Inhibitors of Epidermal Growth Factor Receptor Kinase and of Cyclin-dependent Kinase 2 Activation Induce Growth Arrest, Differentiation, and Apoptosis of Human Papilloma Virus 16-immortalized Human Keratinocytes

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ABSTRACT

Human papilloma virus 16 (HPV 16) is associated with cervical cancer and is therefore considered a major health risk for women. Immortalization of keratinocytes induced by HPV infection is largely due to the binding of p53 and Rb by the viral oncoproteins E6 and E7, respectively, and is driven to a large extent by a transforming growth factor α/amphiregulin/EGFR autocrine loop. In this study, we show that the growth of HPV 16-immortalized human keratinocytes can be blocked by a selective epidermal growth factor receptor kinase inhibitor, AG 1478, and by AG 555, a blocker of cyclin-dependent kinase 2 (Cdk2) activation. AG 1478 induces a massive increase in the Cdk2 protein inhibitors p27 and p21, whereas AG 555 appears to have a different mechanism of action, inhibiting the activation of Cdk2. Growth arrest induced by AG 1478 and AG 555 is accompanied by up to 20% of cells undergoing apoptosis. Following AG 1478 treatment but not AG 555 treatment, up to 50% of cells undergo terminal keratinocyte differentiation as determined by filaggrin expression and by the decline in the expression of cytokeratin 14. The growth-arresting properties of AG 1478 and AG 555 identifies them as possible lead antipapilloma agents.

INTRODUCTION

Papilloma viruses cause benign skin and mucous proliferation (1, 2). Increasing evidence shows that the infection of the cervix by HPV3 16 and HPV 18 is involved in more than 85% of cervical cancer, which ranks second in cancer mortality in women worldwide (3–5). The transforming genes E6 and E7 are most probably responsible for immortalization of the infected cells (6–8). The E6/E7 gene products are barely detectable in basal epithelial layers of low-grade HPV 16-, 18-, or 33-induced squamous intraepithelial lesions compared to normal epithelium, are strongly transcribed in basal cells and throughout the undifferentiated epithelium (9, 10). As the grade of neoplasia increases, they become comparatively strongly transcribed in basal cells and throughout the undifferentiated epithelium (9–11). The viral E6 and E7 oncogenes bind the p53 and Rb tumor suppressor proteins, respectively, loosening cell cycle control, destabilizing the cellular genome, and inducing the immortalization of the infected cells. These cells as well as cervical carcinoma cell lines, contrary to normal epithelium, are stimulated to proliferate by tumor necrosis factor α or interleukin 1, suggesting that proinflammatory cytokines provide a selective growth advantage for abnormal epithelial cells in vivo, thus contributing to carcinogenesis (12, 13). HPV 16-immortalized epithelial cells have also been reported to be driven by the transforming growth factor α/amphiregulin/EGFR autocrine loop (12). Because tyrphostins arrest the growth of normal keratinocytes with no adverse toxic effects (14, 15), we decided to examine their effects on HPV 16-immortalized keratinocytes. We report on the essentially irreversible inhibition of the growth of HPV 16-immortalized keratinocytes by the selective EGFR blocker AG 1478 (16–18) and by AG 555, a tyrphostin that blocks Cdk2 activation, and examine their biological and biochemical effects.

MATERIALS AND METHODS

Materials. Anti-EGFR external domain (clone F4) monoclonal antibody (Boehringer Mannheim), antiphosphotyrosine monoclonal antibody PT-66 (Sigma Chemical Co.), and goat antimouse fluorescent antibody (The Jackson Laboratory) were used to monitor the levels of EGFR and its state of tyrosine phosphorylation. Primary antifilaggrin antibody (Biomedical Technologies Inc., Stoughton, MA) with secondary FITC-conjugated goat antimouse antibody (Sigma) was used for staining differentiated keratinocytes. Fetal bovine serum was from Life Technologies, Inc. (Grand Island, NY); tissue culture media antibiotics and trypsin-EDTA solution were from Biological Industries, Beit Haemek, Israel; and tissue culture reagents and growth supplements were from Sigma.

The HF-1 Line HPV 16-immortalized Keratinocytes. We have introduced the entire HPV 16 genome in secondary cultures of human foreskin keratinocytes from a 7-year-old boy. Five × 10^6 cells in 0.5 KM (19) were transfected with 10 μg of PML 2 plasmid containing the entire genome cloned at the BamHI site (20). Thereafter, the cells were seeded in 0.5–1 cm^2 flasks and incubated at 37°C for 24 h for 5 days with new medium containing the tyrphostin. After the fifth treatment (8–9 days after plating), the medium was removed, and a new

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3 The abbreviations used are: HPV, human papilloma virus; EGFR, epidermal growth factor; EGR, EGFR receptor; Cdk2, cyclin-dependent kinase 2; KM, keratinocyte medium; sKM, starvation KM; DAPI, 4',6-diamidino-2-phenylindole; FAC5C, fluorescence-activated cell sorting.
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Fig. 1. Growth of HPV 16-immortalized HF-1 keratinocytes compared to normal human keratinocytes. The cells were plated in KM at 2 x 10^5 cells/25 mm-culture dish or on the feeder layer of irradiated 3T3 cells, and the medium was replaced every 3 days. At the indicated times, cells were trypsinized, and cell numbers were determined by counting in hemocytometer. Points, means of duplicate determinations.

medium without tyrphostin was added. The cultures were grown for another 7 days (12 days from the beginning of tyrphostin treatment and 14–15 days since seeding). Medium was changed every 3 days. AG 1478 inhibits EGFR autophosphorylation in EGFR overexpresser cells with IC50 values of 2–900 nM (16–18) and blocks the growth of EGFR overexpressers with IC50 values between 0.1 and 10 μM similarly to PD 153035, which is the bromine analogue of AG 1478 (24). AG 555, like its close homologue AG 494 (22, 23), arrests EGF-dependent DNA synthesis at 20 μM but does not block EGFR autophosphorylation in intact cells (23 and this study).

**Tyrophostins and Treatment of Cells.** The synthesis of AG 555 and AG 1478 is described elsewhere (18, 25). Stock solutions were 10 mM in DMSO. For the experiments, the tyrphostins were diluted into the KM with 10% fetal bovine serum (Life Technologies). The following concentrations were examined: 1, 5, 10, and 50 μM.

Cells were grown in KM and in KM with DMSO. The concentration of DMSO in the controls was equal to the concentration in the tyrphostin containing KM. For each tyrphostin concentration the appropriate DMSO concentration was taken as 100%. For each concentration, the control was taken as 100% growth. It can be seen that even 0.5% DMSO had no significant effect on cell growth, whereas 1.0% had a very small effect (not shown). The highest DMSO concentration used in this study was 0.5%.

**Western Blot Analysis for the EGFR and Phosphotyrosine.** HF-1 cells were seeded 5 x 10^5 cells/35-mm plate in KM. After 2–3 days, the cells were washed and fed with sKM (without serum and EGF, sKM) and starved for 48 h. Tyrphostin in sKM at the appropriate concentration was added for 4 h, and then the cells were stimulated with 30 ng/ml EGF for 10 min. The reaction was stopped by placing the culture on ice and washing it with PBS. Whole cells were lysed with hot SDS-PAGE sample buffer, scraped, boiled for 5 min, then run on 7% SDS gel for 4 h, transferred to nitrocellulose paper, and incubated overnight at 4°C with either monoclonal mouse antihuman EGFR antibodies (0.5 mg/ml) or, for phosphotyrosine-containing proteins, with monoclonal antiphosphotyrosine antibody PT-66, following the manufacturer’s recommendations. Fluorescent goat antimouse second antibody was then added for a 2-h incubation at room temperature (1 ml/20 ml). After drying, the gels were concentration, for 10 min at room temperature. After washing, the microplates were stained with 0.1% methylene blue in 0.1 M borate buffer (pH 8.5) for 60 min at room temperature. Thereafter, the plates were extensively and rigorously washed to remove excess dye and then dried. The dye taken up by cells was eluted in 0.1 N HCl for 60 min at 37°C and read at 620 nm.

Titration experiments revealed linear readings for 1 x 10^4 to 4 x 10^4 cells/well. Each point of the growth curve experiments is calculated from 15 wells.

**Calculation of Growth Inhibition.** For each tyrphostin concentration used, the appropriate KM containing only DMSO was used for the control. Thus, for each concentration, the control was taken as 100% growth. It can be seen that even 0.5% DMSO had no significant effect on cell growth, whereas 1.0% had a very small effect (not shown). The highest DMSO concentration used in this study was 0.5%.

4 R. Karni and A. Levitzki, unpublished observations.
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Fig. 2. The inhibitory effect of tyrphostins AG 1478 and AG 555 on the proliferation of HF-1 cells. HF-1 cells (3000 cells/well) were grown in culture for 2–3 days prior to the beginning of treatment. For 5 days, cells were grown in medium containing tyrphostin, and then growth was continued in the absence of the compound (as described in "Materials and Methods"). Cell growth was determined by the automated microculture methylene blue assay (A and B), and the percentage of growth inhibition was calculated from it (C and D). Data are means; bars, SE.

exposed to X-ray films in cassettes to visualize the EGFR or phosphotyrosine-containing proteins. Identical procedures were used to examine the level of EGFR and its state of phosphorylation after prolonged treatment with AG 1478 and AG 555.

Cdk2 Activity in HF-1 Cells. Cells (1–2 × 10⁶) were lysed in 1 ml of cold lysis buffer [50 mM HEPES (pH 7.4); 250 mM NaCl; 5 mM DTT; 0.25% NP-40; 10 mM NaF; and the following protease inhibitors: 10 µg/ml leupeptin, aprotinin, soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride]. Lysates were centrifuged 300,000 × g for 10 min. Supernatants were transferred, and samples containing 600–1000 µg protein were incubated in the presence of 1–2 µg anti-Cdk2 antibodies (SC-163; Santa Cruz Biotechnology). The immunocomplex was collected on protein A agarose beads (50 ml of 10% suspension) for 1 h, washed three times in lysis buffer, and separated into aliquots containing one-third or two-thirds of the bead immunocomplex. The smaller aliquots were washed a fourth time in kinase buffer (50 mM HEPES, 10 mM MgCl₂, 1 mM DTT, and 1 µM nonradioactive ATP). All buffer was then aspirated from the beads, and the reaction was initiated by the addition of a 40 µl/sample reaction mixture [kinase buffer + 10 µl [γ-³²P]ATP + 2.5 µg histone H1 (freshly prepared)]. The reaction was performed for 20 min at 30°C and then stopped by addition of 13.5 µl/assay 4X Laemmli sample buffer. Samples were separated on 10% SDS-PAGE gels and exposed to film for various durations.

Levels of Cdk2, p21, p27, and Cyclin A. Cells (2 × 10⁶) were lysed and assayed for protein. Equal amounts of protein were subjected to immunoprecipitation with 2 µg/sample anti-Cdk2 (SC-163; Santa Cruz) as described earlier. The blot serving for Sections B, C, and D (Fig. 7) was cut to strips encompassing the following molecular weights: M₁ 17,000–25,000, M₂ 25,000–30,000, and M₃ 30,000–46,000, respectively. These strips were treated with anti-p21 (SC-397; Santa Cruz), anti-p27 (K25020, 1:1000; Transduction Laboratories), and anti-Cdk2 (SC-163 1:2500; Santa Cruz), respectively. The levels of cyclin A in the complex were determined using antibodies obtained from Dr. D. Resnitzky (1:1000).

Immunoblot Analysis. Aliquots containing two-thirds of the bead suspension were used following buffer aspiration. The immunocomplexes were released in warm sample buffer (37°C, 10 min), and samples were run on SDS-PAGE 12–17% gradient gels, transferred to nitrocellulose LKB Multiphor II semidry blot electrophoresis unit, and blocked for 45 min in TBST [Tris-buffered saline (pH 7.5), 0.2% Tween 20, and 5% low-fat milk]. The blots were probed with different antibodies, as described in each figure legend (Figs. 7–9), for 2 h at 4°C in TBST; washed three times in TBST; reprobed with horseradish peroxidase conjugated to protein A (1:4000) or with horseradish peroxidase conjugated to goat antirabbit or goat antimouse antibody (1:20,000); and washed three times in TBST. The enhanced chemiluminescence reaction was then performed.

Indirect Immunofluorescence Staining for Filaggrin. For localization of filagrin, the cells were dispersed with trypsin-EDTA solution; 0.1 ml of the solution, containing 1 × 10⁶ cells, was stained in suspension, with antifilagrin antibody for 60 min at room temperature and with a second FITC-conjugated goat antimouse IgG for 30 min. The binding capacity (percentage of cells binding a given antibody), fluorescent intensity, and distribution pattern were examined with a UV microscope (Zeiss, Jena, Germany) and a confocal microscope (Sarastro, Phorbos 1000; Molecular Dynamics, Sunnyvale, CA).

The Effect of Anti-EGFR Antibodies on HF-1 Growth. Cells were plated at 1500 and 3000 cells/well in KM without EGF and cholera tox
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Results

HPV 16-immortalized Keratinocytes versus Normal Keratinocytes. The HPV 16-immortalized keratinocytes (HF-1 cells) are insensitive to density limitation and grow continuously in serial culture (Fig. 1). Normal keratinocytes, on the other hand, show a limited growth potential, and when confluence is reached they stop dividing and die (Fig. 1; see also Ref. 19).

Inhibition of Cell Growth by AG 1478 and AG 555. We have reported previously that tyrphostins block the growth of normal keratinocytes (14) and of psoriatic keratinocytes (15). We therefore tested tyrphostins representing a number of families for their potency to block the proliferation of human keratinocytes immortalized by HPV 16 (HF-1 cells). The two most potent blockers were found to be tyrphostin AG 1478, a selective EGFR kinase blocker (16-18) and AG 555, which we find blocks Cdk2 activation but has no effect on EGFR autophosphorylation in intact cells (Ref. 23 and see below). When cells are treated continuously with the inhibitor, maximal growth-inhibitory effect is observed after 5 days when the drug is washed out. Cessation of treatment at day 5 by AG 1478 results in complete recovery when treatment was performed with 1.0 \( \mu M \) drug, but only 50% of the cells recovered after treatment with 10 \( \mu M \) AG 1478. Exposure to 50 \( \mu M \) AG 1478 resulted in complete growth arrest with no recovery after cessation of treatment at day 5 (Fig. 2, A and C, day 12). AG 555 caused complete suppression of growth of HF-1 cells, and no regain of growth was observed subsequent to treatment with 10 or 50 \( \mu M \). Up to 20% inhibition was observed with 1 \( \mu M \) AG 555 (Fig. 2, B and D). Rescue experiments in which the treated cells were trypsinized and replanted confirm these observations (not shown). After treatment with 1 \( \mu M \) AG 1478 and drug wash, complete rescue was obtained, whereas only 50% of the plated cells regained their proliferative capacity after treatment with 10 \( \mu M \) AG 1478. After treatment with 10 or 50 \( \mu M \) AG 555 replated cells attach but remain arrested and do not regain their proliferative capacity at all (data not shown). Fig. 3 depicts the morphological changes that occur in the cells as a result of AG 1478 and AG 555 treatment. It can be seen that AG 1478 treatment does not result in cell death and that the treatment by AG 555 results in cell shrinkage and cell death.

Points of Arrest in the Cell Cycle and Apoptosis. AG 1478 treatment causes time-dependent accumulation of the cells in the G1 phase of the cell cycle with a concomitant reduction in the number of cells at the G2-M and S phases of the cell cycle (Fig. 4A). Cessation of treatment after 5 days leaves the cells arrested mainly at G1 with up to 15% of cells undergoing apoptosis (Figs. 4A and 5B). AG 555 treatment for 5 days results in cell cycle arrest in G1 and in S with up to 15–20% apoptotic cells (Figs. 4B and 5C).

Terminal Differentiation. The arrested cells that do not undergo apoptosis remain arrested and cannot be induced to divide by replating. We therefore tested the possibility that these cells are terminally differentiated. Indeed, AG 1478-treated cells exhibit increased filaggrin expression and decreased expression of cytokeratin 14 (Table I; Fig. 6), indicating terminal differentiation. A decrease in cytokeratin

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Footnote:

5. H. Ben-Bassat and A. Levitzki, unpublished observations.
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Fig. 4. The effect of AG 1478 and AG 555 tyrphostins on the cell cycle of HF-1 cells. The DNA content of the cells from the treated and untreated cultures was analyzed by FACS analysis as described in "Materials and Methods." Analysis was performed after 5 days of treatment. Other details are given in "Materials and Methods." A, treatment with 10 μM AG 1478. B, treatment with 50 μM AG 555. Data are means; bars, SE.

14 (which stains basal epithelial cells) was also observed mainly on day 8 and was observed for AG 1478 and much less with AG 555 (Table 1). This finding demonstrates that at least a fraction of the cells that do not apoptose are driven by AG 1478 but not by AG 555 to terminal differentiation. These growth-arresting properties of AG 1478 and AG 555 prompted us to examine in more detail the biochemical effects of these two potent blockers to understand better their biological effects.

Biochemical Activities of AG 1478 and AG 555. Treatment of HF-1 cells with AG 1478 results in a dose-dependent inhibition of EGFR autophosphorylation (Fig. 7B) but not in the expression of the EGFR (Fig. 7C). It can be seen that HF-1 cells overexpress EGFR as compared to normal keratinocytes (Fig. 7A). AG 555, in contrast to AG 1478, has no effect on EGFR autophosphorylation (Fig. 7A), although AG 555 is a very potent growth inhibitor (Figs. 2 and 4). For the experiment depicted in Fig. 7, cells were washed extensively to diminish the basal autophosphorylation, which results from the autocrine stimulation (see below). Furthermore, to observe the phosphorylated EGFR in normal keratinocytes, in the present experiment we have used 4 × 10⁵ cells per gel lane as compared to 2.5 × 10⁴ cells per lane for HF-1 cells (Fig. 7A). Neither AG 1478 nor AG 555 has any effect on the high level of expression of the EGFR (Fig 7C).

Fig. 7D shows that HF-1 cells show significant phosphorylation of the receptor even in the absence of added EGF and that the level of phosphorylation is enhanced greatly in the presence of the ligand. These findings strongly support the significant role that the autocrine stimulation of the EGFR in HF-1 cells plays, confirming earlier observations on the role of EGFR autocrine growth stimulation of HPV 16-immortalized keratinocytes (26, 27). In both
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kinase activity of Cdk2 shows that AG 1478 induces a complete shutoff of Cdk2 activity and AG 555 induces a significant decrease in enzyme activity after a 5-day treatment (Fig. 8A). After 3 days of recovery subsequent to the 5-day treatment, Cdk2 remains completely inactive for AG 1478-treated cells and recovers only to a small extent in the AG 555-treated cells (Fig. 8), but growth is not resumed (Fig. 2 and 3). The amount of cyclin A associated with Cdk2 subsequent to treatment with AG 1478 or AG 555 remains essentially unchanged (Fig. 9). Reduction of Cdk2 activity does not result from the direct inhibition of Cdk2 kinase activity, because neither compound blocks Cdk2 activity when directly added to the kinase reaction (Fig. 8E). The level of the Cdk2 protein is reduced somewhat in AG 1478 in 5 day-treated cells but is unaltered in AG 555-treated cells (Fig. 8B). Three days after cessation of treatment, the level of Cdk2 remains essentially unchanged for AG 555-treated cells but increases in AG 1478-treated cells (Fig. 8B). After a 5-day treatment, the level of p27 is high for AG 1478-treated cells and even higher for AG 555-treated cells (Fig. 8C). After 7 days of recovery, the level of p27 remains very high for AG 1478-treated cells, whereas AG 555-treated cells express lower levels of p27. Untreated (control) cells that become confluent at that time point (12 days) also express high levels of p27 (Fig. 8C), and Cdk2 activity is diminished significantly (Fig. 7A). p21 is only expressed significantly by AG 1478-treated cells 3 and 7 days after cessation of treatment. In summary, AG 1478-treated cells express much higher levels of the Cdk2 inhibitors p21 and p27 than AG 555-treated cells (Fig. 8). This may explain the complete absence of Cdk2 kinase activity in AG 1478-treated cells as compared to some activity in AG 555-treated cells after a 3- or 7-day recovery (Fig. 8A).

Because AG 555 does not block EGFR kinase activity (Fig. 7A) but inhibits Cdk2 activation (Fig. 8A), it seems that its mechanism of action is different from that of AG 1478. In contrast to AG 1478, the severe decline in Cdk2 activity cannot be attributed exclusively to the high levels of p27 (Fig. 8).

AG 555 seems therefore to inhibit a yet-unidentified step regulating Cdk2 activation, because it has no direct effect on the intrinsic kinase activity of the enzyme (Fig. 8E) and has no effect on the association of the enzyme with its cyclins (Fig. 9). AG 555 seems to block a step downstream to the EGFR kinase in proximity to the biochemical processes that activate Cdk2. We have thus far been unable to identify its precise molecular target. These differences between AG 1478 and AG 555 probably account for the fact that AG 1478 induces terminal differentiation, whereas AG 555 does not (Fig. 6; Table 1; see also “Discussion”).

Neither AG 1478 nor AG 555 has any effect on the level of E6 or E7 RNA expression as measured by Northern blotting (data not shown).

Table 1 Increased filaggrin expression and decreased cytokeratin 14 expression in HF-1 cells treated with AG 1478 and AG 555a

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<th>Treatment</th>
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<td>AG 1478</td>
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a Cells were examined after 5 days of treatment with 10 μM AG 1478 or 10 μM AG 555 and after an additional 3 days of recovery (total 8 days from plating), as described in “Materials and Methods.” Filaggrin expression was scored by UV microscopy and cytokeratin 14 expression was scored by FACS. The variability between experiments does not vary more than 10–15%.
The Inhibition of HF-1 Cells by Anti-EGFR Antibodies. The strong inhibition of HF-1 cell growth by AG 1478 suggested to us that indeed EGFR plays a key role in HF-1 proliferation. We have therefore examined the effect of the EGFR antibody 225 on the growth of HF-1 cells. Fig. 10 shows that this antibody has a profound inhibitory effect on the growth of these cells. The inhibitory effect of the antibody is independent of added EGF, confirming that the EGFR in HF-1 cells is stimulated by an autocrinic mechanism. An antibody against amphiregulin (12) tested in parallel as a control is without effect on the growth of these cells (Fig. 10). Full-growth inhibition is achieved by AG 1478 as compared to the antibody (Fig. 10), most probably because the blocker is cell permeable through the membrane and can inhibit intracellular EGFR, whereas the antibody is unable to block the active intracellular receptors.

DISCUSSION

The results presented show that the selective EGFR kinase blocker AG 1478 (16, 17) blocks the growth of HPV 16-immortalized human keratinocytes (HF-1 cells). Growth arrest (Figs. 2 and 3) that occurs at G1 (Fig. 4) is accompanied by a decline of Cdk2 kinase activity, which is accompanied by a massive increase in the levels of the Cdk2 inhibitors p21 and p27 (Fig. 8) and a small decline in the levels of...
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Fig. 7. The state of the EGFR phosphorylation. A, level of EGFR phosphorylation in HF-1 cells and normal keratinocytes, the effect of AG 555. B, inhibition of EGFR autophosphorylation by AG 1478. C, effect of AG 1478 and AG 555 on EGFR level. The number of cells per lane were 4 X 10^4 for normal keratinocytes and 2.5 X 10^5 for HF-1 cells. A-C, cells were washed. D, level of receptor autophosphorylation in HF-1 cells as compared to normal keratinocytes. The number of cells per lane was 4 X 10^4 for normal keratinocytes and 2.5 X 10^5 for HF-1 cells. In D, cells were not washed as in A-C, making it possible to observe the basal autophosphorylation in the absence of added EGF.

Cdk2 (Fig. 9). The levels of cyclin A (Fig. 9) or E (not shown) associated with the enzyme are not changed significantly. Cell morphology due to growth inhibition by AG 1478 is normal, whereas in the AG 555-treated cells, growth arrest is accompanied by some rounding up. The key role that EGFR kinase plays in the growth of HF-1 cells is strongly supported by the finding that anti-EGFR antibodies also block the growth of these cells (Fig. 10). HF-1 cells, which harbor the HPV 16 genome, express a large number of EGFRs (Fig. 7C) and also express its ligands (26). This results in sufficient constitutive receptor phosphorylation (Fig. 7D; see also Ref. 27), which allows cell growth with little effect of externally added EGFR ligand. Antibodies to EGFR as well as the selective EGFR blocker AG 1478 can block the growth of these cells (Figs 2 and 10), which suggests that EGFR plays a key role in driving HF-1 cell growth. The inhibition by anti-EGFR antibodies is extensive even in the absence of externally added EGFR ligand. Antibodies to EGFR as well as the selective EGFR blocker AG 1478 can block the growth of these cells (Figs 2 and 10), which suggests that EGFR plays a key role in driving HF-1 cell growth. The inhibition by anti-EGFR antibodies is extensive even in the absence of added external EGFR ligand (Fig. 10). This is due, most probably, to the fact that the extent of receptor activation by the endogenously made ligands (Fig. 7D) is sufficient to sustain full growth for many generations (Fig. 1). Normal keratinocytes do not grow for many generations under the same experimental conditions (Fig. 1).

After prolonged in vitro progression (>100 passages), the HF-1 cells are able to proliferate in the absence of EGF for a limited period (data not shown).

AG 555, which does not inhibit EGFR kinase (Fig. 7) in the intact cells, blocks the activation of Cdk2 (Fig. 8) and induces cell cycle arrest of HF-1 at G_1 and S (Fig. 4). In this case, p27 levels is elevated transiently in treated cells, but the levels of Cdk2 kinase activity remains low (Fig. 8), and cell growth does not resume (Fig. 2). Thus, it seems that AG 555 blocks a step of the cell cycle machinery at the G_1-S early S zone, which is essential for Cdk2 activation and which is yet to be defined (28). AG 1478 and AG 555 induce similar, but not identical, biological phenotypes, although they act at different points of the signaling pathways regulating the proliferation of HPV 16-immortalized cells. This is, most probably, because their actions converge at the inhibition of the cell cycle machinery at G_1 and G_1/early S phases of the cell cycle, respectively. Both compounds arrest growth after 2—5 days of treatment. Whereas AG 1478 induces arrest exclusively at G_1 (Fig. 4), most probably because it blocks the EGFR (Fig. 7), which is the major growth-promoting receptor (Fig. 10),
AG 555 induces arrest at both G₁ and S phases of the cell cycle (Fig. 4), suggesting a more proximal effect of AG 555 to the activation of Cdk2. AG 1478 but not AG 555 induces a massive increase in p27 and p21 (Fig. 8), which is most probably correlated with its superiority over AG 555 in inducing differentiation (Fig. 6; Table 1). Also, the ratio of cyclin A to Cdk2 in the complex is reduced somewhat in AG 1478-treated cells but not in AG 555-treated cells (Fig. 9). Both inhibitors induce 10–20% of the cells to undergo apoptosis (Fig. 5), and up to 50% undergo terminal differentiation in AG 1478-treated cells, whereas the rest remain arrested without being able to resume growth (Fig. 6; Table 1). It is not yet clear why three patterns of fates are induced in these cells by the drugs applied. One possibility is that this heterogeneity in behavior reflects a biological microheterogeneity where the delicate balance between cell cycle progression, the apoptotic machinery, and the differentiation program is not identical in all cells. These aspects are currently under investigation. AG 555 induces persistent growth arrest at 10 and 50 μM with a very low level of differentiation, whereas 10 μM AG 1478 induces persistent growth arrest with a very high fraction of cells undergoing terminal differentiation (Fig. 6; Table 1). This difference most probably reflects the difference in the point of action of the two agents. AG 1478 blocks growth purely at G₁, whereas AG 555 induces growth arrest at G₁ closer to S and within S (Fig. 4). The point at which AG 555 acts may be late for allowing the differentiation program to set in. The molecular target of AG 555 has not been identified yet and is under investigation (28). The likelihood that AG 555 has a well-defined molecular target comes from the very precise struc-

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### Table 1: Treatment and Recovery

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### Fig. 8: The inhibition of Cdk2 activation by AG 1478 and AG 555 and levels of p21 and p27.

For experimental details, see "Materials and Methods." A. Inhibition of Cdk2 activation by 10 μM AG 1478 and 50 μM AG 555. B. Level of Cdk2 expression. C. Level of Cdk2 p27 expression. D. Level of Cdk2 p21 expression. E. Effect of AG 1478 and AG 555 on Cdk2 activity in the kinase assay.

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### Fig. 9: Cyclin A levels in the Cdk2 immunocomplex.

For experimental details, see "Materials and Methods." A. Levels of Cdk2 in the complex. B. Levels of cyclin A associated with Cdk2.
ture activity relationship that identifies the point of action in the cell cycle of AG 555 type compounds.6

Because AG 1478 and AG 555 induce their effects without general cytotoxicity, it make these compounds suitable candidates for the development of drugs against HPV 16 and HPV 18. The fact that these compounds also arrest the growth of normal keratinocytes (14, 15) without adverse toxicity but also have profound inhibitory effects on psoriatic keratinocytes has already qualified them for clinical trials for psoriasis. Indeed, SU 5271 (AG 1517), the bromoanalogue of AG 1478 that is identical to PD 153035 (24) has already entered clinical trials for psoriasis.

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