Mechanisms of Growth Inhibition in Human Papillomavirus Positive and Negative Cervical Cancer Cells by the Chloromethyl Ketone Protease Inhibitor, Succinyl-Alanine-Alanine-Proline-Phenylalanine Chloromethyl Ketone

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Received December 11, 2008; accepted April 27, 2009

ABSTRACT

The chymotrypsin-like serine protease inhibitor, succinyl-alanine-alanine-proline-phenylalanine chloromethyl ketone (AAPFCMK), has been shown to have anticarcinogenic activity in a number of model systems and to be relatively selective for a nuclear protease. This inhibitor also has substantial effects on growth of tumorigenic human papillomavirus (HPV)-infected keratinocytes in organotypic raft cultures. Here, we examined the effects of AAPFCMK on cell growth, cell-cycle kinetics, apoptosis induction, and DNA synthesis in two human cervical carcinoma cell lines: SiHa cells, which have integrated high-risk HPV-16; and C33a cells, which do not contain HPV DNA. AAPFCMK inhibited growth of both cell lines in a time- and dose-dependent manner. Apoptosis studies showed no significant difference in drug-treated versus vehicle-treated cells in the C33a cell line. However, a significant dose-dependent increase in apoptosis occurred at a late time point in SiHa cells. Cell-cycle progression and DNA synthesis assays showed that the cellular mechanisms of growth inhibition by AAPFCMK differ between the HPV16-positive and HPV-negative tumorigenic cell lines. Drug-treated C33a cells showed a significant accumulation of cells in the G2 phase of the cell cycle. In SiHa cells, growth inhibition produced by AAPFCMK seemed to result from a global arrest of the cell cycle. Although the molecular mechanisms involved in AAPFCMK-induced growth inhibition are distinct between the two tumorigenic cell lines, such differences may ultimately prove to have therapeutic utility. Novel therapies for treating established HPV infections are needed, because HPV is a causative agent in the development of multiple types of cancer.

Human papillomavirus (HPV) has been well established as an underlying cause of cervical cancer, which is the second most common cancer in women worldwide (Kamangar et al., 2006). Scientific research has made progress against the development of cervical cancer with the advent of preventative virus-like particle vaccines against HPV-16 and HPV-18, two of the most common high-risk HPVs (Munoz et al., 2003). Despite this progress, established HPV infection remains a serious health problem that warrants research into additional treatment options. The first problem is that at least 15 high-risk types of HPV exist; HPV-16 and -18 are associated with up to only 70% of cervical cancer cases (Munoz et al., 2003). Second, the virus-like particle vaccines are not a viable treatment for women already infected by HPV (Hildesheim et al., 2007) and are not available to most women in less-developed countries where cervical cancer is most prevalent (Kamangar et al., 2006). Third, HPV is a sexually transmitted disease, and precancerous lesions typically arise in women during their childbearing years (Schiffman and Castle, 2005). Current surgical and ablative treatments for cervical intraepithelial neoplasia significantly increase preterm delivery in subsequent pregnancies (Jakobsson et al., 2007). Fourth, in addition to cervical and other anogenital cancers, HPV seems to be an underlying cause of vaginal, vulvar, and anal cancer.

This work was supported by the Jake Gittlen Cancer Research Foundation, Pennsylvania State College of Medicine (Hershey, PA). This work was presented previously: Duncan KJ (2008) Mechanism(s) of growth inhibition of human papillomavirus positive and negative human cervical cancer cells by a chymotrypsin-like serine protease inhibitor, in 2008 American Association for Cancer Research Meeting; 2008 Apr 12–16; San Diego, CA. American Association for Cancer Research, Philadelphia, PA.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.108.149765.

ABBREVIATIONS: HPV, human papillomavirus; AAPFCMK, succinyl-alanine-alanine-proline-phenylalanine chloromethyl ketone; DMSO, dimethyl sulfoxide; EMEM, Eagle’s minimum essential medium; FBS, fetal bovine serum; EGR CMK, glutamic acid-glycine-arginine chloromethyl ketone; GR CMK, glycine-arginine chloromethyl ketone; BrdU, 5-bromo-2’-deoxyuridine; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PI, propidium iodide; ANOVA, analysis of variance.
important causative agent in a subset of head and neck cancers (van Houten et al., 2001; Gillison and Lowy, 2004; Kreimer et al., 2005). The participation of HPV in the development of multiple types of cancer, the prevalence of infected women in less developed countries, and the current negative reproductive consequences of treatment make finding novel and affordable therapies critical to treating established HPV infections.

Succinyl-alanine-alanine-proline-phenylalanine chloromethyl ketone (AAPF<sub>CMK</sub>) is a chymotrypsin-like serine protease inhibitor that has been shown to substantially inhibit growth of organotypic raft cultures infected with high-risk HPV types, with no effect observed on uninfected keratinocyte raft cultures (Drubin et al., 2006). Previous research has shown that AAPF<sub>CMK</sub> is relatively selective for a nuclear protease in rat liver hepatocytes and mouse fibroblasts and has been used to study the link between nuclear protease activity and cancer (Clawson et al., 1992, 1993). AAPF<sub>CMK</sub> produced a significant early decrease of protease activity in the nuclear scaffold of mouse embryo fibroblasts and inhibited transformation of mouse embryo fibroblasts by chemical carcinogens (Clawson et al., 1992, 1993). AAPF<sub>CMK</sub> also inhibited spontaneous transformation in SV40-immortalized rat hepatocytes (Drubin and Clawson, 2004) and selectively inhibited growth of ras-transformed cells, but not the parental SV40-immortalized rat hepatocytes (Clawson et al., 1995). Cells infected with high-risk HPV have also shown a significant increase in nuclear protease activity (Drubin et al., 2006).

To determine potential targets of AAPF<sub>CMK</sub>, mass spectrometer-generated sequences of proteins bound to biotinylated AAPF<sub>CMK</sub>, which was incubated in rat liver nuclear preparations, were analyzed (Dhamne et al., 2007). These investigations identified the serine protease HtrA1 (Gene IDs: 5654 for human, 65164 for rat, and 56213 for mouse) as a biomarker for HPV-negative cells, as well as the serine protease HtrA2 (Gene IDs: 5655 for human and 65165 for rat) as a biomarker for HPV-positive cells. These findings suggest that AAPF<sub>CMK</sub> may be a potential therapeutic target for HPV-negative cancers.

Materials and Methods

Reagents. AAPF<sub>CMK</sub> was obtained from MP Biomedicals (Irvine, CA). As a control, inhibitors of trypsin-like protease activity, glycine-arginine chloromethyl ketone (GR<sub>CMK</sub>) (MP Biomedicals) and glutamic acid-glycine-arginine chloromethyl ketone (EGR<sub>CMK</sub>) (Calbiochem, San Diego, CA; or SM Biochemicals, Yorba Linda, CA), were used. All chloromethyl ketone inhibitors were resuspended in dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich (St. Louis, MO) and stored at −20°C. Immediately before addition to cell culture, the chloromethyl ketone inhibitors were diluted in Eagle’s minimum essential medium (MEM) + 10% fetal bovine serum (FBS). The GI<sub>S</sub> cell-cycle inhibitor mimosine was obtained from Sigma-Aldrich and dissolved at a stock concentration of 10 mM in EMEM without FBS (American Type Culture Collection, Manassas, VA). Staurosporine (Sigma-Aldrich) is an inducer of apoptosis, which was obtained at a stock concentration of 1 mM in DMSO.

Cell Culture. Cervical cancer cell lines SiHa (HPV-16) and C33a (HPV-negative) were obtained from the American Type Culture Collection and maintained in EMEM from the American Type Culture Collection, supplemented with 10% FBS (Sigma-Aldrich). All cultures were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere.

Cell Growth and Inhibition. SiHa and C33a cells were plated at a density of 5 × 10<sup>4</sup> cells per well in a six-well tissue culture plate. After overnight growth, the cells were washed once with PBS, given fresh media, and treated with varying concentrations of AAPF<sub>CMK</sub>, GR<sub>CMK</sub> or EGR<sub>CMK</sub> (trypsin-specific controls), or the equivalent quantity of DMSO. The cells were washed once with PBS and retreated with vehicle/drug and media every other day. The cells were collected at 24, 48, 96, and 144 h after initial drug/treatment. The cells were counted manually by using a hemacytometer, and trypsin blue dye exclusion was used to assess viable cells. The level of growth-inhibitory activity in each lot of AAPF<sub>CMK</sub> was standardized by determining the concentration of AAPF<sub>CMK</sub> required for at least 85% growth inhibition, as a percentage of control after three drug treatments or 144 h after the initial drug treatment, in both cell lines. From this point, we refer to this AAPF<sub>CMK</sub> concentration as the growth inhibitory dose (1.0 GI<sub>50</sub>). Three different lots of AAPF<sub>CMK</sub> were used during the course of this study. Six independent cell growth assays were performed incorporating the results from each lot of AAPF<sub>CMK</sub> using 0.25, 0.50, and 1.0 GI<sub>50</sub> doses, a trypsin-specific inhibitor at an equivalent amount to the 1.0 GI<sub>50</sub> AAPF<sub>CMK</sub> dose, and an equivalent quantity of DMSO.

Apopotosis Assay. Cells were plated and treated as described above. The cells were then collected at various time points and labeled with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Martin et al., 1995). The ApoAlert Annexin V kit was used according to the manufacturer’s instructions (Clontech, Mountain View, CA). The labeled cells were analyzed by using a FACScan flow cytometer (BD Biosciences, San Jose, CA). DNA histogram thresholds for each experiment were set to control samples of PI-stained only (necrotic cells), Annexin V-FITC-stained only (early apoptotic cells), no-dye negative control (viable cells), and dual-stained PI and Annexin V-FITC (late apoptotic cells). As a positive control for apoptosis, cells were treated for 24 h with 1 μM staurosporine (Bernard et al., 2001). The data were analyzed from DNA histograms by using the BD Cell Quest software (Becton Dickinson Canada, Oakville, ON). Independent experiments were performed in triplicate for each cell line.

Cell-Cycle Progression Assays. SiHa and C33a cells were plated at a density of 7.5 × 10<sup>4</sup> cells per 100-mm tissue culture dish. After overnight growth, each dish was treated for 24 h with 0.5 mM mimosine to synchronize the cells in G1/S phase (Krude, 1999). After 24 h, cells were washed twice with PBS and treated with varying concentrations of AAPF<sub>CMK</sub>, GR<sub>CMK</sub>, or EGR<sub>CMK</sub> or an equivalent volume of DMSO (solvent control). The cells were washed twice with PBS and retreated with vehicle/drug and media every other day. At various time points, cells were collected, pelleted, and stored in ethanol at −20°C until analysis. On the day of cell-cycle analysis, samples were thawed and centrifuged at 250g for 5 min at 4°C. The supernatant was removed, and 1 ml of DNA staining buffer (0.1% sodium citrate, 0.3% Triton X-100, 100 μg/ml propidium iodide, 20 μg/ml RNase A) was added to the cell pellet for 30 min on ice before being analyzed by flow cytometry (Krishan, 1975). The data were analyzed by set-
ting DNA content at 2N for G1 and 4N for G2, with the use of ModFit LT 3.0 (Verity Software House, Topsham, ME). Independent experiments were performed in triplicate. The BrdU Cell Proliferation assay kit (Calbiochem, La Jolla, CA) was used to determine S-phase content of the cell populations. SiHa and C33a cells were plated at a density of 500 cells per well in 96-well plates. After growth overnight, the cells were washed once with PBS and given fresh medium and treated with varying concentrations of AAPFCMK or DMSO. SiHa cells were pulse-labeled with 5-bromo-2’-deoxyuridine (BrdU) for 24 h, and C33a cells were pulse-labeled for 12 h. The assay was performed according to the manufacturer’s instructions. Each plate was read by using a Synergy HT microplate reader and KC4 version 3.4 (BioTek Instruments, Winooski, VT). An endpoint reading was taken using an absorbance test wavelength of 450 nm and a reference wavelength of 540 nm. Three independent experiments were each performed in triplicate. To optimize the assay, cells were initially plated at 2000, 1000, 500, and 250 cells per well in a 96-well plate and treated with varying concentrations of AAPFCMK or an equivalent quantity of the solvent DMSO. Linear BrdU incorporation was observed at 500 cells per well for both cell lines (data not shown).

**Statistical Analysis.** Statistical analyses were performed using the GraphPad Prism 4 software (GraphPad Software, San Diego, CA). All of the data were analyzed using two-way analysis of variance (ANOVA) with a Bonferroni post-test for comparison with the control group. In the cell-cycle assay, only the G2 phase of the cell cycle was compared with the control to analyze a time-dependent trend.

**Results**

**Growth Inhibition of C33a Cells by AAPFCMK.** Previous studies have demonstrated that AAPFCMK has anticarcinogenic activity (Clawson et al., 1993; Drubin and Clawson, 2004) and selectively inhibits transformed cells without inhibiting the immortalized parental control cell line (Clawson et al., 1995). To investigate the mechanism of inhibition in its natural context and without viral interference, the human cervical cancer cell line C33a was examined.

To standardize between different lots of AAPFCMK, the concentration of AAPFCMK required for at least 85% growth inhibition, as a percentage of control, after three drug treatments or 144 h after the initial drug treatment, in both cell lines was determined. We refer to this AAPFCMK concentration as the growth inhibitory dose (1.0 GI85). In general, each lot of AAPFCMK produced a 1.0 GI85 at a concentration of approximately 100 μM, with the exception of one very-low-activity lot that produced a 1.0 GI85 at a concentration of 800 μM. Although the absolute concentration of AAPFCMK required for the growth inhibitory dose differed for each lot of drug, the concentration of drug required for inhibition was always the same for both cell lines.

In C33a cells, six independent cell growth assays were performed incorporating the results from three lots of AAPFCMK. A significant difference was observed for the 1.0 GI85 dose at both 96 h (p < 0.01, two-way ANOVA; Bonferroni post test) and 144 h (p < 0.001) compared with the vehicle control (Fig. 1A). An equivalent dose of the trypsin-specific inhibitors, GRcmk and EGRcmk, had no significant effect relative to the vehicle control (Fig. 1A). However, it should be noted that the trypsin-specific inhibitory activity of each lot used was undetermined. Lower AAPFCMK concentrations resulted in partial growth inhibition. This dose-dependent growth inhibition was significantly different from the vehicle control 144 h after the initial drug treatment at 0.25 GI85 (p < 0.01).
and 0.50 GI$_{50}$ ($p < 0.001$) (Fig. 1A). From the untreated growth curve, we estimated that C33a cells doubled every 18 h. After removal of AAPF$_{CMK}$, C33a cells resumed normal growth rates (data not shown).

**No Apoptosis Induced in C33a Cells by AAPF$_{CMK}$.** To determine whether the observed growth inhibition is caused by apoptosis, the cells were treated with multiple concentrations of AAPF$_{CMK}$ and stained by using Annexin V and PI at 12, 24, and 36 h after drug treatment. Late apoptotic cells, as measured by positive staining for both Annexin V and PI, showed no significant difference relative to vehicle control (Fig. 1B). As a positive control, the C33a cells were treated for 24 h with 1 μM staurosporine to induce apoptosis (Bernard et al., 2001). The staurosporine treatment induced a 13-fold increase of cells in late apoptosis (Fig. 1B). Early apoptotic cells (Annexin V-only staining) showed no significant difference for AAPF$_{CMK}$-treated versus vehicle control (data not shown). Staurosporine treatment induced an 8-fold increase of cells in early apoptosis relative to vehicle control (data not shown).

**G$_{2}$/M Phase Accumulation in C33a Cells by AAPF$_{CMK}$.** C33a cells were treated with 0.5 mM mimosine to synchronize the cells in G$_{1}$/S phase. The 24-h mimosine treatment resulted in 62% of the cells in G$_{1}$ phase and 38% in S phase. The synchronized cells were released, treated with vehicle, EGR$_{CMK}$, or AAPF$_{CMK}$, and collected at 12, 18, 24, and 36 h. A highly significant increase in the cells accumulating within the G$_{2}$/M phase of the cell cycle was seen after treatment with the 1.0 GI$_{50}$ dose of AAPF$_{CMK}$ ($p < 0.001$, two-way ANOVA; Bonferroni post test), whereas an equivalent dose (100 μM) of the trypsin-specific inhibitor, EGR$_{CMK}$, showed no significant difference from the control group (Fig. 2A; Table 1). Treatment with 0.25 or 0.50 GI$_{50}$ doses of AAPF$_{CMK}$ did not significantly alter the cell cycle (data not shown).
BrdU incorporation was measured to determine the rate of progression of cells through S phase. Unsynchronized C33a cells were treated with varying concentrations of AAPFCMK or vehicle control. After drug treatment, BrdU was pulse-labeled every 12 h. We observed no significant differences in BrdU incorporation between solvent and AAPFCMK-treated cultures (Fig. 2B).

**Growth Inhibition of SiHa Cells by AAPFCMK** - Analogous to the C33a cell line, SiHa cells were also derived from cervical cancer tissue. However, SiHa cells contain integrated HPV-16 DNA (Yee et al., 1985) that express the E6 and E7 viral oncoproteins (Pater and Pater, 1985). HPV-16 is the most prevalent HPV type in cervical cancer (Muñoz et al., 2003, 2004), and AAPFCMK has been shown to substantially inhibit growth of organotypic raft cultures infected with high-risk HPV types (Drubin et al., 2006). Duplication of the C33a growth inhibition studies with the HPV-16-infected SiHa cells showed similar results. Six independent cell growth assays were performed incorporating the results from each lot of AAPFCMK. At the 1.0 GI85 dose, a significant difference was observed for both 96 (p < 0.01) and 144 h (p < 0.001) compared with the vehicle control (Fig. 3A). No significant effect was seen relative to the vehicle control in an equivalent dose of the trypsin-specific inhibitors, GR CMK and EGR CMK (Fig. 3A). Dose-dependent partial growth inhibition was also observed at lower AAPFCMK concentrations. This dose-dependent growth inhibition was significantly different from the vehicle control at 0.25 (p < 0.01) and 0.50 GI85 (p < 0.001) at 144 h after the initial drug treatment (Fig. 3A).

**Dose-Dependent Apoptosis Induced in SiHa Cells by AAPFCMK** - To determine whether the observed growth inhibition of SiHa cells is accompanied by apoptosis, the cells were treated with AAPFCMK, collected at 24, 48, 72, and 96 h after initial drug treatment, and analyzed as described above. A dose-dependent increase in late apoptotic cells was observed at 96 h. This was significant for both 0.75 GI85 AAPFCMK, with a 5-fold increase (p < 0.05), and 1.0 GI85 AAPFCMK, with an 8-fold increase (p < 0.01), in late apoptotic cells compared with DMSO (Fig. 3B). Treatment for 24 h with 1 μM staurosporine induced a 14-fold increase of cells in late apoptosis (Fig. 3B). No significant difference was observed in early apoptotic cells for AAPFCMK-treated versus vehicle control (data not shown). Staurosporine (1 μM) induced a 6-fold increase in early apoptotic cells relative to vehicle control (data not shown).

**Global Cell-Cycle Arrest Induced in SiHa Cells by AAPFCMK** - SiHa cells synchronized with mimosine showed 79% of the cells in G1 phase and 21% in S phase. After synchronization, cells were released into media containing AAPFCMK, EGR CMK, or vehicle control. At 24, 48, 72, and 96 h after initial vehicle/drug treatment, cells were retreated with vehicle/drug and media every other day. Data represent the mean ± S.D. of six independent experiments; **, p < 0.01; ***, p < 0.001. Significance was determined by two-way ANOVA with Bonferroni post-test of vehicle treatment. The cells were treated with vehicle/drug and media every other day. Data represent the mean ± S.D. of three independent experiments; *, p < 0.05; **, p < 0.01.
96 h after initial drug or vehicle treatment, DNA content was analyzed by flow cytometry. In contrast to the C33a cells, at 1.0 GI85 AAPF\textsubscript{CMK}, we observed no significant change in the cell-cycle distributions of SiHa cells treated with AAPF\textsubscript{CMK} relative to the vehicle control (Fig. 4A). The equivalent dose (100 \mu M) of EGR\textsubscript{CMK} also showed no significant difference from the control group (Fig. 4A).

BrdU incorporation was measured to determine whether AAPF\textsubscript{CMK} affected cell progression through the cell cycle. SiHa cells were pulse-labeled every 24 h with BrdU to determine the rate of progression through the S phase. We observed a significant decrease in BrdU incorporation in the AAPF\textsubscript{CMK} treatment groups (up to $p < 0.001$), relative to vehicle control, that was both time- and dose-dependent (Fig. 4B). Before the BrdU pulse-labeling, the cells were not synchronized; therefore, the rapid and significant decrease in DNA synthesis of the cell population caused by drug treatment suggests that the cells have halted cell-cycle progression. These data, combined with the observation that the distribution of the cells in specific phases of the cell cycle is not altered after AAPF\textsubscript{CMK} treatment, is evidence that the SiHa cells have undergone a global arrest of the cell cycle induced by drug treatment.

**Discussion**

We have used established cell culture models to study the antiproliferative mechanisms of the chymotrypsin-like serine protease inhibitor, AAPF\textsubscript{CMK}, using HPV-16-positive (SiHa) or HPV-negative (C33a) human tumorigenic cervical cell...
lines. AAPFCMK significantly inhibited growth in a time- and dose-dependent manner in both the C33a and SiHa cells (Figs. 1A and 3A, respectively). The growth inhibition seems to have chymotrypsin-like specificity as an equivalent dose of the two trypsin-specific inhibitors, GRMK and EGRMK, had no significant impact on cellular growth in either cell line. However, the trypsin-specific inhibitory activity of each lot of GRMK and EGRMK was unknown. To understand the mechanism behind this drug-induced growth inhibition, we examined the effects of AAPFCMK on apoptosis induction, cell-cycle progression, and DNA synthesis in both cell lines. Overall, our data indicate that AAPFCMK inhibits growth of HPV-positive SiHa cells and HPV-negative C33a tumor-derived cells by different mechanisms that alter cell-cycle progression. In the C33a cell line, the cells accumulate in the G2/M phase of the cell cycle, whereas in the SiHa cell line, they stop cycling together after the addition of AAPFCMK, with a small portion of the cells undergoing apoptosis.

During the course of this study, three different lots of both AAPFCMK and FBS were used. The level of growth inhibitory activity in each lot of AAPFCMK was standardized by determining the concentration of AAPFCMK required for at least 85% growth inhibition, as a percentage of control after three drug treatments or 144 h after the initial drug treatment, in both cell lines (referred to as the growth inhibitory dose or 1.0 GI85). FBS has inherent antichymotrypsin activity, and previous experiments have demonstrated variability in the concentration and abundance of proteins in different lots of FBS (Zheng et al., 2006). Regardless of the lot of FBS used, no change in the 1.0 GI85 dose of AAPFCMK was observed (data not shown). Each lot of AAPFCMK in general produced a 1.0 GI85 dose at a concentration of approximately 100 μM, with the exception of one very-low-activity lot that produced a 1.0 GI85 dose at a concentration of 800 μM. We conclude that an unintended error was made in the manufacturing process of this one lot of AAPFCMK, which affected its level of chymotrypsin-specific inhibitory activity. Nevertheless, the absolute concentration of AAPFCMK required for the growth inhibitory dose differed for each lot of drug, the concentration of drug required for inhibition was always the same for both cell lines.

Gene expression analyses have shown that HPV-positive cells from cervical cancers and from head and neck cancers have cell-cycle pathways that are deregulated in mechanistically different ways compared with HPV-negative cancer cells of the same tissue type (Pyeon et al., 2007). In synchronized C33a cells, after AAPFCMK treatment, we observed a significant accumulation of cells in the G2/M phase of the cell cycle relative to vehicle control (Fig. 2A; Table 1). This G2/M phase accumulation was not accompanied by a significant effect on DNA synthesis (Fig. 2B) or an increase in apoptosis (Fig. 1B). In contrast to C33a cells, synchronized SiHa cells showed no significant difference in the distribution of cells in each cell-cycle phase between the AAPFCMK-treated and control groups (Fig. 4A). However, in unsynchronized SiHa cells, we observed a significant decrease in the rate of cell progression through S phase after AAPFCMK treatment that is both time- and dose-dependent (Fig. 4B). The significant decrease in the rate of progression through S phase, combined with no accumulation in one phase of the cell cycle, demonstrated that the SiHa cells undergo a global arrest of the cell cycle after the addition of AAPFCMK. After removal of AAPFCMK, the SiHa cells resumed a normal growth rate (data not shown); however, a dose-dependent increase in late apoptotic cells, at 96 h after initial drug treatment, is observed (Fig. 3B).

SiHa cells contain integrated HPV-16, and continued expression of the E6 and E7 viral oncoproteins is necessary for their continued growth (Jones and Münger, 1996). In an important function, the E6 and E7 proteins bind to and trigger degradation of two crucial tumor suppressors, p53 and retinoblastoma (pRb), respectively (Hebner and Laimins, 2006). SiHa cells exhibit low levels of wild-type p53 and hypophosphorylated pRb, the form known to inhibit replication (Scheffner et al., 1991). C33a cells exhibit high levels of mutant p53. The hypophosphorylated pRb can be detected in C33a cells, but it also appears in an inactive mutated form (Scheffner et al., 1991). Although both the p53 and pRb proteins are inactivated in both cell lines, the pathways of inactivation are distinct. Therefore, the genetic differences between the two cell lines may be a potential reason for the differential response to the inhibitor. Alternatively, the mechanism by which AAPFCMK inhibition occurs may depend on the HPV status of the cell line. Future experiments are needed to distinguish between these two models.

Cellular proteases are involved in the control of many functions, such as replication, cycle progression, and death (Turk, 2006). Because many proteases have been shown to be overexpressed in cancer, protease inhibitors have become strong candidates for use in cancer therapeutics and are also being developed as antiviral therapies (Turk, 2006). Serine protease inhibitors, such as the Bowman-Birk Inhibitor (which has mainly chymotrypsin-specific activity), have been in clinical trials for their anticarcinogenic activity against oral cancer (Turk, 2006). A phase IIa clinical trial on oral leukoplakia, with use of Bowman-Birk Inhibitor Concentrate, showed a complete or partial reduction in the treated lesions, a reduction in protease activity, and no detectable side effects (Armstrong et al., 2000). The Hepatitis C NS3/4a serine protease is also targeted by selective serine protease inhibitors in clinical trials (Abbenante and Fairlie, 2005; Fear et al., 2007). In our study, the inhibitory activity of the chymotrypsin-like serine protease inhibitor AAPFCMK highlights its ability to affect cells that are tumorigenic with or without integrated HPV DNA.

It is also recognized that protease inhibitors can have pleiotropic targets within the cell (Turk, 2006). AAPFCMK was initially shown to be relatively selective for a nuclear protease; however, a number of nonprotease targets have subsequently been identified (Dhamne et al., 2007). Therefore, mechanisms in addition to protease inhibition may be responsible for the growth inhibition observed in this study. Future research is necessary to examine the potential of AAPFCMK as a therapeutic agent or as part of an individually tailored chemotherapeutic cocktail in HPV-positive carcinomas. Understanding the mechanisms of growth inhibition with AAPFCMK in HPV-positive cells should enhance anti-HPV therapeutics, and we hypothesize that expression of HPV E6/E7 proteins in differentiated cells may preferentially alter susceptibility of cells to AAPFCMK effects. The prevalence of HPV in multiple types of cancers and the current negative repercussions of treatment make finding novel and affordable therapies a high priority.
Acknowledgments

We thank Nate Sheaffer and Dave Stanford (Cell Science/Flow Cytometry Core Facility, Section of Research Resources, Pennsylvania State University, College of Medicine, Hershey, PA) for help with the flow cytometry work and Neil Christensen and Craig Meyers for critical reading of the manuscript.

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Cytometry Core Facility, Section of Research Resources, Pennsylvania State University, College of Medicine, Hershey, PA) for help with the critical reading of the manuscript.

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