have elicited significant interest in recent years, largely due to their apparent ability to kill tumor cells while sparing most normal cells. Previous studies have reported on the effectiveness of both TRAIL and α-TRAIL R1 and R2 in combination with other sensitizing agents such as

doxorubicin, bortezomib, adriamcin, 5-fluorouracil, irinotecan hydrochloride, paclitaxel, carboplatin, gemcitabine, cisplatin and radiation in both cell and animal model systems [83-86]. Currently, the challenge with using these approaches to target HPV-associated cancers is similar to the challenge noted above; namely, that E6 targets the mediating molecules for rapid degradation.

Work from our laboratory over the past decade has demonstrated that E6 can bind to many components involved in extrinsic apoptosis, including tumor necrosis factor receptor 1 (TNF R1) [75], the adaptor molecule Fas-associated death domain (FADD) [70], and procaspase 8 [87]. The binding sites on FADD and caspase 8 were identified using targeted mutagenesis, and a 23 amino acid peptide corresponding to the E6 binding site on FADD was designed and synthesized. We then demonstrated that this peptide could block the interaction between HPV E6 and FADD (and between E6 and caspase 8), thus re-sensitizing SiHa cells to TRAIL- and Fas-induced apoptosis [74] and providing proof-of-principle for our proposed approach. However, turning peptides into therapeutic agents is quite challenging due to well-known issues related to delivery, stability, etc.

Small molecules represent alternative approach, as they have been used successfully to inhibit important signal transduction pathways involved in breast, colon, pancreatic and lung cancer formation [88]. Comparing to peptide inhibitors, small molecules are relatively stable, penetrate more easily into target cells, and are easier to modify for drug development. Once small molecules block the inhibitory activity of E6, known triggers of apoptosis, such as TRAIL, could initiate apoptosis of HPV-positive cells. Such findings suggest that targeting HPV E6 (and/or other HPV oncoproteins)

could prove an excellent therapeutic strategy to treat HPV-associated cancers, and that small molecules could prove an appropriate approach to reach this goal.

Purpose of This Study

As previously mentioned, expression of HPV E6 blocks apoptotic signal transduction by binding to and accelerating the degradation of several apoptotic proteins including p53, caspase 8, and FADD. This ability of HPV E6 makes cancer therapies that function by inducing apoptosis ineffective for the treatment of HPV-associated malignancies. Therefore, in order to discover and develop novel and effective therapeutic approaches for these malignancies, we sought to identify agents that can block E6 function by addressing the following questions: 1) What kinds of small molecules have the ability to block the interactions between E6 and its apoptotic protein partners? 2) Can those small molecules re-sensitize E6-expressing cells to normal apoptotic stimuli?

Results from this study will lay the groundwork for making apoptotic therapies that function by activating apoptotic pathways, such as TRAIL or other chemotherapy drugs, available to patients suffering from HPV-associated malignancies. Our contribution to the field will be the identification of small molecules that, by inhibiting the binding of E6 to its cellular partners, sensitize tumor cells to apoptosis. This contribution is significant because it will lay the foundation for developing a novel class of therapeutic agents effective in HPV-infected individuals. The proposed work will also lead to insights beyond the field of HPV therapeutics by enhancing our current understanding of protein/protein interactions and how they can be blocked, HPV virology, virus/host interactions, and how the cellular pathways affected by HPV normally function.

CHAPTER TWO

SMALL MOLECULE INHIBITORS OF THE HPV16-E6 INTERACTION WITH CASPASE 8

Chung-Hsiang Yuan, Maria Filippova, Sandy S. Tungteakkhun, Penelope J. Duerksen-Hughes, John L. Krstenansky

Published in Bioorganic & Medicinal Chemistry Letters, 2011 [89]

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.

Introduction

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) [90]. 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 [91-94]. 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 the retinoblastoma (Rb) protein 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 [12, 13, 95, 96].

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 [70, 72, 75, 87]. 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 [70, 75, 87].

Although two vaccines designed to prevent cervical cancer are now available [97-99], 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. 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 [78, 88]. 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 [12, 70, 72, 87, 96] 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.

Experimental Methods and Results

The construction of the pGEX-E6 and pTriEx-Caspase-8 DED plasmids has been reported [12]. Expression and purification of GSTE6, His-FADD and His-Caspase-8 DED were carried out as previously described [12, 74]. 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 (Figure 1).

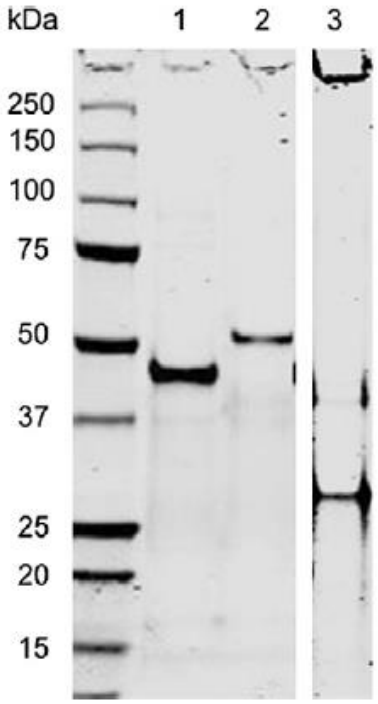


Figure 1. Purity of isolated proteins. Purified proteins (10 ug) were separated by SDS–PAGE. The gel was then stained using the Coomassie reagent. Lane 1: GST-E6, Lane 2: GST-caspase 8, Lane 3: His-caspase 8.

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 (Figure 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.

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) clogP: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 [100]. 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' [101] ($100 < MW < 350$ & $1 < clogP < 3$) 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.

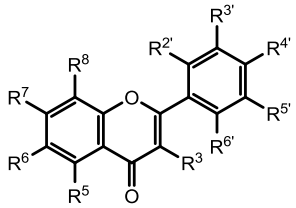
Among the most potent of the 19 were a flavonol, kaempferol, and a flavone, chrysin 7-methyl ether. Notably, flavone and apigenin were in the original library and did not exhibit sufficient potency for selection. These data indicate that this class of compounds exhibits clear SAR at this binding site. Additionally, the literature contained several descriptions of this class of compounds having potential antitumor activity [87, 102-105].

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 [74].

Consistent with these findings, we were able to verify that kaempferol could indeed inhibit both the His-FADD and His-caspase 8 interactions 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 our assay results, and; (2) using this assay allowed us to perform analogous counter-screening to demonstrate specificity, by asking whether candidate molecules did or did not inhibit the binding between His-caspase 8 and GST-caspase 8.

To follow up on the flavone/flavonol hits, 19 flavones and flavonol compounds representing systematic substitution of the ring system were selected and purchased, and then tested for inhibition of the E6/caspase 8 interaction (Table 1). We sought to determine the SAR for these ligands with regards to their binding to E6, and also asked whether the SAR corresponded to the prior literature or was unique.

Table 1. Structures of the flavones and 3-flavonols tested for their ability to inhibit the interaction between HPVE6 and caspase 8. Position numbers are given on the structure as the superscript.



Compound	3	5	6	7	8	2′	3′	4′	5′	6′
Myricetin (**1**)	OH	OH	H	OH	H	H	OH	OH	OH	H
Morin (**2**)	OH	OH	H	OH	H	OH	H	OH	H	H
Quercetin (**3**)	OH	OH	H	OH	H	H	OH	OH	H	H
Kaempferol (**4**)	OH	OH	H	OH	H	H	H	OH	H	H
Galangin (**5**)	OH	OH	H	OH	H	H	H	H	H	H
Apigenin (**6**)	H	OH	H	OH	H	H	H	OH	H	H
7-Hydroxyflavonol (**7**)	OH	H	H	OH	H	H	H	H	H	H
Rhamnetin (**8**)	OH	OH	H	OMe	H	H	OH	OH	H	H
Isorhamnetin (**9**)	OH	OH	H	OH	H	H	OMe	OH	H	H
Geraldol (**10**)	OH	H	H	OH	H	H	OMe	OH	H	H
Datiscetin (**11**)	OH	OH	H	OH	H	OH	H	H	H	H
Fisetin (**12**)	OH	H	H	OH	H	H	OH	OH	H	H
6-Hydroxyflavonol (**13**)	OH	H	OH	H	H	H	H	H	H	H
7-Methoxyflavonol (**14**)	OH	H	H	OMe	H	H	H	H	H	H
(**15**)	OH	H	CH_2COOH	H	Cl	H	H	H	H	H
(**16**)	OH	H	CH_2COOH	H	Cl	H	H	Cl	H	H
(**17**)	OH	H	CH_2COOH	H	Cl	H	H	$CH(Me)_2$	H	H
Flavonol (**18**)	OH	H	H	H	H	H	H	H	H	H
Flavone (**19**)	H	H	H	H	H	H	H	H	H	H

AlphaScreen™ technology (Perkin–Elmer) was used to detect the interaction between GST-E6 and His-caspase 8 using the same methodology and incubation times described above. The binding reaction was performed in a 384-well plate format in a total volume of 25 μl per well. Each well contained 5 μl GST-E6 protein (4.37 ng), 5 μl His-caspase 8 protein (50 ng), 5 μl blocking buffer (0.5 mg BSA, 0.5% Tween 20 in PBS), 5 μl chemical compounds at different concentrations diluted in PBS, and 5 μl of the acceptor and donor beads solution.

The structural characteristics of tested flavones are presented in Table 1. Each of these compounds was tested at four different concentrations, from 20 nM to 20 μM, and the results demonstrated that six of them, including kaempferol, inhibited the interaction between E6 and caspase 8. These data of relative potency gave the following ranking: 1 > 2 > 3 > 4 = 5>>(6–19). The structure– activity relationship (SAR) that these data suggest are that the 3,5,7-OH groups are all important:

3-OH **4** > **6** 5-OH **5** > **7** 7-OH **3** > **8**

and that in the prime ring hydroxyl groups affect potency:

3’, 4’, 5’ (**1**) > 2’,4’ (**2**) > 3’,4’ (**3**) > 4’ (**4**) = none (**5**)

3’-methoxy vs 3’-OH eliminates affinity (**9** vs **3**)

Additionally, we determined the IC_{50} for **1**–**5** and **13** (Fig. 2). For kaempferol and quercetin the IC_{50} was greater than 20 μM, while for morin, 50% inhibition was achieved at 4 μM. The compound with the lowest IC50 concentration was myricetin with an IC_{50} of approximately 850 nM.

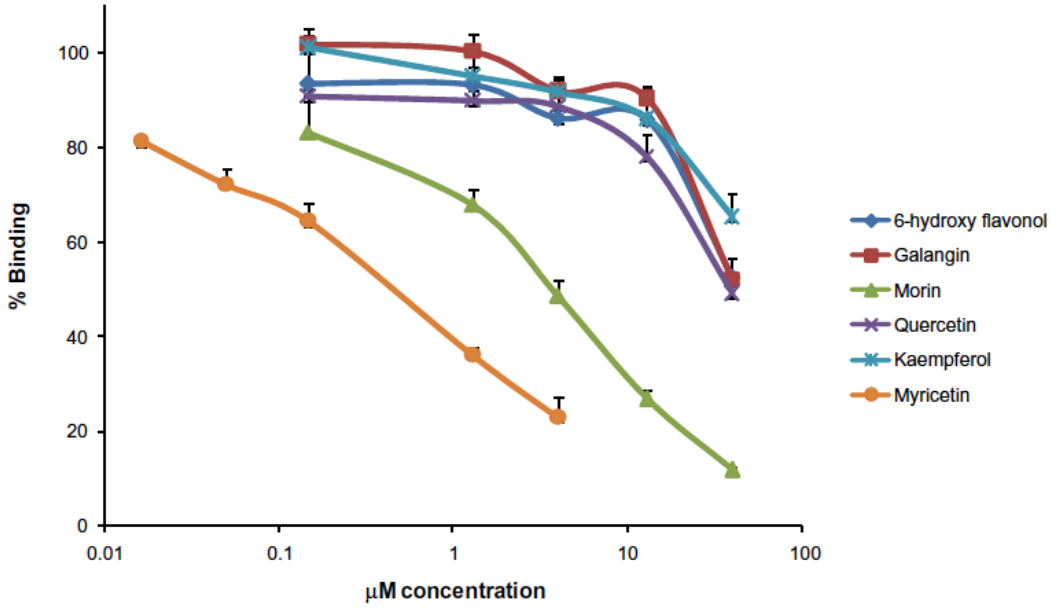


Figure 2. IC_{50} determination of selected compounds. The binding between GST-E6 and His-caspase 8 DED was used to determine the IC_{50} of each test chemical. 4.37 ng of GST-E6 and 50 ng His-caspase 8 were mixed in each well and the indicated concentrations of the test chemicals were applied. Binding in the presence of DMSO only was set at 100%. The p-values were < 0.05 when morin was compared to 6-hydroxy flavonol, galangin, kaempferol, and quercetin at 40 µM and 13 µM, and to 6-hydroxy flavonol, galangin, kaempferol, quercetin, and myricetin at 4 µM and 1.3 µM. The p-values were <0.05 when myricetin was compared to 6-hydroxy flavonol, galangin, kaempferol, quercetin, and morin at 40 µM, 13 µM, 4 µM and 1.3 µM. The p-values were obtained by multiple comparisons using the Bonferroni correction [101].

The mammalian two-hybrid system has previously been used to show that caspase 8 binds to itself [87]. For this reason, we were able to use this system to determine the specificity of the inhibitors, as compounds that inhibited the caspase 8/caspase 8 binding were likely to either interact with caspase 8 rather than E6, or to influence the assay itself in a non-specific manner. Therefore, to determine whether the observed inhibitions were indeed specific, or whether they might instead reflect some nonspecific mechanism related to the assay method, candidate compounds were tested for their ability to inhibit binding between GST-caspase 8 and His-caspase 8. As shown in Figure 3, we found that myricetin, morin and 6-hydroxy flavonol exhibited no inhibition of caspase 8/caspase binding over a range of concentrations.

The structure–activity relationship we observe for the inhibition of HPV16 E6 binding to caspase 8 is distinctly different from prior reports of flavones in a variety of *in vivo* and *in vitro* anticancer assays [103-107]. The reducing environment of the assays, the distinct SAR, and the specificity experiments all indicate that this is a real inhibition by the flavonols and not a non-specific artifact from flavonol oxidation products. Therefore, these results represent the first report of the potential of flavonols for the prevention or reversal of HPV-induced apoptotic blockade. Preliminary *in vivo* studies (data not reported here) indicate a weak ability for myricetin to suppress TRAIL induced tumor growth. Poor cellular penetration is likely the reason for the modest result due to the highly polar nature of the compound. Since the polar groups are an important feature of the SAR, optimization of this series of compounds would be difficult without resorting to a prodrug approach, further complicating the optimization process. Therefore, we are

also engaged in additional screening efforts with libraries containing more ‘lead-like’ members that will enable optimization [108].

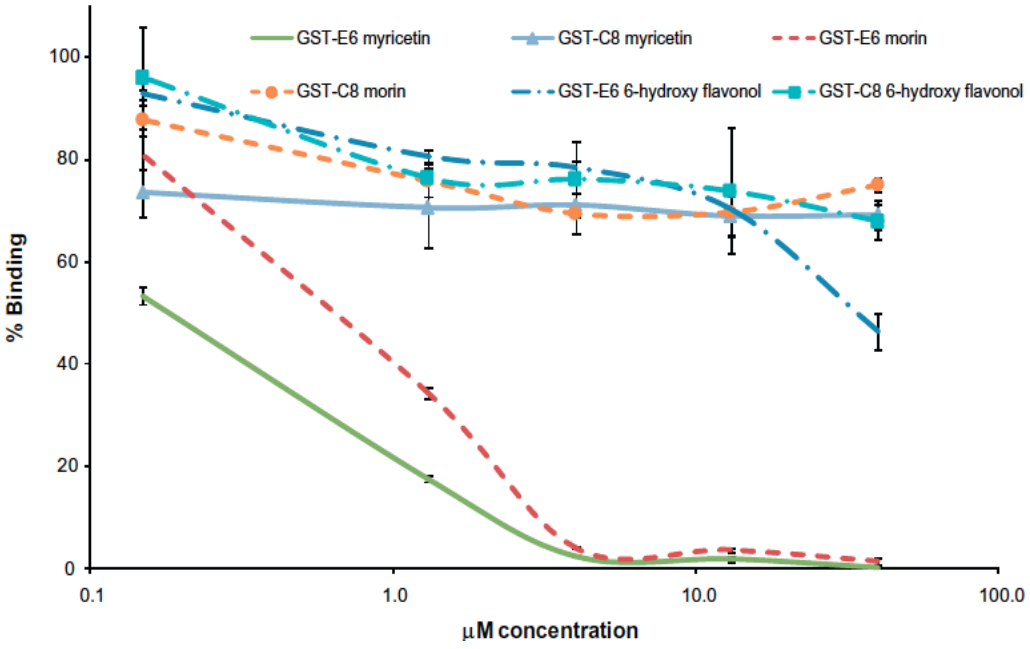


Figure 3. Specificity of the inhibition of E6/caspase 8 binding. The ability of myricetin, morin, and 6-hydroxy flavonol to inhibit binding between His-caspase 8 and either GST-E6 or GST-caspase 8 was assessed using the bead-based binding assay, as described in the legend of Figure 2. Where indicated, 13.5 ng GST-caspase 8 was added to each well. The p-values were <0.001 when myricetin and morin were compared to 6-hydroxy flavonol only in GST-E6/His-caspase 8 group. The p-values were obtained by multiple comparisons using the Bonferroni correction.

CHAPTER THREE

FLAVONOL AND BENZIMIDAZOLE DERIVATIVES BLOCK HPV16 E6 FUNCTIONS AND REACTIVATE APOPTOSIS PATHWAYS IN HPV$^+$ CELLS

Introduction

High-risk types of human papillomavirus (HPV), especially types 16 (species 9) and 18 (species 7), 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 (for example, vaginal and penile cancers) [109]. Cervical cancer is the second most common cancer in women worldwide. Approximately 400,000 new cases of this disease are diagnosed each year, and approximately half of these patients will die. HPV are small, double stranded DNA viruses that infect epithelial tissues, including those of the anogenital tract. The HPV-encoded oncogenes E6 and E7 are responsible for cellular immortalization and transformation, and consequently, for the development of HPV-associated cancer. While E7 is best known for the inactivation of Rb and thus for the immortalization of differentiated cells, E6 induces degradation of several molecules involved in apoptosis.

Recently, two HPV vaccines, Gardasil (MSD) and Cervarix (GSK), have been developed and approved for the prevention of infection with either two or four types of HPV. These vaccines are therefore predicted to reduce the incidence of cervical cancer. However, while these vaccines can prevent HPV infection, they offer no benefit to an

individual who has already been infected with HPV. Also, the vaccines will not have a significant effect on human health for decades, because most women are infected in their late teens/early twenties, while cancer appears in their late forties/early fifties. Furthermore, the vaccines only protect against HPV-16 and -18, leaving 25–30% of high-risk infections unaffected by an individual's vaccination status. An additional issue is the high cost of these vaccines, making it difficult to deliver them to areas and populations with low resources.

Surgical treatment is often employed to cure cervical cancer in the early stages. However, it can be difficult to apply this approach to head and neck cancers or to laryngeal papillomatosis, due to the need to maintain the normal structure of the airway and to avoid pulmonary spread. Furthermore, even following surgical excision, HPV-associated cancers frequently return, especially in the immunocompromised population. Combined chemoradiotherapies have been assessed, with variable results [110]. Topical treatments, such as trichloroacetic acid, liquid nitrogen, imiquimod, interferon-α injections and podophyllin resin, are also available [11, 111-113]. However, repeated treatments are necessary and are not always effective. Furthermore, imiquimod has not been approved for use in the oral cavity. Even when a wart or papilloma can be removed by such approaches, the HPV infection has not necessarily been cured and may return. In conclusion, there is no effective antiviral agent currently available.

Most currently employed chemotherapies, such as cisplatin and doxorubicin, focus on activation of the intrinsic (p53-mediated) apoptosis pathway. Cisplatin has been used for cancer treatment since 1978, and is considered the most active single agent for the treatment of cervical cancer [114-116]. Cisplatin carries a positive charge when

entering cells, and binds to nucleophilic molecules such as DNA, RNA, and proteins. By interacting with DNA and forming adducts, cisplatin blocks DNA replication and causes DNA damage, p53 expression, and apoptosis. Doxorubicin was first discovered as an anthracycline antibiotic, and has been used as chemotherapy drug to treat many different cancers [117]. The mechanism of action proposed for doxorubicin is that it can intercalate into the DNA molecules, thus causing the inhibition of topoisomerase-II, DNA damage, and p53 up-regulation during DNA replication [118].

On the other hand, activation of the TRAIL-mediated, extrinsic apoptotic pathway shows promise in the treatment of several types of tumors. These therapies have elicited significant interest in recent years, largely due to their apparent ability to kill tumor cells while sparing most normal cells. TRAIL receptors 1 and 2 (TRAIL R1 and TRAIL R2, also known as DR4 and DR5, respectively) are highly expressed on a large number of solid and hematologic cancers, and many of these tumors are sensitive to the induction of apoptosis induced by both TRAIL itself and by antibodies to the receptor [83, 119-123]. Previous studies have reported on the effectiveness of both TRAIL and α-TRAIL R1 and R2 in combination with other sensitizing agents such as doxorubicin, bortezomib, adriamcin, 5-fluorouracil, irinotecan hydrochloride, paclitaxel, carboplatin, gemcitabine, cisplatin and radiation in both cell and animal model systems [83-86]. Initial data suggests that this antibody is well tolerated, has a half-life of 13-19 days, and in some patients, is associated with a reduction in tumor size [124, 125].

Unfortunately, therapies that function by activating apoptosis, including those based on p53 or TRAIL, are unlikely to effectively treat HPV-associated malignancies, as high-risk E6 proteins subvert both extrinsic and intrinsic apoptotic pathways. E6 proteins

from high-risk types of human papillomavirus, such as HPV 16 and HPV 18, are well known for their ability to mediate the rapid degradation of p53, a tumor suppressor that engages the intrinsic apoptotic pathway [61, 126, 127]. The p53 tumor suppressor is the first-described and best-known target of HPV E6 [61]. p53 acts as a transcriptional factor, and can trigger cell cycle arrest or apoptosis in response to cellular stress or DNA damage. Under normal conditions, triggers such as DNA damage cause increases in the level of p53, which then lead to downstream effects such as cell cycle arrest and/or apoptosis, depending on the intensity or amplitude of the damage or stimulus. The presence of E6 from high-risk types of HPV interferes with this process, because E6 binds to both p53 and E6-associated protein ligase (E6AP), causing ubiquitinylation and the subsequent degradation of p53. This loss then prevents p53 from inducing either growth arrest or apoptosis of infected cells [62]. This E6-mediated inhibition of p53 activity can therefore increase the survival of transformed cells [63, 64]. Interestingly, a recent study showed that E6 could also interact with ubiquitin ligases other than E6AP in order to promote p53 degradation [65]. In addition to p53, E6 also interacts with other partner proteins that play a variety of roles in the cell. Our laboratory has discovered that HPV 16 E6 also blocks engagement of the extrinsic, receptor-mediated apoptotic pathways by binding to and inactivating several molecules involved in these signaling complexes, including TNF R1 [128], FADD [129] and caspase 8 [130]. For example, our lab has shown that HPV 16 E6 can protect HCT116 cells from apoptosis triggered by TRAIL by accelerating the degradation of both FADD and caspase 8 [131]. As a result of these E6 activities, therefore, engagement of either the extrinsic or the intrinsic apoptotic pathways does not 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 tools for the elimination of HPV-associated malignancies, it will be necessary to restore the missing signaling molecules.

In our previous work [89], we identified myricetin as a compound that can inhibit the E6/caspase 8 interaction. Unfortunately, myricetin is known to also inhibit number of cellular proteins, including several tyrosine kinases, and its structure makes modification difficult. Furthermore, our laboratory found that myricetin has a much lower efficiency in a cell-based system than in *in vitro*, suggesting difficulty in entering cells. Therefore, the identification of additional, more tractable inhibitors of E6 /procaspase 8 interaction is necessary.

In this study, we proposed to begin overcoming this challenge by identifying one or more additional small molecules that can inhibit HPV E6 function and re-sensitize HPV^+ cells to apoptosis. Screening an ActiPribe 2K library enabled us to identify benzimadole derivatives as a class of interest, and we further examined the ability of one particular derivative, 6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid (DIPC), to inhibit specific interactions between E6 and its partner proteins. We found that DIPC was able to block E6 binding to both E6AP and to caspase 8 *in vitro* with IC_{50} values of 20 μM and 17 μM, respectively. Within cells, we found that DIPC, as well as the flavonol myricetin, can re-sensitize HPV^+ cells to apoptosis triggered by apoptotic inducers such as TRAIL, cisplatin, and doxorubicin. Furthermore, we found that both DIPC and myricetin could also increase caspase 3/7 activity and restore 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 cervical cancer.

Materials and Methods

Protein Purification

The construction of the pGEX-E6, pTriEx-E6AP, and pTriEx-Caspase-8 DED plasmids has been reported [12]. Expression and purification of GSTE6, His-E6AP, and His-Caspase-8 DED were carried out as previously described [12, 74]. 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 Hepes pH 7.4, 150 mM NaCl, 2 mMKCl, 5% glycerol, 2 mM DTT), respectively. 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.

Small Molecule Library and Acquisition of Additional Compounds

The 2,000-compound small molecule library (ActiProbe 2K) from TimTec, LLC (Newark, DE) was to encompass a highly diverse selection of lead-like compounds. Five additional benzimidazole derivatives were purchased from Sigma (Benzimidazole, 2-(methoxymethyl)-1H-benzimidazole, and 3-(1H-Indol-1-yl)propan-1-amine methanesulfonate) and TimTec (1H-benzimidazole-1-methanol and 6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid)

Screening of Small Molecular Library

Alpha-Screen™ technology was used to assess the interactions between GST-E6, His-E6AP, and His-caspase 8. Binding assays were performed in white 384-well plates (Perkin-Elmer) in a total volume of 25 μl as previously described [12]. Briefly, 5 μl (50 ng) of GST-E6 and 5 μl (87.5 ng) of His-caspase 8 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. Members of the library were present at 10 μM in DMSO. 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).

Cell Culture

U2OS, SiHa and C33A cells were obtained from the America Type Culture Collection (Manassas, VA) and cultured in Eagle minimal essential medium (Invitrogen, Carlsbad, CA) supplemented as described previously [12].

Cell Viability Assay

To measure cell survival after treatment with TRAIL and chemotherapy drugs, SiHa ($2x10^4$/well) and C33A ($1x10^4$/well) cells were seeded into 96-well plates and allowed to adhere overnight. Small molecules at the desired concentration were added

and incubated at 37ºC for 4 h. TRAIL, cisplatin or doxorubicin was then added in the presence of cycloheximide (5 μg/ml) to inhibit *de novo* protein synthesis, and the cells were incubated for 16 h prior to measuring cell viability by the MTT assay preformed as described previously [70].

Caspase Activity Assay

Cells were plated into 96-well plates at a density of 2×10^4 cells per well and incubated overnight. Small molecules were added and incubated at 37 ºC for 4 h. TRAIL (100 ng/ml) was then added, along with cycloheximide (5 μg/ml) at the indicated time points. Caspase 3/7 activity was measured using the flourogenic substrate CellTitier-Glo for caspase 3/7 activity (Promega) following the manufacturer's instructions. Briefly, cells were lysed by the addition of 20 • l of 5X passive lysis buffer (Promega). The plate was put on an orbital shaker and incubated for 10 min at room temperature. 20 μl of cell lysates were transferred to white plates, and either substrate alone or substrate plus the caspase 3/7 inhibitor was added to the appropriate wells. After 10 min incubation, the released fluorophore was measured using a plate-reading fluorimeter (Flx800, Bio-Tek Instrument Co). The activity in wells treated with inhibitor was subtracted from the activity in wells lacking inhibitor. The resulting difference was expressed as a percentage of the caspase activity of the untreated cells.

Results

Benzimidazole Derivatives Specifically Inhibit the Interaction of HPV E6 with Caspase 8

Previously, we have reported that HPV E6 binds to caspase 8 [72] and that myricetin can block the E6/caspase 8 interaction *in vitro* [89]. In this study, we screened

the ActiProbe 2K (2,000 compounds) library, using AlphaScreen^{TM} technology from Perkin-Elmer, to look for inhibitors of the E6/caspase interaction. In our primary screen, 118 (5.9%) compounds demonstrated an ability to inhibit the E6/caspase 8 interaction. 79 of these 118 compounds presented IC_{50} values lower than 10 · M, and were therefore chosen for further experiments. 23 out 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 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 benzimidazole derivatives which were then purchased from Sigma and TimTec (Benzimidazole, 2-(methoxymethyl)-1H-benzimidazole, and 3-(1H-Indol-1-yl)propan-1-amine methanesulfonate were purchased from Sigma; 1H-benzimidazole-1-methanol and 6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid were purchased from TimTec) (Figure 1**)**.

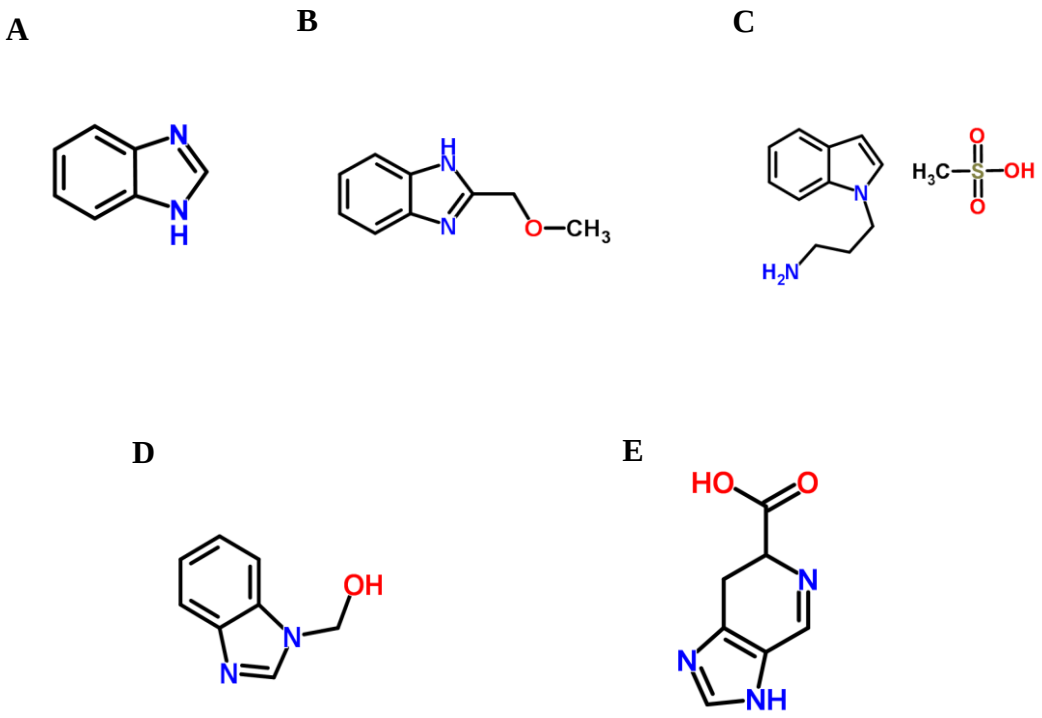


Figure 1. Five analyzed benzimidazole derivatives (A) Benzimidazole (B) 2-(methoxymethyl)-1H-benzimidazole (C) 3-(1H-Indol-1-yl)propan-1-amine methanesulfonate (D) 1H-benzimidazole-1-methanol (E).6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid

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 binding. The results showed that both 3-(1H-Indol-1-yl)propan-1-amine methanesulfonate and 6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid (DIPC) 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 E6AP. Inhibition of one or more of these additional E6 interactions has the potential to enhance the therapeutic effectiveness of agents developed from those chemical compounds. The E6/E6AP interaction has been studied extensively, and is required for the E6-mediated acceleration of p53 degradation. We therefore tested the ability of these five benzimidazole derivatives to inhibit E6/E6AP binding, and found that both 3-(1H-Indol-1-yl)propan-1-amine methanesulfonate and DIPC, the same two 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 (Figure 2B). 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. 3-(1H-Indol-1-yl)propan-1-amine methanesulfonate was able to inhibit the caspase 8/caspase 8 interaction about as well as it inhibited the E6/caspase 8 interaction, indicating that its action was non-specific. However, DIPC 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, DIPC displayed the lowest IC_{50} value for the inhibition of E6/caspase 8 and E6/E6AP interactions, but did not inhibit caspase 8/caspase 8 binding.

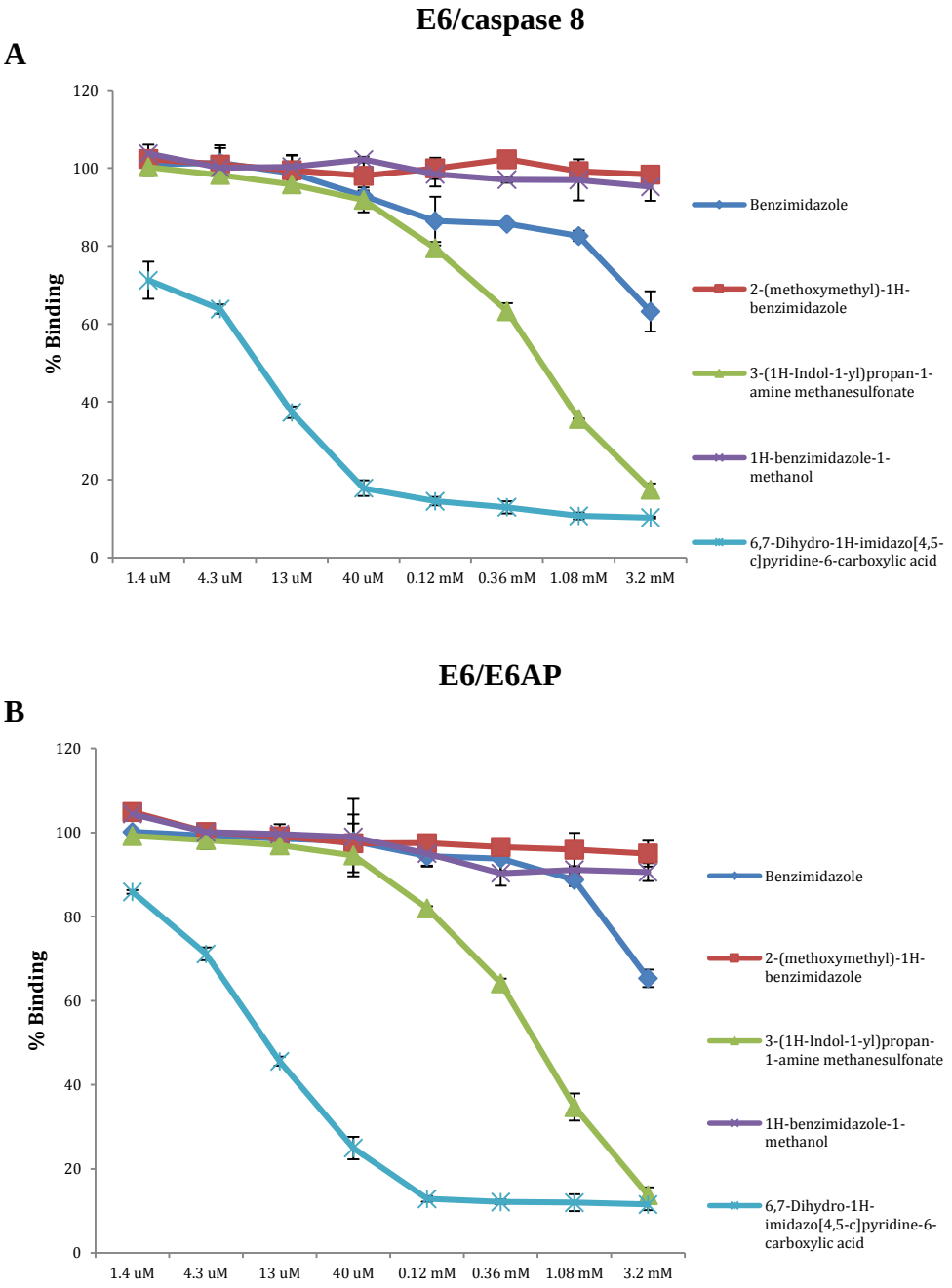


Figure 2. Benzimidazole derivatives inhibit protein-protein interactions. Five compounds, at the indicated concentrations (1.4 uM to 3.2 uM) were tested for their ability to inhibit three different protein/protein interactions: (A) GST-E6/His-caspase 8 (B) GST-E6/HisE6AP

caspase 8/caspase 8

C

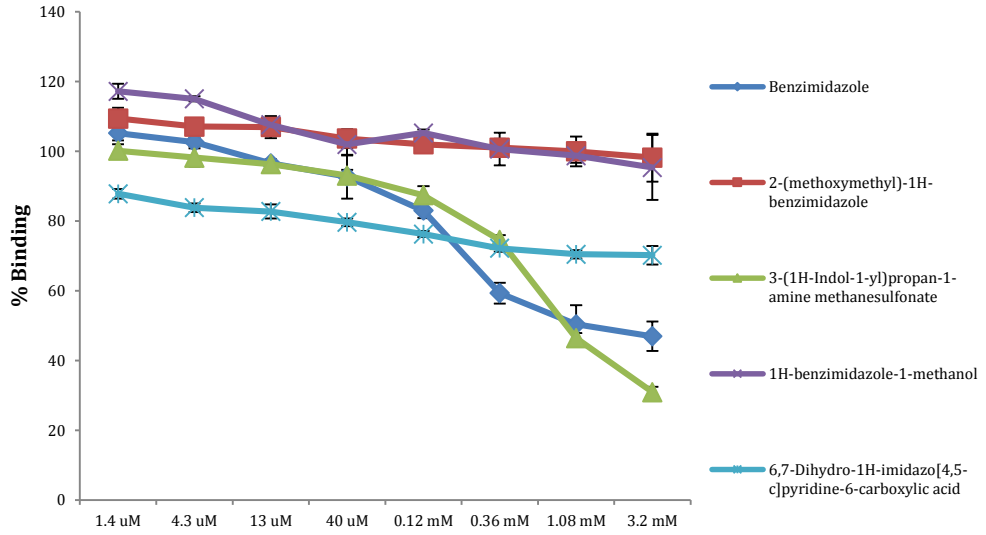


Figure 2 ***continued.*** (C) GST-caspase 8/His-caspase 8. Binding in the presence of DMSO only was set at 100%. Experiments were performed in triplicate, and error bars indicate the standard deviation.

Table 1. The IC_{50} values of tested small molecules for the indicated protein-protein interactions.

IC_{50} (µM)	Benzimidazole	2-(methoxymethyl) -1H-benzimidazole	3-(1H-Indol-1-yl) Propan-1-amine methanesulfonate	1H-benzimidazole-1 -methanol	6,7-Dihydro-1H-imidazo[4,5c] pyridine-6-carboxylic acid
GST-E6/His-Caspase 8	1.038	No Inhibition	0.632	No Inhibition	0.017
GST-E6/His-E6AP	1.481	9.436	0.670	No Inhibition	0.020
GST-Caspase8/His-Caspase8	1.051	3.309	0.832	No Inhibition	No inhibition

Myricetin and DIPC Sensitize SiHa Cells to TRAIL-Induced Apoptosis

Following identification of molecules that can block E6/caspase 8 binding *in vitro*, the next step was to determine which of these molecules could also act in the context of a cell. SiHa cells are an HPV^+ cell line, derived from a cervical carcinoma, that serves as a commonly-used model for HPV-associated malignancies. SiHa cells are resistant to TRAIL-induced apoptosis as a result of their expression of HPV E6 (Figure 3). In this experiment, U2OS cells display the TRAIL-sensitive phenotype. We selected 1H-benzimidazole-1-methanol, which showed no inhibition *in vitro* (Table 1), as our negative control.

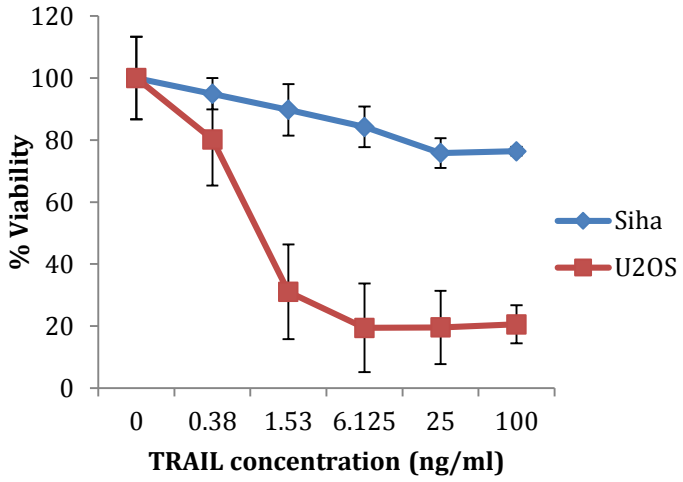


Figure 3. HPV^{+} SiHa cells are resistant to TRAIL-induced apoptosis as compared to the human osteosarcoma cell line U2OS. The viability of cells untreated with TRAIL was set at 100% for each group. Experiments were performed in triplicate, and error bars indicate the standard deviation.

We first determined the non-specific toxicity of our small molecules of interest. SiHa cells were treated with several concentrations of these molecules (0 ・ M, 15.6 μM, 31 μM, 62.5 μM, and 125 μM) for 16 hours, and cell viability was assessed using the MTT assay. A loss of viability of about 20%-30% following treatment with 62.5 μM for 16 h was observed with all compounds (Figure 4A). We next asked whether myricetin and DIPC would be able to sensitize HPV^+ cells to TRAIL, an inducer of extrinsic apoptosis. SiHa cells ($2x10^4$ per well) in culture were seeded into a 96-well plate for overnight incubation. The indicated concentrations (0 μM to 125 μM) of myricetin, 1H-benzimidazole-1-methanol, and DIPC were then added to the appropriate wells, and the cells allowed to pre-incubate for 4 hours. Next, cells were treated with cycloheximide only (5 □g/ml) (control) or with cycloheximide plus TRAIL (100 ng/ml) for 16 hours. Cell death was measured using the MTT assay. Both myricetin and DIPC induced significantly more cell death (an increase of 3.5 fold for myricetin and 8.6 fold for DIPC) in combination with TRAIL (Figure 4B) (Table 2). These results provide strong evidence that inhibiting the E6/caspase 8 interaction can restore the sensitivity of HPV-positive cells to apoptotic signals.

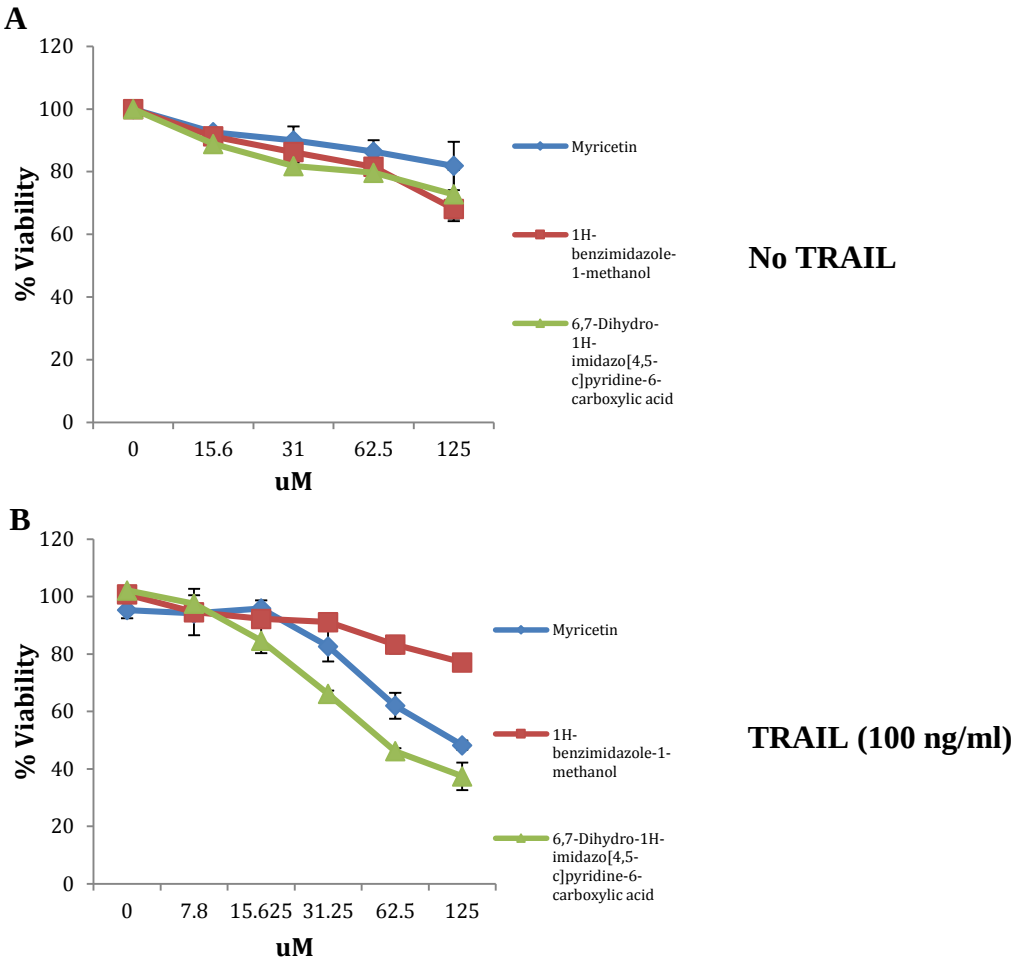


Figure 4. (A) Both myricetin and DIPC display low toxicity, with a viability loss of approximately 20% observed following treatment with 62.5 μM of either compound. (B) The addition of either myricetin or DIPC re-sensitizes these HPV^+ cells to TRAIL, inducing a loss of viability of more than 40% following treatment with 62.5 μM of either compound. Experiments were performed in triplicate, and error bars indicate the standard deviation.

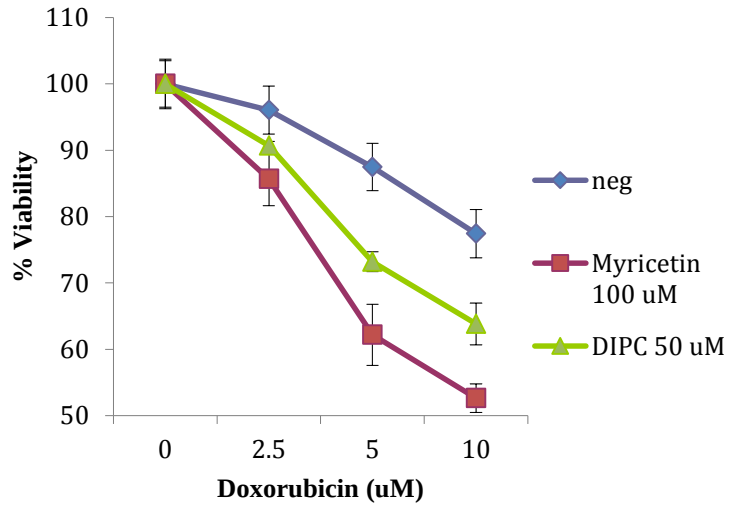
Table 2. The IC_{50} value of the indicated compounds for cell toxicity and for TRAIL-induced apoptosis.

IC_{50} (µM)	Myricetin	1H-benzimidazole-1-methanol	6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid
Cell Toxicity	539.073	284.888	268.506
TRAIL-induced apoptosis	154.7728	No Inhibition	31.251

Myricetin and DIPC Increase the Sensitivity of SiHa Cells to Doxorubicin and Cisplatin

As noted earlier, both myricetin and DIPC can inhibit the binding of E6 to E6AP, an E3 ligase involved in the degradation of p53, *in vitro*, One prediction stemming from this observation is that these two chemicals should increase the sensitivity of SiHa cells to chemotherapy drugs that act through the p53 pathway. To test this prediction, we asked whether myricetin and/or DIPC would increase the sensitivity of SiHa cells to two such drugs, doxorubicin and cisplatin. SihH cells (2×10^4 per well) were seeded into a 96-well plate. After overnight incubation, 100 μM myricetin or 50 μM DIPC were added to the indicated groups, and cells allowed to incubate for 4 hours. The indicated concentrations of doxorubicin (0-10 μM) and cisplatin (0-100 μM) were then added for a 16-hour treatment, and cell death was assessed using the MTT assay. As compared to the negative control, myricetin increased the sensitivity of SiHa cells to doxorubicin and cisplatin by approximately 20-30% (Figure 5A). DIPC displayed a similar effect, in that it increased the sensitivity by approximately 30-40% (Figure 5B). These results demonstrate that both myricetin and DIPC can inhibit the interaction between E6 and E6AP within cells, thus reducing the degradation of p53 and increasing the sensitivity of HPV^+ cells to inducers of p53-mediated apoptosis.

A



B

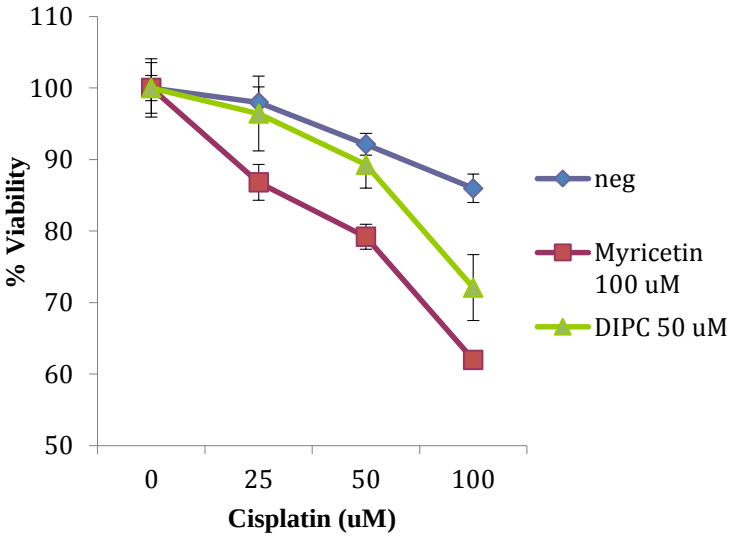


Figure 5. Myricetin and DIPC increase the sensitivity of SiHa cells to chemotherapy drugs. Pretreatment with either myricetin or DIPC increases the sensitivity of SiHa cell to doxorubicin (A) and cisplatin (B) by approximately 20% (DIPC) to 30% (myricetin). The viability of cells untreated with either myricetin or DIPC was set at 100%. Experiments were performed in triplicate, and error bars indicate the standard deviation.

Myricetin and DIPC Re-sensitize HPV^+ Cells to TRAIL-Induced Apoptosis by Blocking the Binding of HPV E6 to Caspase 8

To determine whether myricetin and DIPC re-sensitize SiHa cells to TRAIL-induced apoptosis by specifically blocking the binding of E6 to caspase 8 or through some other mechanism, we chose to employ the HPV^- human cervical carcinoma cell, C33A, for comparison, as these cells do not express E6. Both SiHa and C33A cells were either left untreated or pre-treated with either myricetin (100 μM) or DIPC (50 μM) for 4 hours, then treated with cycloheximide plus TRAIL (0-100 ng/ml) for 16 hours. Cell death was measured using the MTT assay. Although the HPV^+ SiHa cells displayed resistance to TRAIL at concentrations up through 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 6A). Similar results were also found following pre-treatment with 50 μM DIPC (Figure 6B). Together, these results demonstrate that myricetin and DIPC can re-sensitize HPV^+ cells to TRAIL-mediated apoptosis by specifically blocking the binding of HPV E6 to apoptotic proteins such as caspase 8, but likely not through other apoptotic mechanisms.

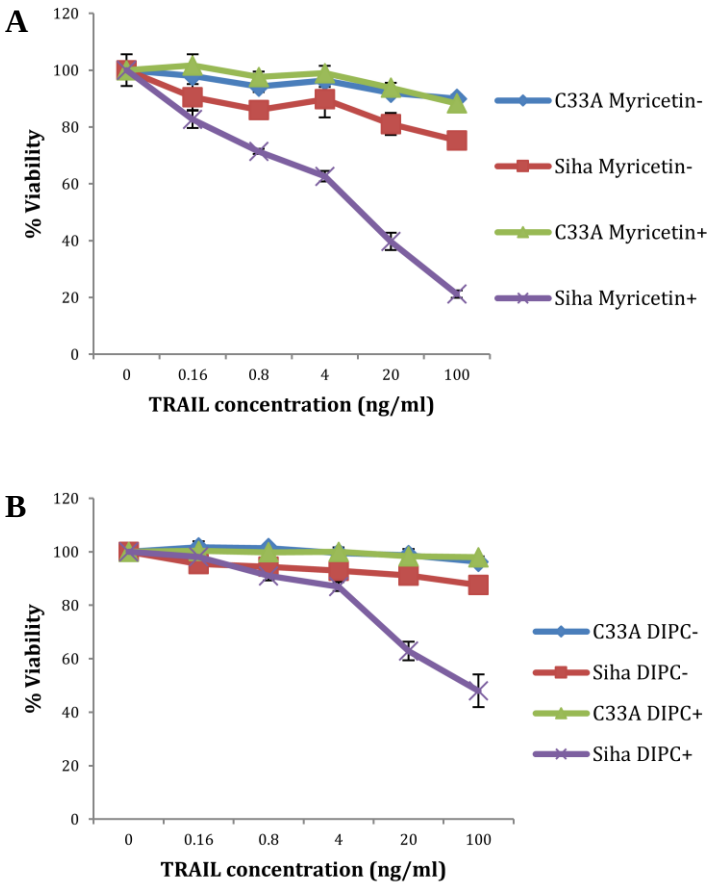


Figure 6. Myricetin and DIPC can re-sensitize HPV^+, but not HPV^-, cells to treatment with TRAIL. Pre-treatment with myricetin resulted in an 80% loss of cell viability (A) while pre-treatment with DIPC caused a 60% loss of cell viability (B) in SiHa cells treated with 100 ng/ml TRAIL. No significant decrease in cell viability was observed in C33A cells or in the absence of myricetin/DIPC. The viability of cells untreated with TRAIL was set as 100% for each group. Experiments were performed in triplicate, and error bars indicate the standard deviation.

Myricetin and DIPC 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 the increase in cell death observed when TRAIL, cisplatin or doxorubicin combined with myricetin or DIPC indeed occurs through the apoptotic pathway. Our previously described data (Figure 2) demonstrates that both myricetin and DIPC can inhibit the E6/caspase 8 and E6/E6AP interactions *in vitro*. One prediction following from this data is that pre-treatment with either myricetin or DIPC 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 TRAIL, cisplatin or doxorubicin treatment in the presence or absence of myricetin or DIPC. As shown in Figure 7A, we found that the caspase 3/7 activity increased approximately 2 fold when myricetin or DIPC were combined with TRAIL as compared to cells not exposed to myricetin or DIPC. Similar results were obtained following treatment with cisplatin and doxorubicin **(**Figure 7B, 7C). These data demonstrate that the increased cell death induced by the combination of apoptotic inducers and myricetin/DIPC occurs through the apoptotic pathway.

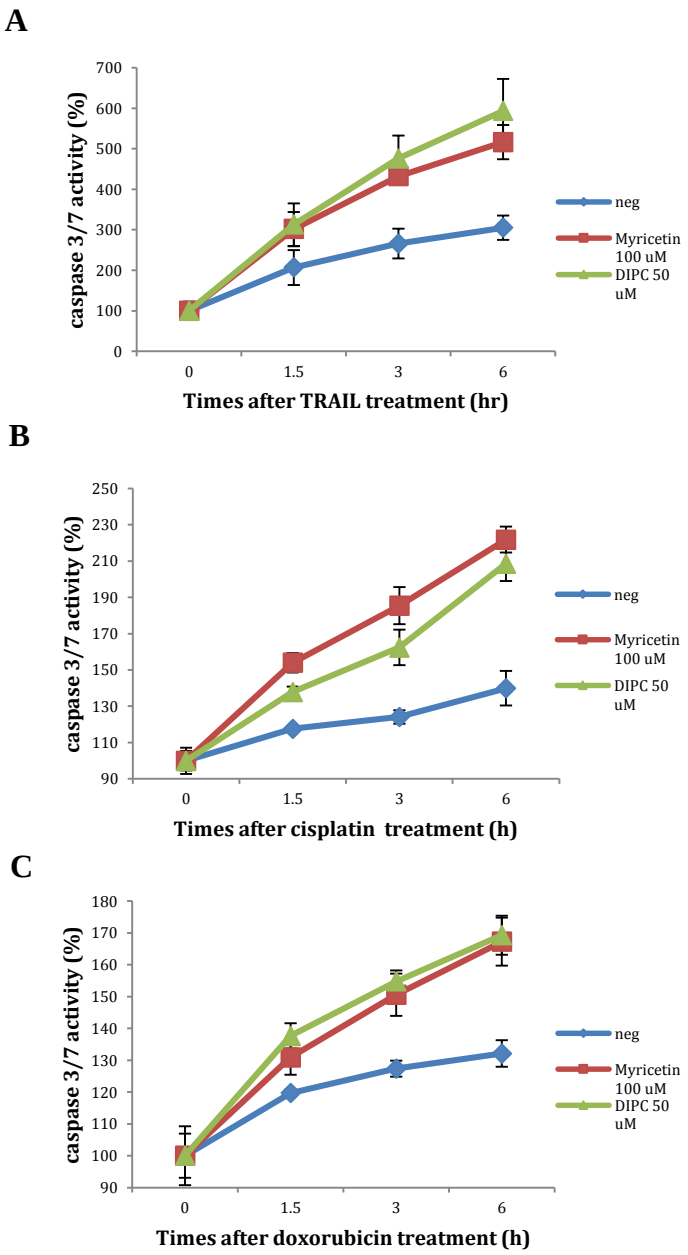


Figure 7. Myricetin and DIPC increase caspase 3/7 activity of SiHa cells after TRAIL and chemotherapy drugs treatment. SiHa cells were pre-treated with myricetin or DIPC 4 hours prior to application of the indicated inducers of apoptosis. 100 ng/ml TRAIL (A), 50 • M cisplatin (B), or 2 • M doxorubicin (C) were added as indicated, and caspase 3/7 activity was measured after 0, 1.5, 3, and 6 hours. Activity at 0 h of treatment was set at 100% for each group. Experiments were performed in triplicate, and error bars indicate the standard deviation.

Discussion

Currently, no small molecules targeting any of the HPV proteins are available for clinical use. However, a number of groups are currently examining the possibility of developing these sorts of small, inhibitory molecules in order to expand and enhance the limited therapeutic options currently available for HPV-associated malignancies [78, 132]. HPV E1 and E2 are enzymes involved in replication and gene expression, and thus were some of the initial targets for the development of small, inhibitory molecules. As one example, biphenylsulfonacetic acid has been shown to inhibit E1 ATPase activity, and the optimized compound has an IC_{50} value of 4.0 nM [133]. However, E1 and E2 are often lost during the development of cancer, making them less-than-optimal targets for cancer treatment. In contrast, E6 and E7 oncogenes are expressed at relatively high levels during cancer development and therefore have the potential to serve as useful targets for small molecule inhibitors. For example, inhibition of E6 is predicted to lead to more cell death, as E6 normally functions to block both intrinsic and extrinsic apoptotic pathways. The prevention of E6-mediated p53 degradation, perhaps by inhibiting formation of the E6/E6AP complex, represents one promising approach for the development of small molecule inhibitors of HPV-associated cancer [134]. Previous studies have identified several compounds that could inhibit the interaction between E6 and E6AP, thus reducing the degradation of p53 [78, 79]. However, these findings have not yet led to clinically useful interventions.

Therapies based on TRAIL-mediated apoptosis are attractive possibilities for the treatment of many different types of cancer., For example, previous studies have demonstrated the effectiveness of both TRAIL and ・-TRAIL R1 and R2 in combination

with other sensitizing agents such as doxorubicin, bortezomib, adriamcin, 5-fluorouracil, irinotecan hydrochloride, paclitaxel, carboplatin, gemcitabine, cisplatin and radiation in both cell and animal model systems to treat a variety of cancers [83-86]. However, these treatments are unlikely to be helpful in the context of HPV on their own, due to the ability of E6 to compromise apoptotic pathways. The studies described here, focused on the identification of E6-inhibitory molecules, have the potential to make this type of therapy available to patients suffering from HPV-associated malignancies. It is the first reported that our study demonstrated the combination of TRAIL with small molecules could re-sensitize E6-expressing cells to TRAIL-induced apoptosis.

Our laboratory previously reported that the flavonol myricetin can inhibit interactions between E6 and caspase 8 *in vitro*. We confirmed this binding, and demonstrated a direct interaction between E6 and myricetin by isothermal titration calorimetry (ITC), an approach that can detect and characterize protein-protein and protein-molecular interactions. Preliminary results (data not shown) indicate that myricetin binds to E6 at a ratio of 1:1 (one molecule of myricetin to one molecule of E6). Unfortunately, myricetin is known to inhibit a number of cellular proteins (including several kinases), and in addition, the high IC_{50} value observed in the context of a cell-based model system (Table 2) suggested that its ability to negatively affect tumors may be limited. Because the compound is highly polar, difficulty in entering cells may be the explanation for these modest results. Since polar groups are an important feature in assessing structure-activity relationships (SAR), optimization of this series of compounds would be difficult without resorting to a pro-drug approach, further complicating the optimization process. Therefore, we engaged in additional screening efforts with libraries

containing more ‘lead-like’ members that are expected to provide better platforms for optimization. This effort identified the benzimidazole family as a chemical group of interest for drug development. Benzimidazoles are a large chemical family, and several of these compounds have already been employed for medicinal chemistry due to their various bioactivities. The core structure of benzimidazole is a heterocyclic ring (Figure 1), and these compounds display a broad range of pharmacological effects, bioactivities, and functions that are dependent on the functional groups that surround this core structure. Previously, benzimadoles have been identified as useful for their antifungal, antioxidant [135], antiallergic [136], and antimicrobial [137-139] properties. In recent years, they were also found to display antitumor [140] and antiviral activities against HCV (Hepatitis C Virus) [141] and HIV (Human Immunodeficiency Virus) [142].

Of the five tested benzimidazole derivatives, 6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6- carboxylic acid (DIPC) was best able to specifically inhibit the interactions of E6 with both caspase 8 and E6AP (Figure 2), demonstrating the lowest IC_{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). To characterize the ability of DIPC, as well as myricetin, to function within cells, the ability of the two chemicals to cause cell toxicity and to enhance TRAIL-induced apoptosis were tested in the HPV^{+} SiHa cell line. The IC_{50} values of 154.8 μM for myricetin and 31.2 μM for DIPC, respectively, demonstrate that myricetin in combination with TRAIL is approximately 3.5 fold as toxic as myricetin alone, while DIPC in combination with TRAIL is approximately 8.6 fold as toxic as DIPC alone (Table 2). These results indicate that, as

compared to myricetin, DIPC may be better able to penetrate into cells and thus re-sensitize cells more efficiently to TRAIL-induced apoptosis.

As mentioned previously, the interaction between E6 and E6AP accelerates degradation of p53, a mediator of intrinsic apoptosis. Our *in vitro* results demonstrated that DIPC could inhibit the interaction between E6 and E6AP, and thus has the potential to increase the sensitivity of HPV^+ 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. When we combined myricetin or DIPC with those chemotherapy drugs, we observed a loss of cell viability of more than 30% as compared to that seen in the absence of these sensitizing agents, indicating that indeed, myricetin and DIPC can both increase the sensitivity of HPV^+ cells to chemotherapy drugs that work through the p53 pathway. Interestingly, DIPC appeared to be somewhat more effective than myricetin when combined with TRAIL, while myricetin appeared more effective than DIPC when combined with either cisplatin or doxorubicin. However, we note that myricetin required the presence of a higher concentration (100 μM) than did DIPC (50 μM) to cause inhibition of binding. Thus, we suggest that DIPC may actually demonstrate a higher efficiency in sensitizing HPV^+ cells to both TRAIL- and p53-mediated apoptosis.

Although we began by searching for inhibitors of extrinsic apoptosis, we found that DIPC was able to block the binding of E6 to both caspase 8 and to E6AP, and thus sensitize HPV^+ cells to both extrinsic and intrinsic apoptosis. This may 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 DIPC binds to a region on E6 required by both E6AP and caspase 8.

In order to determine whether myricetin and DIPC re-sensitize cells to TRAIL-induced apoptosis by specifically blocking the binding of HPV E6 to caspase 8, C33A, a HPV^- cell line that does not express E6, was compared to SiHa. Each cell line was untreated or pre-treated with myricetin or DIPC, then different concentrations of TRAIL were added and cell viability was measured after overnight incubation. Figure 6 demonstrates that SiHa cells pretreated with either myricetin or DIPC showed a greater loss of cell viability than did the other groups. These results indicate that TRAIL-induced apoptosis in SiHa was restored due to the ability of myricetin and DIPC to inhibit the E6/caspase 8 interaction.

Finally, caspase 3/7 activity was assessed to determine whether the loss of cell viability occurred through activation of the apoptotic pathway. Caspase 3/7 can be activated through both the extrinsic and intrinsic apoptosis pathways, as induced by TRAIL or chemotherapy drugs, respectively. We found that both TRAIL and chemotherapy drug treatment, in combination with myricetin or DIPC, led to a significant and enhanced time-dependent activation of caspase 3/7 activity in SiHa cells (Figure 7). Taken together, these data indicate that myricetin and DIPC can block the 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. These activities suggest that myricetin and DIPC may be able to inhibit multiple functions of E6, and therefore provide the basis for developing an effective therapeutic strategy to treat HPV-mediated cancers.

Overall, this study identified a new candidate, the benzimidazole derivative DIPC, which effectively blocks the interactions of E6 with apoptotic proteins such as caspase 8 and E6AP. Furthermore, both DIPC and myricetin were able to re-sensitize the HPV^+ cell line SiHa, but not the HPV^- cell line C33A, to TRAIL-induced apoptosis, indicating that they most likely work by blocking the binding between E6 and its apoptotic protein partners. In addition to re-sensitizing HPV^+ cells to TRAIL-induced apoptosis, DIPC and myricetin could also increase the sensitivity of SiHa cells to two chemotherapy drugs, doxorubicin and cisplatin, by blocking the E6/E6AP interaction and restoring the level of p53. The observed increase in caspase 3/7 activity also provides evidence that sensitization of SiHa cells by DIPC occurred through activation of apoptosis.

Previous work from our laboratory had identified peptide inhibitors that can specifically inhibit the interactions between E6 and caspase 8 and FADD, but not the interaction between E6 and E6AP [12]. In contrast, both myricetin and DIPC inhibit the interactions of E6 with both the caspase 8 and E6AP protein partners. As compared to peptide inhibitors, small molecules such as DIPC have numerous advantages because they are more stable, penetrate target cells more easily, and can be modified and optimized by organic chemists during drug development. Because DIPC has the potential to restore both the intrinsic and extrinsic apoptotic pathways, it may prove to be an ideal candidate for making apoptosis-inducing therapies available to patients suffering from HPV-associated malignancies.

CHAPTER FOUR

DISSCUSION

Summary of Findings

High-risk strains of human papillomavirus (HPV), especially types 16 and 18, are the causative agents of nearly all cases of human cervical cancer (approximately 400,000 new cases each year, with a 50% mortality rate), between 20% and 30% of head and neck cancer cases, and some cases of other cancers (for example, vaginal and penile cancers) [109]. Current treatment options are frequently inadequate, providing a sense of urgency to the development of better, more effective clinical interventions.

The experiments described in chapter 2 demonstrate that myricetin, a flavonol, can block HPV E6 interactions *in vitro*. To ask whether this inhibition was specific, we developed 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-screen, myricetin showed no inhibition to the binding of caspase 8 to itself, providing evidence of its specificity for E6. These promising results demonstrated that, indeed, small molecules capable of blocking E6/caspase 8 binding in a specific manner existed, and that our bead-based assay could find them.

The results of experiments from chapter 3 identified a new class of chemicals, benzimidazoles, which also showed the potential to block the binding of E6 to both caspase 8 and E6AP. Since myricetin is not a suitable candidate for drug development due to its inhibition of a number of cellular proteins as well as inherent difficulties in

optimizing its structure, the identification of other inhibitors of E6/caspase 8 was deemed to be necessary. Following the testing of five benzimidazole derivatives for dose-dependency and specificity, we found that 6,7-Dihydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid (DIPC) showed the greatest ability to inhibit both E6/caspase 8 and E6/E6AP interactions while not blocking the binding of caspase 8 to itself, thereby demonstrating both specificity for E6 and the ability to inhibit multiple E6 interactions.

Next, we performed a cell-based analysis designed to determine which of our small molecules of interest could function well within cells. SiHa cells are a cervical cancer cell line that maintains the entire genome of HPV and expresses E6, and therefore functions well as a model system. We found that, as expected, SiHa cells are resistant to TRAIL at concentrations up to 100 ng/ml. We also found that neither myricetin nor DIPC were significantly toxic to SiHa cells. However, when TRAIL was combined with either myricetin or DIPC, we observed a significant loss of cell viability (up to 50%), indicating that both myricetin and DIPC are able to re-sensitize HPV^+ cells to TRAIL-induced apoptosis, DIPC more efficiently than myricetin. A comparison between SiHa and C33A cells demonstrated that re-sensitization to TRAIL-induced apoptosis was due to specific blocking of the binding of E6 to caspase 8, as no sensitization of C33A cells was observed. In addition to its inhibition of E6/caspase 8 binding, DIPC was also able to inhibit E6/E6AP binding, an interaction involved in p53 degradation. Myricetin and DIPC increased the sensitivity of SiHa to the chemotherapy drugs doxorubicin and cisplatin, agents that can induce p53-mediated apoptosis, consistent with the prediction from our *in vitro* data. Finally, the increase of caspase 3/7 activity in both TRAIL- and

chemotherapy drug-induced experiments shows that the loss of cell viability was due to apoptosis rather than to other mechanisms of cell death.

Conclusion

Cancer therapies that function by triggering apoptosis have been used widely in the treatment of different types of cancers for decades. Unfortunately, many types of cancer cells are resistant to those treatments due to mutations or loss of apoptotic proteins. For example, mutant p53 has been found in more than 50% of cancers [143]. Many researches have shown that restoration of p53 has the potential to reduce certain types of cancer without damaging normal cells [144]. Activation of TRAIL-induced apoptosis pathway has also shown promise in the treatment of several types of cancers, as described previously. In particular, several agonistic monoclonal antibodies directed against DR4 and DR5 are currently in Phase I and II clinical trials designed to evaluate their safety, pharmacokinetics and therapeutic efficacy. For example, GS-ETR1 DR4 and HGS-ETR2 DR5 (Human Genome Sciences) are in Phase I/II trials, and HGS-TR2J DR5 (also from Human Genome Sciences) is in Phase I. Also, as of 2008, CS-1008 (TRA-8) DR5 (Daiichi Sankyo) is in Phase I and showed no dose-limiting toxicity at doses up to 8 mg/kg [145], AMG 655 DR5 (Amgen) is in Phase I/II [146], Apomab DR5 (Genentech) is in Phase I/II [147], and LBY 135 DR5 (Novartis) is in Phase I/II in patients with advanced solid tumors [148]. Unfortunately, in the case of HPV-mediated cancers, E6 can bind to and accelerate the degradation of apoptotic proteins, such as FADD, caspase 8, and p53, rendering treatments that work through these pathways ineffective. By

eliminating those apoptotic proteins and blocking apoptotic signal transduction, HPV ensures its persistence and propagation.

Because E6 plays a key role in preventing apoptosis and promoting cancer development, blocking its interactions with partner proteins carries with it the promise of developing effective and novel treatments for HPV-mediated cervical cancer. Two questions were posed at the beginning of these studies. 1) What kinds of small molecules have ability to block interactions between E6 and apoptotic proteins? This question was answered in Chapters 2 and 3. 2) Can those small molecules re-sensitize E6-expressing cells to normal apoptotic stimuli? This question was also answered in Chapter 3. Results from the experiments described in this dissertation demonstrate that the binding between E6 and its partner proteins, caspase 8 and E6AP, can be blocked by at least two different classes of small molecule inhibitors. Second, the blocking of E6 interactions with caspase 8 and E6AP can indeed restore the functionality of apoptosis pathways in an HPV^+ cell line. Therefore, the overall findings of this study demonstrate that small molecules targeting E6 may indeed prove to be a potential approach to the treatment of cervical cancer as well as other HPV-associated cancers.

Future Directions

Because DIPC can block multiple functions of HPV E6, the development of one or more clinically-useful drugs based on this compound has great potential. As mentioned previously, the benzimidazole family includes a number of derivatives with diverse bioactivities, which provides a precedent for optimizing DIPC to obtain one or more chemical compounds that can specifically block E6 functions. The next step in this

process will be to test DIPC for function *in vivo*. A xenograft mouse has already been developed in our laboratory in order to test the ability of small molecules to reduce the development of cervical cancer. This model can be used to test DIPC as well as additional candidates, once identified. To obtain additional and potentially more promising candidates, we will continue searching for additional classes of small molecule inhibitors. We have already developed collaborations with Kansas University and with the National Center for Advancing Translational Science (NCATS) to adapt our bead-based assay to a high throughput screening (HTS) format, and look forward to obtaining data from a screening of libraries totaling approximately 1.4 million compounds. Those hits from HTS will be re-tested for dose-dependency in the E6/caspase 8 binding assay, subjected to the caspase 8/caspase 8 counter-screening assay, and examined in our cell model as described previously. Following these steps, we hope to further characterize and optimize the best candidates, perform the appropriate *in vivo* studies, and move toward clinical trials.

REFERENCES

1. Stanley, M.A., *Genital human papillomavirus infections: current and prospective therapies.* The Journal of general virology, 2012. **93**(Pt 4): p. 681-91.

2. Brink, A.A., et al., *Clinical relevance of human papillomavirus testing in cytopathology.* Cytopathology : official journal of the British Society for Clinical Cytology, 2005. **16**(1): p. 7-12.

3. Longworth, M.S. and L.A. Laimins, *Pathogenesis of human papillomaviruses in differentiating epithelia.* Microbiology and molecular biology reviews : MMBR, 2004. **68**(2): p. 362-72.

4. Parkin, D.M., *The global health burden of infection-associated cancers in the year 2002.* International journal of cancer. Journal international du cancer, 2006. **118**(12): p. 3030-44.

5. Widdice, L.E., *Human papillomavirus disease in adolescents: management and prevention.* Adolescent medicine: state of the art reviews, 2012. **23**(1): p. 192-206, xii-xiii.

6. Goldstein, M.A., et al., *Case records of the Massachusetts General Hospital. Case 10-2009. A 23-year-old woman with an abnormal Papanicolaou smear.* The New England journal of medicine, 2009. **360**(13): p. 1337-44.

7. Frazer, I.H., G.R. Leggatt, and S.R. Mattarollo, *Prevention and treatment of papillomavirus-related cancers through immunization.* Annu Rev Immunol, 2011. **29**: p. 111-38.

8. Garland, S.M., et al., *Quadrivalent vaccine against human papillomavirus to prevent anogenital diseases.* N Engl J Med, 2007. **356**(19): p. 1928-43.

9. Paavonen, J., et al., *Efficacy of a prophylactic adjuvanted bivalent L1 virus-like-particle vaccine against infection with human papillomavirus types 16 and 18 in young women: an interim analysis of a phase III double-blind, randomised controlled trial.* Lancet, 2007. **369**(9580): p. 2161-70.

10. Villa, L.L., et al., *Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial.* Lancet Oncol, 2005. **6**(5): p. 271-8.

11. Scheinfeld, N. and D.S. Lehman, *An evidence-based review of medical and surgical treatments of genital warts.* Dermatology online journal, 2006. **12**(3): p. 5.

12. Tungteakkhun, S.S., et al., *The interaction between human papillomavirus type 16 and FADD is mediated by a novel E6 binding domain.* Journal of virology, 2008. **82**(19): p. 9600-14.

13. Moody, C.A. and L.A. Laimins, *Human papillomavirus oncoproteins: pathways to transformation.* Nature reviews. Cancer, 2010. **10**(8): p. 550-60.

14. Stubenrauch, F., H.B. Lim, and L.A. Laimins, *Differential requirements for conserved E2 binding sites in the life cycle of oncogenic human papillomavirus type 31.* Journal of virology, 1998. **72**(2): p. 1071-7.

15. Thomas, J.T., et al., *Human papillomavirus type 31 oncoproteins E6 and E7 are required for the maintenance of episomes during the viral life cycle in normal human keratinocytes.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(15): p. 8449-54.

16. Park, R.B. and E.J. Androphy, *Genetic analysis of high-risk e6 in episomal maintenance of human papillomavirus genomes in primary human keratinocytes.* Journal of virology, 2002. **76**(22): p. 11359-64.

17. De Geest, K., et al., *Growth and differentiation of human papillomavirus type 31b positive human cervical cell lines.* Gynecologic oncology, 1993. **49**(3): p. 303-10.

18. Stanley, M., *The immunology of genital human papilloma virus infection.* European journal of dermatology : EJD, 1998. **8**(7 Suppl): p. 8-12; discussion 20-2.

19. Doorbar, J., *The papillomavirus life cycle.* Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology, 2005. **32 Suppl 1**: p. S7-15.

20. Munger, K., et al., *Biological activities and molecular targets of the human papillomavirus E7 oncoprotein.* Oncogene, 2001. **20**(54): p. 7888-98.

21. McBride, A.A., J.G. Oliveira, and M.G. McPhillips, *Partitioning viral genomes in mitosis: same idea, different targets.* Cell cycle, 2006. **5**(14): p. 1499-502.

22. Peh, W.L., et al., *The viral E4 protein is required for the completion of the cottontail rabbit papillomavirus productive cycle in vivo.* Journal of virology, 2004. **78**(4): p. 2142-51.

23. Genther, S.M., et al., *Quantitative role of the human papillomavirus type 16 E5 gene during the productive stage of the viral life cycle.* Journal of virology, 2003. **77**(5): p. 2832-42.

24. Fehrmann, F. and L.A. Laimins, *Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation.* Oncogene, 2003. **22**(33): p. 5201-7.

25. Hummel, M., J.B. Hudson, and L.A. Laimins, *Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes.* Journal of virology, 1992. **66**(10): p. 6070-80.

26. Ozbun, M.A. and C. Meyers, *Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b.* Journal of virology, 1997. **71**(7): p. 5161-72.

27. Davy, C., et al., *A novel interaction between the human papillomavirus type 16 E2 and E1--E4 proteins leads to stabilization of E2.* Virology, 2009. **394**(2): p. 266-75.

28. McIntosh, P.B., et al., *E1--E4-mediated keratin phosphorylation and ubiquitylation: a mechanism for keratin depletion in HPV16-infected epithelium.* Journal of cell science, 2010. **123**(Pt 16): p. 2810-22.

29. Venuti, A., et al., *Papillomavirus E5: the smallest oncoprotein with many functions.* Molecular cancer, 2011. **10**: p. 140.

30. Doorbar, J., *Molecular biology of human papillomavirus infection and cervical cancer.* Clinical science, 2006. **110**(5): p. 525-41.

31. Grm, H.S., et al., *Crosstalk between the human papillomavirus E2 transcriptional activator and the E6 oncoprotein.* Oncogene, 2005. **24**(33): p. 5149-64.

32. Rudin, C.M. and C.B. Thompson, *Apoptosis and disease: regulation and clinical relevance of programmed cell death.* Annual review of medicine, 1997. **48**: p. 267-81.

33. Mundt, B., et al., *Involvement of TRAIL and its receptors in viral hepatitis.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2003. **17**(1): p. 94-6.

34. White, S. and A. Rosen, *Apoptosis in systemic lupus erythematosus.* Current opinion in rheumatology, 2003. **15**(5): p. 557-62.

35. Chen, W., et al., *Development of a human neuronal cell model for human immunodeficiency virus (HIV)-infected macrophage-induced neurotoxicity: apoptosis induced by HIV type 1 primary isolates and evidence for involvement of*

the Bcl-2/Bcl-xL-sensitive intrinsic apoptosis pathway. J Virol, 2002. **76**(18): p. 9407-19.

36. Fulda, S. and K.M. Debatin, *Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy*. Oncogene, 2006. **25**(34): p. 4798-811.

37. Taylor, J.M. and M. Barry, *Near death experiences: poxvirus regulation of apoptotic death.* Virology, 2006. **344**(1): p. 139-50.

38. Gaur, U. and B.B. Aggarwal, *Regulation of proliferation, survival and apoptosis by members of the TNF superfamily.* Biochemical pharmacology, 2003. **66**(8): p. 1403-8.

39. Ashkenazi, A., P. Holland, and S.G. Eckhardt, *Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL).* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2008. **26**(21): p. 3621-30.

40. Scaffidi, C., et al., *Two CD95 (APO-1/Fas) signaling pathways.* The EMBO journal, 1998. **17**(6): p. 1675-87.

41. Schmitz, I., et al., *Differences between CD95 type I and II cells detected with the CD95 ligand.* Cell death and differentiation, 1999. **6**(9): p. 821-2.

42. Wang, J., et al., *Caspase-10 is an initiator caspase in death receptor signaling.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(24): p. 13884-8.

43. Tinel, A. and J. Tschopp, *The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress.* Science, 2004. **304**(5672): p. 843-6.

44. Henshall, D.C., et al., *Caspase-2 activation is redundant during seizure-induced neuronal death.* Journal of neurochemistry, 2001. **77**(3): p. 886-95.

45. Sohn, D., W. Budach, and R.U. Janicke, *Caspase-2 is required for DNA damage-induced expression of the CDK inhibitor p21(WAF1/CIP1).* Cell death and differentiation, 2011. **18**(10): p. 1664-74.

46. Rich, T., R.L. Allen, and A.H. Wyllie, *Defying death after DNA damage.* Nature, 2000. **407**(6805): p. 777-83.

47. Vermeulen, K., D.R. Van Bockstaele, and Z.N. Berneman, *Apoptosis: mechanisms and relevance in cancer.* Annals of hematology, 2005. **84**(10): p. 627-39.

48. Li, P., et al., *Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade.* Cell, 1997. **91**(4): p. 479-89.

49. Zou, H., et al., *An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9.* The Journal of biological chemistry, 1999. **274**(17): p. 11549-56.

50. Hill, M.M., et al., *Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes.* The EMBO journal, 2004. **23**(10): p. 2134-45.

51. Kothakota, S., et al., *Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis.* Science, 1997. **278**(5336): p. 294-8.

52. Kato, K., et al., *Caspase-mediated protein kinase C-delta cleavage is necessary for apoptosis of vascular smooth muscle cells.* American journal of physiology. Heart and circulatory physiology, 2009. **297**(6): p. H2253-61.

53. Yuan, C.H., M. Filippova, and P. Duerksen-Hughes, *Modulation of apoptotic pathways by human papillomaviruses (HPV): mechanisms and implications for therapy.* Viruses, 2012. **4**(12): p. 3831-50.

54. Zilli, D., et al., *The adenovirus E3 region 14.7 kDa protein, heat and sodium arsenite inhibit the TNF-induced release of arachidonic acid.* Biochemical and biophysical research communications, 1992. **188**(1): p. 177-83.

55. Rahman, M.M., A.R. Lucas, and G. McFadden, *Viral TNF inhibitors as potential therapeutics.* Advances in experimental medicine and biology, 2009. **666**: p. 64-77.

56. Bertin, J., et al., *Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(4): p. 1172-6.

57. Zhang, Y., et al., *Effects of Epstein-Barr virus infection on the development of multiple myeloma after liver transplantation.* Science China. Life sciences, 2012. **55**(8): p. 735-43.

58. Cai, Q., et al., *Epstein-Barr virus nuclear antigen 3C stabilizes Gemin3 to block p53-mediated apoptosis.* PLoS pathogens, 2011. **7**(12): p. e1002418.

59. Gandarillas, A., et al., *Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes.* Experimental dermatology, 1999. **8**(1): p. 71-9.

60. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

61. Scheffner, M., et al., *The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53.* Cell, 1990. **63**(6): p. 1129-36.

62. Huibregtse, J.M., M. Scheffner, and P.M. Howley, *A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18.* The EMBO journal, 1991. **10**(13): p. 4129-35.

63. Crook, T., J.A. Tidy, and K.H. Vousden, *Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation.* Cell, 1991. **67**(3): p. 547-56.

64. Lechner, M.S. and L.A. Laimins, *Inhibition of p53 DNA binding by human papillomavirus E6 proteins.* Journal of virology, 1994. **68**(7): p. 4262-73.

65. Massimi, P., et al., *HPV E6 degradation of p53 and PDZ containing substrates in an E6AP null background.* Oncogene, 2008. **27**(12): p. 1800-4.

66. Shai, A., H.C. Pitot, and P.F. Lambert, *E6-associated protein is required for human papillomavirus type 16 E6 to cause cervical cancer in mice.* Cancer research, 2010. **70**(12): p. 5064-73.

67. Thomas, M. and L. Banks, *Inhibition of Bak-induced apoptosis by HPV-18 E6.* Oncogene, 1998. **17**(23): p. 2943-54.

68. Gross-Mesilaty, S., et al., *Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(14): p. 8058-63.

69. Filippova, M., et al., *The human papillomavirus 16 E6 protein can either protect or further sensitize cells to TNF: effect of dose.* Cell death and differentiation, 2005. **12**(12): p. 1622-35.

70. Filippova, M., L. Parkhurst, and P.J. Duerksen-Hughes, *The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis.* The Journal of biological chemistry, 2004. **279**(24): p. 25729-44.

71. Filippova, M., et al., *Complexes of human papillomavirus type 16 E6 proteins form pseudo-death-inducing signaling complex structures during tumor necrosis factor-mediated apoptosis.* Journal of virology, 2009. **83**(1): p. 210-27.

72. Garnett, T.O., M. Filippova, and P.J. Duerksen-Hughes, *Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis.* Cell death and differentiation, 2006. **13**(11): p. 1915-26.

73. Du, J., et al., *Resistance to apoptosis of HPV 16-infected laryngeal cancer cells is associated with decreased Bak and increased Bcl-2 expression.* Cancer letters, 2004. **205**(1): p. 81-8.

74. Tungteakkhun, S.S., et al., *The full-length isoform of human papillomavirus 16 E6 and its splice variant E6* bind to different sites on the procaspase 8 death effector domain.* Journal of virology, 2010. **84**(3): p. 1453-63.

75. Filippova, M., et al., *The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis.* The Journal of biological chemistry, 2002. **277**(24): p. 21730-9.

76. Borbely, A.A., et al., *Effects of human papillomavirus type 16 oncoproteins on survivin gene expression.* The Journal of general virology, 2006. **87**(Pt 2): p. 287-94.

77. Jabbar, S.F., et al., *Cervical cancers require the continuous expression of the human papillomavirus type 16 E7 oncoprotein even in the presence of the viral E6 oncoprotein.* Cancer research, 2012. **72**(16): p. 4008-16.

78. D'Abramo, C.M. and J. Archambault, *Small molecule inhibitors of human papillomavirus protein - protein interactions.* The open virology journal, 2011. **5**: p. 80-95.

79. Baleja, J.D., et al., *Identification of inhibitors to papillomavirus type 16 E6 protein based on three-dimensional structures of interacting proteins.* Antiviral research, 2006. **72**(1): p. 49-59.

80. Liu, Y., et al., *Design and characterization of helical peptides that inhibit the E6 protein of papillomavirus.* Biochemistry, 2004. **43**(23): p. 7421-31.

81. Scheffner, M. and N.J. Whitaker, *Human papillomavirus-induced carcinogenesis and the ubiquitin-proteasome system.* Seminars in cancer biology, 2003. **13**(1): p. 59-67.

82. Thomas, M.C. and C.M. Chiang, *E6 oncoprotein represses p53-dependent gene activation via inhibition of protein acetylation independently of inducing p53 degradation.* Molecular cell, 2005. **17**(2): p. 251-64.

83. Bellail, A.C., et al., *TRAIL agonists on clinical trials for cancer therapy: the promises and the challenges.* Rev Recent Clin Trials, 2009. **4**(1): p. 34-41.

84. El-Zawahry, A., J. McKillop, and C. Voelkel-Johnson, *Doxorubicin increases the effectiveness of Apo2L/TRAIL for tumor growth inhibition of prostate cancer xenografts.* BMC Cancer, 2005. **5**: p. 2.

85. Mom, C.H., et al., *Mapatumumab, a fully human agonistic monoclonal antibody that targets TRAIL-R1, in combination with gemcitabine and cisplatin: a phase I study.* Clin Cancer Res, 2009. **15**(17): p. 5584-90.

86. Naka, T., et al., *Effects of tumor necrosis factor-related apoptosis-inducing ligand alone and in combination with chemotherapeutic agents on patients' colon tumors grown in SCID mice.* Cancer Res, 2002. **62**(20): p. 5800-6.

87. Filippova, M., et al., *The large and small isoforms of human papillomavirus type 16 E6 bind to and differentially affect procaspase 8 stability and activity.* Journal of virology, 2007. **81**(8): p. 4116-29.

88. Yi, C., et al., *Development of small-molecule inhibitors of the group I p21-activated kinases, emerging therapeutic targets in cancer.* Biochemical pharmacology, 2010. **80**(5): p. 683-9.

89. Yuan, C.H., et al., *Small molecule inhibitors of the HPV16-E6 interaction with caspase 8.* Bioorganic & medicinal chemistry letters, 2012. **22**(5): p. 2125-9.

90. Munoz, N., et al., *Chapter 1: HPV in the etiology of human cancer.* Vaccine, 2006. **24 Suppl 3**: p. S3/1-10.

91. Garland, S.M., et al., *Cervical cancer--what role for human papillomavirus?* The Medical journal of Australia, 1992. **156**(3): p. 204-12.

92. Kreimer, A.R., et al., *Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2005. **14**(2): p. 467-75.

93. Alam, M., J.B. Caldwell, and Y.D. Eliezri, *Human papillomavirus-associated digital squamous cell carcinoma: literature review and report of 21 new cases.* Journal of the American Academy of Dermatology, 2003. **48**(3): p. 385-93.

94. Ragin, C.C. and E. Taioli, *Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis.* International journal of cancer. Journal international du cancer, 2007. **121**(8): p. 1813-20.

95. Ghittoni, R., et al., *The biological properties of E6 and E7 oncoproteins from human papillomaviruses.* Virus genes, 2010. **40**(1): p. 1-13.

96. Tungteakkhun, S.S. and P.J. Duerksen-Hughes, *Cellular binding partners of the human papillomavirus E6 protein.* Archives of virology, 2008. **153**(3): p. 397-408.

97. Romanowski, B., et al., *Sustained efficacy and immunogenicity of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine: analysis of a randomised placebo-controlled trial up to 6.4 years.* Lancet, 2009. **374**(9706): p. 1975-85.

98. Garland, S.M. and J.S. Smith, *Human papillomavirus vaccines: current status and future prospects.* Drugs, 2010. **70**(9): p. 1079-98.

99. Scarinci, I.C., et al., *Cervical cancer prevention: new tools and old barriers.* Cancer, 2010. **116**(11): p. 2531-42.

100. Lipinski, C.A., et al., *Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings.* Adv Drug Deliv Rev, 2001. **46**(1-3): p. 3-26.

101. Curtin, F. and P. Schulz, *Multiple correlations and Bonferroni's correction.* Biol Psychiatry, 1998. **44**(8): p. 775-7.

102. Teague, S.J., et al., *The Design of Leadlike Combinatorial Libraries.* Angew Chem Int Ed Engl, 1999. **38**(24): p. 3743-3748.

103. Ren, W., et al., *Flavonoids: promising anticancer agents.* Med Res Rev, 2003. **23**(4): p. 519-34.

104. Lee, H.G., et al., *Inhibitory effect of jaceosidin isolated from Artemisiaargyi on the function of E6 and E7 oncoproteins of HPV 16.* J Ethnopharmacol, 2005. **98**(3): p. 339-43.

105. Ramanouskaya, T.V., V.V. Smolnykova, and V.V. Grinev, *Relationship between structure and antiproliferative, proapoptotic, and differentiation effects of flavonoids on chronic myeloid leukemia cells.* Anticancer Drugs, 2009. **20**(7): p. 573-83.

106. Ichimatsu, D., et al., *Structure-activity relationship of flavonoids for inhibition of epidermal growth factor-induced transformation of JB6 Cl 41 cells.* Mol Carcinog, 2007. **46**(6): p. 436-45.

107. Moridani, M.Y., G. Galati, and P.J. O'Brien, *Comparative quantitative structure toxicity relationships for flavonoids evaluated in isolated rat hepatocytes and HeLa tumor cells.* Chem Biol Interact, 2002. **139**(3): p. 251-64.

108. Hann, M.M. and T.I. Oprea, *Pursuing the leadlikeness concept in pharmaceutical research.* Curr Opin Chem Biol, 2004. **8**(3): p. 255-63.

109. Parkin, D.M. and F. Bray, *Chapter 2: The burden of HPV-related cancers.* Vaccine, 2006. **24 Suppl 3**: p. S3/11-25.

110. Oehler-Janne, C., et al., *HIV-specific differences in outcome of squamous cell carcinoma of the anal canal: a multicentric cohort study of HIV-positive patients receiving highly active antiretroviral therapy.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2008. **26**(15): p. 2550-7.

111. Hagensee, M.E., et al., *Human papillomavirus infection and disease in HIV-infected individuals.* The American journal of the medical sciences, 2004. **328**(1): p. 57-63.

112. Beutner, K.R. and A. Ferenczy, *Therapeutic approaches to genital warts.* The American journal of medicine, 1997. **102**(5A): p. 28-37.

113. Beutner, K.R., et al., *Imiquimod, a patient-applied immune-response modifier for treatment of external genital warts.* Antimicrobial agents and chemotherapy, 1998. **42**(4): p. 789-94.

114. Bonomi, P., et al., *Randomized trial of three cisplatin dose schedules in squamous-cell carcinoma of the cervix: a Gynecologic Oncology Group study.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 1985. **3**(8): p. 1079-85.

115. Thigpen, T., et al., *Cis-platinum in treatment of advanced or recurrent squamous cell carcinoma of the cervix: a phase II study of the Gynecologic Oncology Group.* Cancer, 1981. **48**(4): p. 899-903.

116. Thigpen, J.T., et al., *A randomized comparison of a rapid versus prolonged (24 hr) infusion of cisplatin in therapy of squamous cell carcinoma of the uterine cervix: a Gynecologic Oncology Group study.* Gynecologic oncology, 1989. **32**(2): p. 198-202.

117. Minotti, G., et al., *Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity.* Pharmacological reviews, 2004. **56**(2): p. 185-229.

118. Gewirtz, D.A., *A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin.* Biochemical pharmacology, 1999. **57**(7): p. 727-41.

119. Buchsbaum, D.J., et al., *Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model.* Clin Cancer Res, 2003. **9**(10 Pt 1): p. 3731-41.

120. Younes, A. and M.E. Kadin, *Emerging applications of the tumor necrosis factor family of ligands and receptors in cancer therapy.* J Clin Oncol, 2003. **21**(18): p. 3526-34.

121. Ichikawa, K., et al., *Tumoricidal activity of a novel anti-human DR5 monoclonal antibody without hepatocyte cytotoxicity.* Nat Med, 2001. **7**(8): p. 954-60.

122. Chuntharapai, A., et al., *Isotype-dependent inhibition of tumor growth in vivo by monoclonal antibodies to death receptor 4.* J Immunol, 2001. **166**(8): p. 4891-8.

123. Tanaka, S., et al., *Expression and antitumor effects of TRAIL in human cholangiocarcinoma.* Hepatology, 2000. **32**(3): p. 523-7.

124. Doi, T., et al., *Phase 1 study of conatumumab, a pro-apoptotic death receptor 5 agonist antibody, in Japanese patients with advanced solid tumors.* Cancer Chemother Pharmacol, 2011. **68**(3): p. 733-41.

125. Herbst, R.S., et al., *A first-in-human study of conatumumab in adult patients with advanced solid tumors.* Clin Cancer Res, 2010. **16**(23): p. 5883-91.

126. Huibregtse, J.M., M. Scheffner, and P.M. Howley, *A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18.* EMBO J, 1991. **10**(13): p. 4129-35.

127. Scheffner, M., et al., *The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53.* Cell, 1993. **75**(3): p. 495-505.

128. Tungteakkhun, S.S., et al., *The full-length isoform of human papillomavirus 16 E6 and its splice variant E6* bind to different sites on the procaspase 8 death effector domain.* J Virol, 2010. **84**(3): p. 1453-63.

129. Filippova, M., L. Parkhurst, and P.J. Duerksen-Hughes, *The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis.* J Biol Chem, 2004. **279**(24): p. 25729-44.

130. Filippova, M., et al., *The large and small isoforms of human papillomavirus type 16 E6 bind to and differentially affect procaspase 8 stability and activity.* J Virol, 2007. **81**(8): p. 4116-29.

131. Garnett, T.O., M. Filippova, and P.J. Duerksen-Hughes, *Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis.* Cell Death Differ, 2006. **13**(11): p. 1915-26.

132. Phelps, W.C., J.A. Barnes, and D.C. Lobe, *Molecular targets for human papillomaviruses: prospects for antiviral therapy.* Antiviral chemistry & chemotherapy, 1998. **9**(5): p. 359-77.

133. Faucher, A.M., et al., *Discovery of small-molecule inhibitors of the ATPase activity of human papillomavirus E1 helicase.* Journal of medicinal chemistry, 2004. **47**(1): p. 18-21.

134. Hebner, C., M. Beglin, and L.A. Laimins, *Human papillomavirus E6 proteins mediate resistance to interferon-induced growth arrest through inhibition of p53 acetylation.* Journal of virology, 2007. **81**(23): p. 12740-7.

135. Kus, C., et al., *Antioxidant and antifungal properties of benzimidazole derivatives.* Z Naturforsch C, 2010. **65**(9-10): p. 537-42.

136. Nakano, H., et al., *Synthesis and biological activities of novel antiallergic agents with 5-lipoxygenase inhibiting action.* Bioorg Med Chem, 2000. **8**(2): p. 373-80.

137. Ozden, S., et al., *Synthesis and potent antimicrobial activity of some novel methyl or ethyl 1H-benzimidazole-5-carboxylates derivatives carrying amide or amidine groups.* Bioorg Med Chem, 2005. **13**(5): p. 1587-97.

138. Goker, H., et al., *Synthesis and potent antibacterial activity against MRSA of some novel 1,2-disubstituted-1H-benzimidazole-N-alkylated-5-carboxamidines.* Eur J Med Chem, 2005. **40**(10): p. 1062-9.

139. Andrzejewska, M., et al., *Synthesis, antiprotozoal and anticancer activity of substituted 2-trifluoromethyl- and 2-pentafluoroethylbenzimidazoles.* Eur J Med Chem, 2002. **37**(12): p. 973-8.

140. Wang, X.J., et al., *Newly synthesized bis-benzimidazole derivatives exerting anti-tumor activity through induction of apoptosis and autophagy.* Bioorg Med Chem Lett, 2012. **22**(19): p. 6297-300.

141. Hirashima, S., et al., *Benzimidazole derivatives bearing substituted biphenyls as hepatitis C virus NS5B RNA-dependent RNA polymerase inhibitors: structure-activity relationship studies and identification of a potent and highly selective inhibitor JTK-109.* J Med Chem, 2006. **49**(15): p. 4721-36.

142. Miller, J.F., et al., *Synthesis and SAR of novel isoquinoline CXCR4 antagonists with potent anti-HIV activity.* Bioorg Med Chem Lett, 2010.

143. Hollstein, M., et al., *p53 mutations in human cancers.* Science, 1991. **253**(5015): p. 49-53.

144. Ventura, A., et al., *Restoration of p53 function leads to tumour regression in vivo.* Nature, 2007. **445**(7128): p. 661-5.

145. Forero-Torres, A., et al., *Phase I trial of weekly tigatuzumab, an agonistic humanized monoclonal antibody targeting death receptor 5 (DR5).* Cancer biotherapy & radiopharmaceuticals, 2010. **25**(1): p. 13-9.

146. Kindler, H.L., et al., *A randomized, placebo-controlled phase 2 study of ganitumab (AMG 479) or conatumumab (AMG 655) in combination with gemcitabine in patients with metastatic pancreatic cancer.* Annals of oncology :

official journal of the European Society for Medical Oncology / ESMO, 2012. **23**(11): p. 2834-42.

147. Ashkenazi, A., *Targeting the extrinsic apoptosis pathway in cancer.* Cytokine & growth factor reviews, 2008. **19**(3-4): p. 325-31.

148. Sharma, S., et al., *Safety, pharmacokinetics, and pharmacodynamics of the DR5 antibody LBY135 alone and in combination with capecitabine in patients with advanced solid tumors.* Investigational new drugs, 2013.