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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ABSTRACT
The chymotrypsin-like serine protease inhibitor, succinyl-alanine-alanine-proline-phenylalanine chloromethyl ketone (AAPF<sub>CMK</sub>), 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 AAPF<sub>CMK</sub> 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. AAPF<sub>CMK</sub> 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 AAPF<sub>CMK</sub> differ between the HPV16-positive and HPV-negative tumorigenic cell lines. Drug-treated C33a cells showed a significant accumulation of cells in the G<sub>2</sub> phase of the cell cycle. In SiHa cells, growth inhibition produced by AAPF<sub>CMK</sub> seemed to result from a global arrest of the cell cycle. Although the molecular mechanisms involved in AAPF<sub>CMK</sub>-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 (Muñoz 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
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 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 nuclear protease target of AAPF<sub>CMK</sub> (Dhamne et al., 2007). A number of additional nuclear nonprotease targets were also identified in our initial studies; these included helicases, cohesion complex components, and nuclear scaffolding proteins (Dhamne et al., 2007). Understanding how AAPF<sub>CMK</sub> works at a molecular level is an integral part of elucidating possible treatment options, including its prospect as a topical treatment for HPV.

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 suspended 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 (EMEM) + 10% fetal bovine serum (FBS). The G1/S cell-cycle inhibitor mimosine was obtained from Sigma-Aldrich and dissolved at a stock concentration of 10 mM in EMEM without PBS (American Type Culture Collection, Manassas, VA). Staurosporine (Sigma-Aldrich) is an inducer of apoptosis, 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>/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 effects of AAPF<sub>CMK</sub> on apoptosis induction, cell-cycle progression, and protein synthesis were determined using a multiparameter FACScan flow cytometer (BD Biosciences, San Jose, CA). DNA histograms were used to assess DNA content, and the percentage of cells in each phase of the cell cycle was calculated. 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>). Differences in AAPF<sub>CMK</sub> concentrations 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.

**Apoptosis 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, Mountview, CA, Australia). 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>/EGR<sub>CMK</sub> or an equal 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 determined by two-way ANOVA with 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)

**Fig. 1.** AAPFCMK induced growth inhibition in a time- and dose-dependent manner without causing apoptosis of C33a cells. A, C33a cells were treated with the indicated doses of AAPFCMK (1.0 GI85 is the concentration of AAPFCMK required for at least 85% growth inhibition, as a percentage of control, 144 h after the initial drug treatment), an equivalent dose of the trypsin-specific controls, GRcmk and EGRcmk, and the DMSO vehicle control. Viable cells were determined by manual counting and trypan blue exclusion at the indicated times. 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 control. B, percentage of late apoptotic C33a cells treated with DMSO, staurosporine (stauro) at 24 h only, or an AAPFCMK GI85 dose of 0.25, 0.50, 0.75, or 1.0 and analyzed by flow cytometry at 12, 24, and 36 h after initial drug/vehicle treatment. Data represent the mean ± S.D. of three independent experiments.
and 0.50 GI85 (p < 0.001) (Fig. 1A). From the untreated growth curve, we estimated that C33a cells doubled every 18 h. After removal of AAPFCMK, C33a cells resumed normal growth rates (data not shown).

No Apoptosis Induced in C33a Cells by AAPFCMK. To determine whether the observed growth inhibition is caused by apoptosis, the cells were treated with multiple concentrations of AAPFCMK 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 AAPFCMK-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).

G2/M Phase Accumulation in C33a Cells by AAPFCMK. C33a cells were treated with 0.5 mM mimosine to synchronize the cells in G1/S phase. The 24-h mimosine treatment resulted in 62% of the cells in G1 phase and 38% in S phase. The synchronized cells were released, treated with vehicle, EGRCMK, or AAPFCMK, and collected at 12, 18, 24, and 36 h. A highly significant increase in the cells accumulating within the G2/M phase of the cell cycle was seen after treatment with the 1.0 GI85 dose of AAPFCMK (p < 0.001, two-way ANOVA; Bonferroni post test), whereas an equivalent dose (100 μM) of the trypsin-specific inhibitor, EGRCMK, showed no significant difference from the control group (Fig. 2A; Table 1). Treatment with 0.25 or 0.50 GI85 doses of AAPFCMK did not significantly alter the cell cycle (data not shown).

Fig. 2. AAPFCMK induced G2/M cell-cycle accumulation in C33a cells. A, flow cytometry cell cycle data were analyzed by ModFit LT 3.0 by setting DNA content at 2N for G1 and 4N for G2, represented by the two filled peaks, with the stripped peak in between corresponding to S phase. Each histogram represents data from one independent experiment. B, 12-h incorporation of BrdU in cells treated with DMSO or an AAPFCMK GI85 dose of 0.25, 0.50, 0.75, or 1.0 showed no significant difference from the vehicle control. Data represent the mean ± S.D. of three independent experiments, each performed in triplicate.
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).

**Table 1**

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<th>Time (h)</th>
<th>Vehicle</th>
<th>100 μM EGR&lt;sub&gt;CMK&lt;/sub&gt;</th>
<th>1.0 GI&lt;sub&gt;85&lt;/sub&gt; AAPFCMK</th>
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<td>12</td>
<td>70 ± 4</td>
<td>72 ± 2</td>
<td>68 ± 5</td>
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<td>18</td>
<td>25 ± 7</td>
<td>27 ± 4</td>
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<td>24</td>
<td>25 ± 9</td>
<td>26 ± 9</td>
<td>46 ± 4&lt;sup&gt;***&lt;/sup&gt;</td>
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<td>36</td>
<td>10 ± 8</td>
<td>14 ± 2</td>
<td>47 ± 8&lt;sup&gt;***&lt;/sup&gt;</td>
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<sup>*** p < 0.001</sup>

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<sub>CMK</sub>, or vehicle control. At 24, 48, 72, and 96 h after initial 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/drug and media every other day.

**Fig. 3.** AAPFCMK induced growth inhibition in a time- and dose-dependent manner and dose-dependent apoptosis in SiHa cells. A, SiHa cells were treated with the indicated doses of AAPFCMK (1.0 GI<sub>85</sub> is the concentration of AAPFCMK required for at least 85% growth inhibition, as a percentage of control, 144 h after the initial drug treatment), an equivalent dose of the trypsin-specific controls, GR<sub>CMK</sub> and EGR<sub>CMK</sub>, and the DMSO vehicle control. Viable cells were determined by manual counting and trypan blue exclusion at the indicated times. 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 control.

**Fig. 4.** Percentage of late apoptotic SiHa cells treated with DMSO, staurosporine (stauro) at 24 h only, or 0.25, 0.50, 0.75, or 1.0 GI<sub>85</sub> AAPFCMK and analyzed by flow cytometry at 24, 48, 72, and 96 h after initial drug/vehicle treatment. The cells were retreated 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_CMK, we observed no significant change in the cell-cycle distributions of SiHa cells treated with AAPF_CMK relative to the vehicle control (Fig. 4A). The equivalent dose (100 μM) of EGR_CMK also showed no significant difference from the control group (Fig. 4A).

BrdU incorporation was measured to determine whether AAPF_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_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_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_CMK, using HPV-16-positive (SiHa) or HPV-negative (C33a) human tumorigenic cervical cell
AAPF<sub>CMK</sub> 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, GR<sub>CMK</sub> and EGR<sub>CMK</sub>, had no significant impact on cellular growth in either cell line. However, the trypsin-specific inhibitory activity of each lot of GR<sub>CMK</sub> and EGR<sub>CMK</sub> was unknown. To understand the mechanism behind this drug-induced growth inhibition, we examined the effects of AAPF<sub>CMK</sub> on apoptosis induction, cell-cycle progression, and DNA synthesis in both cell lines. Overall, our data indicate that AAPF<sub>CMK</sub> 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 altogether after the addition of AAPF<sub>CMK</sub>, with a small portion of the cells undergoing apoptosis.

During the course of this study, three different lots of both AAPF<sub>CMK</sub> and FBS were used. 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 (referred to as the growth inhibitory dose or 1.0 GI<sub>50</sub>). 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 GI<sub>50</sub> dose of AAPF<sub>CMK</sub> was observed (data not shown). Each lot of AAPF<sub>CMK</sub> in general produced a 1.0 GI<sub>50</sub> dose at a concentration of approximately 100 μM, with the exception of one very-low-activity lot that produced a 1.0 GI<sub>50</sub> dose at a concentration of 800 μM. We conclude that an unintended error was made in the manufacturing process of this one lot of AAPF<sub>CMK</sub>, which affected its level of chymotrypsin-specific inhibitory activity. Nevertheless, although the absolute concentration of AAPF<sub>CMK</sub> 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 AAPF<sub>CMK</sub> 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 AAPF<sub>CMK</sub>-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 AAPF<sub>CMK</sub> 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 AAPF<sub>CMK</sub>. After removal of AAPF<sub>CMK</sub>, 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 AAPF<sub>CMK</sub> 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 leukoplasia, 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 AAPF<sub>CMK</sub> 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). AAPF<sub>CMK</sub> 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 AAPF<sub>CMK</sub> as a therapeutic agent or as part of an individually tailored chemotherapeutic cocktail in HPV-negative carcinomas. Understanding the mechanisms of growth inhibition with AAPF<sub>CMK</sub> 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 AAPF<sub>CMK</sub> 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.
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References


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