Tyrphostins That Suppress the Growth of Human Papilloma Virus 16-Immortalized Human Keratinocytes[1]

H. BEN-BASSAT, S. ROSENBAUM-MITRANI, Z. HARTZSTARK, R. LEVITZKI, M. CHAOUAT, Z. SHLOMAI, B. Y. KLEIN, N. KLEINBERGER-DORON, A. GAZIT, R. TSVIELI, and A. LEVITZKI

Laboratory of Experimental Surgery (H.B.-B., Z.H., R.L., M.C., Z.S., B.Y.K.), Unit for Molecular Biology, Hadassah University Hospital (S.R.-M., R.T.); Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem (N.K.-D., A.L.); and Department of Organic Chemistry, Institute of Chemistry, The Hebrew University of Jerusalem (A.G.), Jerusalem, Israel

ABSTRACT

Human papilloma virus 16 (HPV16) is considered to be the causative agent for cervical cancer, which ranks second to breast cancer in women's malignancies. In an attempt to develop drugs that inhibit the malignant transformation of HPV16-immortalized epithelial cells, we examined the effect of tyrphostins on such cells. We examined the effect of tyrphostins from four different families on the growth of HPV16-immortalized human keratinocytes (HF-1) cells. We found that they alter their cell cycle distribution, their morphology, and induce cell death by apoptosis. The effects of tyrphostins on HF-1 cells are different from their effects on normal keratinocytes. Growth suppression by AG555 and AG1478 is accompanied by 30% apoptosis in HF-1 cells, but this is not observed in normal keratinocytes. Tyrphostin treatment produces distinctive morphological changes in HF-1 cells and in normal keratinocytes; however, the culture organization of normal keratinocytes is less disrupted. These differential effects of the tyrphostins on HPV16-immortalized keratinocytes compared with their effects on normal keratinocytes suggests that these compounds are suitable candidates for the treatment of papilloma. Previous and present results indicate that group 1 tyrphostins, which inhibit Cdk2 activation, and group 2 tyrphostins, represented by AG1478, a potent epidermal growth factor receptor kinase inhibitor, induce cell cycle arrest; and, in the case of HF-1 cells, apoptosis and differentiation. Cells accumulate in the G_1 phase of the cell cycle at the expense of S and G_2 + M. These compounds block the growth of normal keratinocytes without inducing apoptosis or differentiation, causing them to accumulate in G_1. AG17, which belongs to group 4, exerts its antiproliferative effect mainly by increasing the fractions of cells in G_1 with a concomitant decrease in the fraction of cells in S and G_2 + M.

Carcinoma of the cervix remains one of the most common malignancies in women (Braly, 1996). After breast cancer, it is the second most common malignancy in both incidence and mortality (Parker et al., 1996). Cervical cancer is unique in that it is the first major solid tumor that has been shown to be virally induced in essentially every case (Howley, 1991; Zur Hausen, 1994). Human papilloma virus (HPV) DNA is found in virtually all cervical cancers and precursor lesions (Sha and Howley, 1990). Epidemiological studies indicate that HPV infection is the major risk factor of squamous intraepithelial lesions and invasive cancers (zur Hausen, 1991, 1994). Of the 70 types of HPV, only 23 have been found to infect the uterine cervix, the high-risk types being 16, 18, 31, and 35 (Sha and Howley, 1990; zur Hausen, 1994). The viral E6 and E7 oncoproteins bind p53 (Werness et al., 1990) and the Rb (Dyson et al., 1989) proteins, respectively, induc-

ing immortalization of the cells. The HPV16 E5 oncogene is unable to transform keratinocytes by itself, but it increases the efficiency of cell immortalization by E6/E7 (Stopler et al., 1996). The E5 protein inhibits the down-regulation of epidermal growth factor receptor (EGFR) in the presence of the ligand, resulting in a 2- to 5-fold increase in the steady-state level of EGFR on the cell surface (Straight et al., 1993). The transformed cells overexpress the receptor for epdermal growth factor (EGF) and the receptor is constitutively autophosphorylated because of the persistent autocrine stimulation (Woodworth et al., 1995). These molecular events destabilize the cellular genome and eventually lead to cellular transformation and to the development of cervical cancer (Woodworth et al., 1988; Pecoraro et al., 1989). The enhanced activity of the EGFR in HF-1 suggested to us that inhibitors of EGFR kinase may be useful as blockers of their growth. Such inhibitors, if successful, would prevent the malignant transformation of HPV16-infected epithelial cells. We have previously demonstrated that inhibitors of the EGFR kinase

ABBREVIATIONS: HPV, human papilloma virus; HF-1, HPV16-immortalized human keratinocytes; KGM, keratinocyte growth medium; sKGM, starvation medium (KGM without EGF and without serum); DMSO, dimethyl sulfoxide; FACS, fluorescence-activated cell-sorting analysis.

are effective in blocking the growth of psoriatic keratinocytes where enhanced growth is also predominantly driven by the EGFR system (Ben-Bassat et al., 1995). We have also recently shown that tyrphostins AG494 and AG555, which block Cdk2 activation, induce growth arrest of immortalized cells at G_1-S and early S and are very effective in arresting the growth of EGFR overexpressor cells (Osherov and Levitzki., 1997; Kleinberger-Doron et al., 1998). We have already reported that the EGFR kinase blocker AG1478 and the Cdk2 activation inhibitor, blocker AG555, inhibit the proliferation of HPV16-immortalized human keratinocytes and induce in them terminal differentiation, as well as apoptosis (Ben-Bassat et al., 1997). These findings are significant because they suggest that tyrphostins can suppress the growth of HPV16-immortalized cells and cancer cells by three mechanisms: inhibition of cell cycle progression, induction of differentiation, and induction of apoptosis. In the present article, we expand these observations and describe in detail the effects of three classes of tyrphostins on the growth of HPV16-immortalized human foreskin keratinocytes compared with untransformed cells. The HPV16-immortalized keratinocytes are used as a model for the infected genital epithelium, normally the natural host of HPV16. We show that the tyrphostins have differential effects on HPV16-immortalized cells compared with normal keratinocytes. The differential effects of these compounds suggest that they may be considered for further development as antipapilloma agents and as preventive agents that block the malignant transformation of these immortalized cells.

Experimental Procedures

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

The HF-1 Line: HPV16-Immortalized Foreskin Keratinocytes. We introduced the entire HPV16 genome in secondary cultures of human foreskin keratinocytes as the natural host of HPVs. Five $\times 10^6$ cells in 0.5 keratinocyte growth medium (KGM) (Rheinwald et al., 1988) were transfected with 10 μg of a PML 2 plasmid containing the entire genome cloned at the *Bam*HI site (Mitrani-Rosenbaum et al., 1992). Thereafter, the cells were seeded in 25 flasks and incubated at 37°C for 20 min. Fresh medium was added and the cultures maintained until a permanent line was established. The growth conditions chosen allowed long-term cultivation and formation of multilayer epithelia. The cultures were split at a ratio of 1:1, and no selection was applied. Extended life span of the cells was the criterion for selection of transformants. The permanent cell line HF-1 was established within 2 months. Analysis of the DNA content of the cells at passage 7 and 50 by Southern blot showed that the HPV16 genome was present and integrated at one single locus in the cellular DNA (Mitrani-Rosenbaum et al., 1992). Northern blot analysis showed that HPV16 was expressed, specifically from the early region of the virus (E6/E7 open reading frames). Late gene expression was not detected. The status of the *p53* gene in the HF-1 cells was analyzed by reverse trascription-polymerase chain reaction, which amplified the entire coding region (Scheffner et al., 1991). Direct analysis of the amplified fragment showed that the *p53* gene was wild-type.

Collection of Biopsies and Keratinocyte Cultures. Keratinocyte cultures were initiated from small biopsy specimens (about 1 cm²) of split-thickness skin (Ben-Bassat et al., 1992, 1995). The biopsy specimens from control healthy donors were obtained under local anesthesia with 1% lignocain after Helsinki approval and signed informed consent. The biopsy was incubated in trypsin-EDTA at 4°C for 18 to 20 h. Thereafter, the epidermis was separated and the epithelium disaggregated in trypsin-EDTA to form a single-cell suspension. Trypsin 0.125%-EDTA 0.025% in Puck's saline with 10× antibiotics; namely, 1000 U/ml penicillin, 1000 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Biological Industries, Bet Haemek, Israel), and 40 μg/ml gentamicin (Rousell Laboratories, Ltd., Uxbridge, UK) were used for these procedures. Trypsin solutions were prepared from trypsin 1:250 strength. The cell suspension was inoculated ($3-6 \times 10^6$ cells) into 25-cm² Falcon flasks containing 2×10^6 lethally irradiated 3T3 mouse fibroblasts (Rheinwald and Green, 1975). When primary cultures were about 80% confluent (8–10 days), cells were released by trypsin 0.25%-EDTA, 0.05% EDTA (1:1) without antibiotics and inoculated into secondary cultures 3000 cells/well in 96-well microplates without feeder layers in keratinocyte growth medium (KGM) according to Rheinwald and Green (1988).

Tyrphostins and Treatment of Cells. The tyrphostins used in these experiments were of four different structural groups: group 1, AG555, AG494, AG974, AG1387, and AG18; group 2, AG1478; group 3, AG17; and group 4, AG814. The structures of these compounds are depicted in Fig. 1 The synthesis of these tyrphostins is described elsewhere (Gazit et al., 1989, 1991, 1994, 1996a–c). Stock solutions of 10 mM tyrphostins in dimethyl sulfoxide (DMSO) were kept at −70°C for a long period of time without decomposition. For the experiments, the tyrphostins were diluted into the KGM with 10% fetal bovine serum. The following concentrations were examined: 1, 5,10, 50, and 100 μM. Control cells were grown in KGM and in KGM with the appropriate concentration of DMSO. The concentration of DMSO in the controls was equal to the concentration in the tyrphostin containing KGM. For each tyrphostin concentration, the appropriate DMSO concentration was taken as 100%. The highest concentration of DMSO was 1%, corresponding to the solvent concentration in KGM medium containing 100 μM drug. Cells were treated for 5 days, then the compound was withdrawn, and the cells were monitored for an additional week.

Experimental Design. HF-1 cells and secondary cultures of keratinocytes from healthy donors were seeded on microplates and grown in KGM for 2 to 3 days. Thereafter, the medium was replaced with KGM containing the tyrphostin in the appropriate concentration. Medium was changed every 24 h with new medium containing the tyrphostins for 5 days. After an additional 3 to 4 days (8–9 days after planting), the medium was removed, and new medium without tyrphostin was added. The cultures were grown for another 12 days from the beginning of tyrphostin treatment, 14 to 15 days after seeding. Medium was changed every 3 days.

Automated Microculture Methylene Blue Assay. Cell growth was determined by the automated microculture methylene blue assay (Goldman et al., 1979). The tyrphostin-treated cultures and controls were fixed in glutaraldehyde, 0.05% final concentration, for 10 min at room temperature. After washing, the microplates were stained with 0.1% methylene blue in 0.1 M borate buffer, for 60 min at room temperature. The microplates were extensively and rigorously washed to remove excess dye and dried. The dye taken up by cells is eluted in 0.1N HCl for 60 min at 37°C, and read at 620 nm. In preliminary titration experiments, linear readings were obtained for 1×10^3 to 4×10^4 cells/well. Each point of the growth curve experiments is calculated from 15 wells, because keratinocytes grow in islands and do not form uniform monolayers.
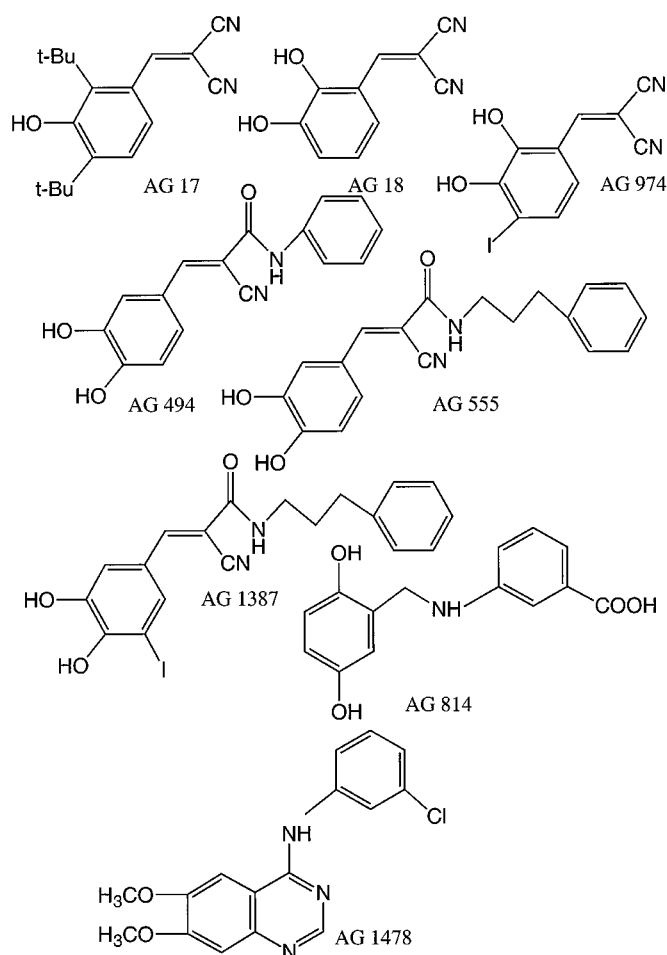
Fig. 1. Structures of tyrphostins.

Calculation of Growth Inhibition. For each tyrphostin concentration used, the appropriate KGM medium 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 and 1%, DMSO had a very small effect (Fig. 2-5). These high concentrations were used only when 50 mM or 100 μM tyrphostin, respectively, were used.

Fluorescence-Activated Cell-Sorting Analysis (FACS) of DNA Content and Determination of Apoptotic Cells. Selected samples of the HF-1 and normal keratinocyte cultures were FACS analyzed for DNA content. Samples of cells treated with tyrphostin concentrations for predetermined periods were dispersed with 0.25% trypsin: 0.05% EDTA (1:1) and stained with ethidium iodide in suspension. Cell cycle analysis and determination of the apoptotic cell fraction of the cell populations were carried out with FACS FPAR PLUS (Becton-Dickinson, Mountain View, CA).

Western Blot Analysis for EGFR and Phosphotyrosine. HF-1 cells and normal keratinocytes were seeded 5×10^5 cells/ 35-mm plates in KGM. After 2 to 3 days the cells were washed, fed with medium without serum and without EGF (sKGM), and starved for 48 h. Tyrphostin in starvation medium (sKGM) at the appropriate concentration was added for 4 h. Cells were then stimulated with 30 ng/ml EGF for 10 min. The reaction was stopped by placing the culture on ice and washing with ice-cold PBS. Whole cells were lysed with hot buffer, scraped and boiled for 5 min, and then run on 7% SDS for 4 h, transferred to nitrocellulose paper, and incubated overnight at 4°C with either monoclonal mouse antihuman EGFR antibodies (0.5 μg/ml) or with monoclonal antiphosphotyrosine antibody PT-66, following the manufacturer's recommendations. Goat anti-

mouse fluorescent antibody was added for a 2-h incubation at room temperature (1 ml/20 ml). After drying, the gels were exposed to X-ray film in cassettes.

Results

Tyrphostins Suppresses Cell Proliferation

To evaluate the ability of tyrosine kinase inhibitors to suppress the proliferation of HPV16-immortalized keratinocytes (HF-1), we analyzed, quantitatively, four structural groups of tyrphostin (Fig. 1). In parallel, we examined the effect of these compounds on the growth of normal human keratinocytes. Cells grown in KGM and in KGM with DMSO in concentrations equal to that of the tyrphostin solution served as controls. Previous and present results (Figs. 2–5) demonstrate that these tyrphostins inhibit the growth of HPV16-immortalized cells (Ben-Bassat et al., 1997). From group 1 tyrphostins, AG555 and AG494 were already effective at 10 μM (IC$_{50}$ = 6.4 and 4.7 μM, respectively), and the cells remained arrested after withdrawal of the compound on day 5 as monitored on days 8 and 12. At 50 μM AG974 and AG1387 completely blocked cell proliferation, and the cells remained arrested after withdrawal of the compound. AG18, at 100 μM caused about 40% inhibition of proliferation which progressed to 80% after withdrawal of the compound (Fig. 2). A similar pattern of growth inhibition was obtained for human keratinocytes (Fig. 3). The potency ratio of the tyrphostins from group 1 for both cell types was found to be AG555 > AG494 > AG974 > AG1387 > AG18. However, the IC$_{50}$ values of AG555 and AG494 for normal keratinocytes was higher (9.4 and 9.8 μM, respectively) than for HF-1 cells (6.4 and 4.7 μM, respectively).

For AG1478, AG17, and AG814 (groups 2, 3, and 4, respectively), the results show (Fig. 4) that 10 μM AG1478 caused about 50% growth inhibition, and 50 μM AG1478 completely blocks proliferation of HF-1 cells. AG17 (group 3) is extremely potent: 0.2 μM completely and irreversibly suppressed cell proliferation. AG814 (group 4) was not effective at 10 μM and slightly inhibitory (20–30%) at 100 μM. The pattern of growth inhibition by these tyrphostins for normal keratinocytes is quite similar but not identical (Fig. 5 and see below). One micromolar AG 1478 caused about 50% inhibition of proliferation of both cell types. The inhibition of normal keratinocytes progressed to 80% after withdrawal of the compound. The potency ratio for these tyrphostins is AG17> AG1478 > AG814 for both HF-1 cells and normal keratinocytes.

Rescue after Treatment

For these experiments, after 5 days of tyrphostin treatment, the cells were washed, segregated, replated, and their proliferative capacity measured. The self-renewal capacity-rescue of HF-1 and normal keratinocytes after treatment with AG555, AG494, AG1387, AG1478, and AG17 is illustrated in Fig. 6. After treatment of HF-1 cells with 0.1 μM AG17 or with 1 μM AG555, AG494, AG1387, and AG1478, washing, and replating, cells resumed growth and complete survival was obtained. With 1 μM AG17, normal keratinocytes did not resume growth and remained suppressed. The rescue experiments confirm the results obtained in the growth inhibition experiments (Fig. 5).

In summary, normal keratinocytes were found not to re-
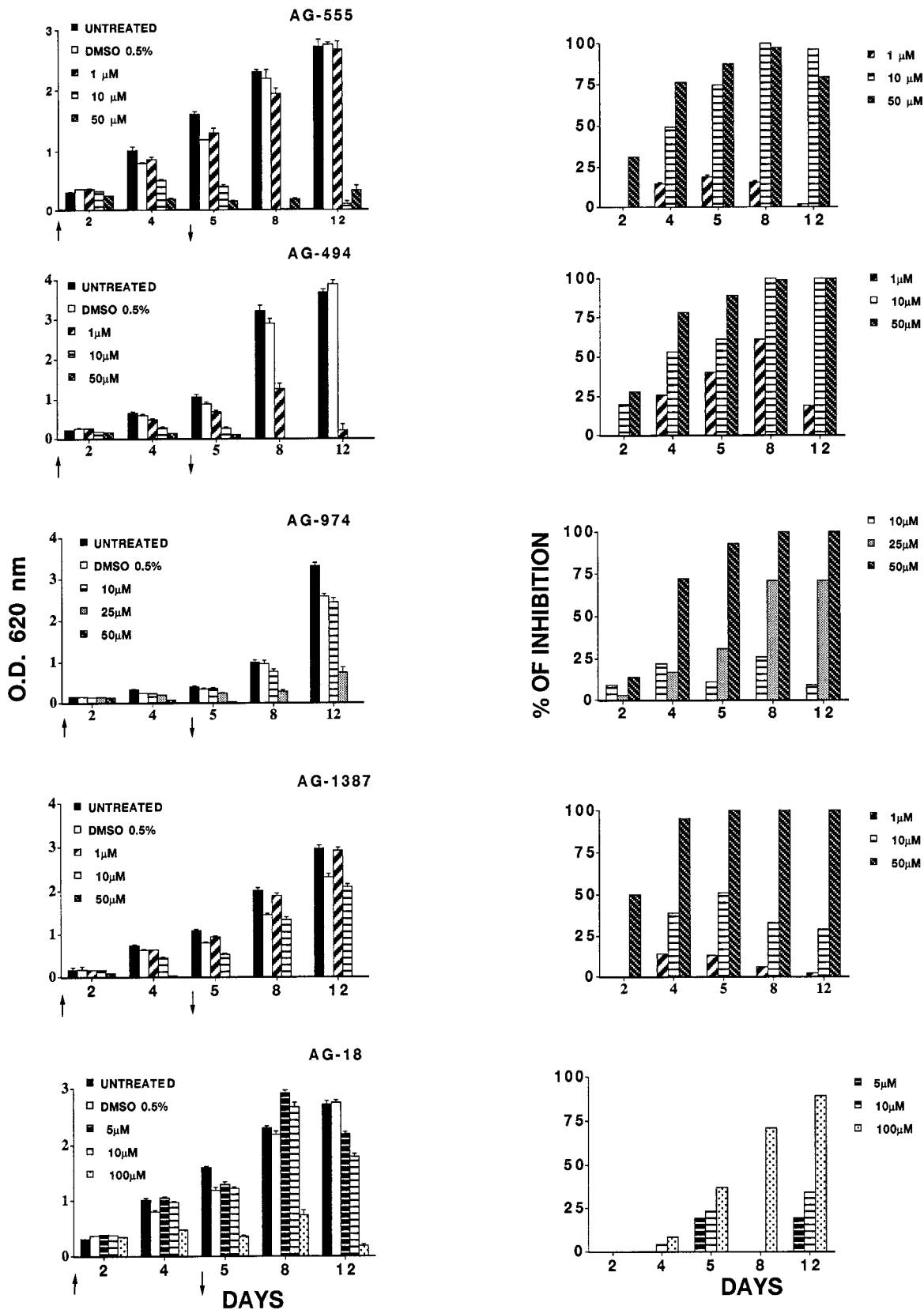
HF-1

Fig. 2. The inhibitory effect of group 1 tyrphostins on the proliferation of HF-1 cells. HF-1 cells (3000/cells/well) were grown in culture for 3 days before the beginning of treatment. The cells were grown in medium containing the compound for 5 days 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 (left columns) and the percentage of growth inhibition calculated from it (right columns).
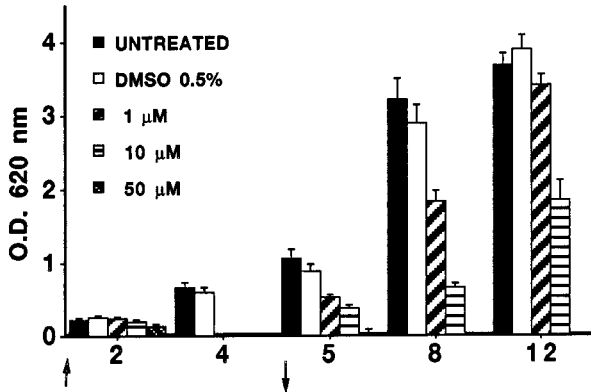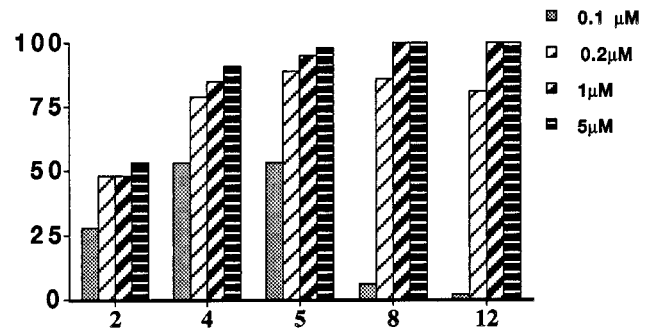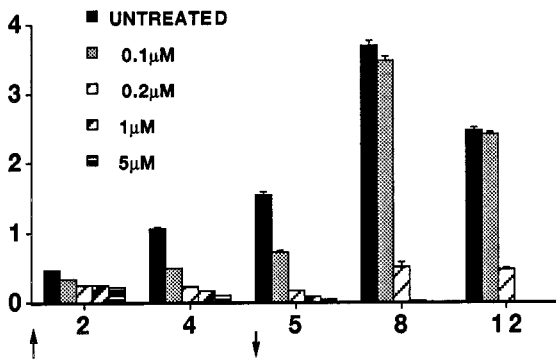
KERATINOCYTES
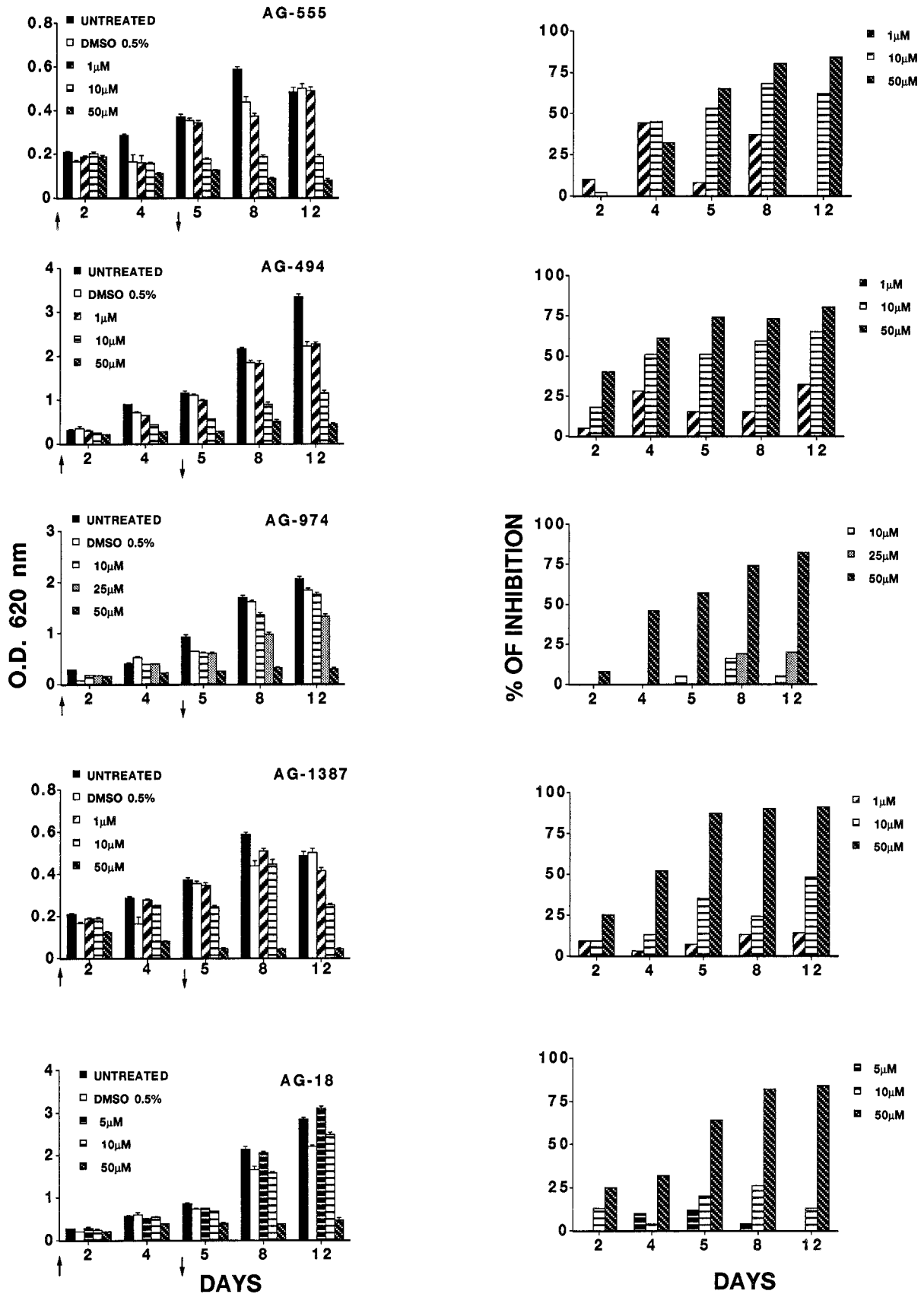
Fig. 3. The inhibitory effects of group 1 tyrphostins on the proliferation of normal keratinocytes. Experimental details are given in the text.
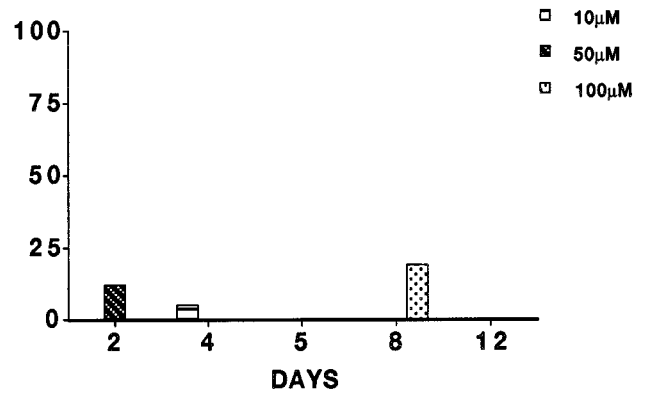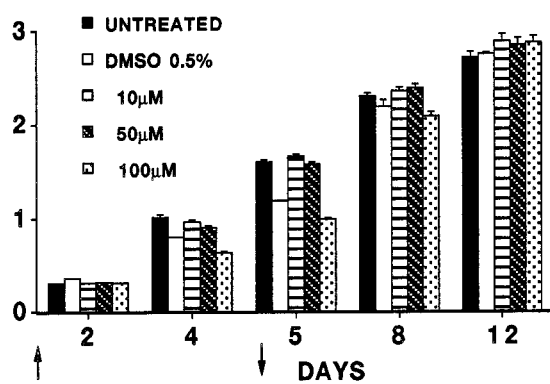
HF-1
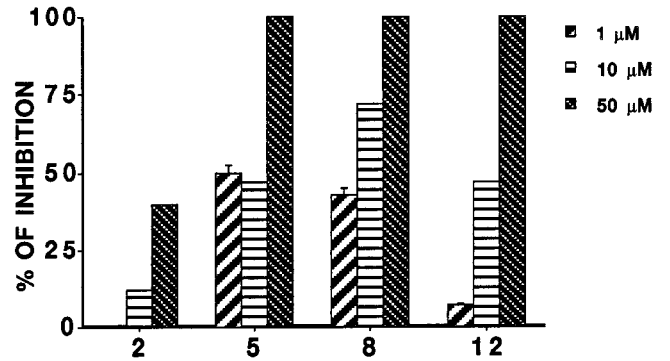
Fig. 4. The inhibitory effect of tyrphostins AG1478 and AG814 and on the proliferation of HF-1 cells. Experimental details are given in the text.

KERATINOCYTES

AG-1478
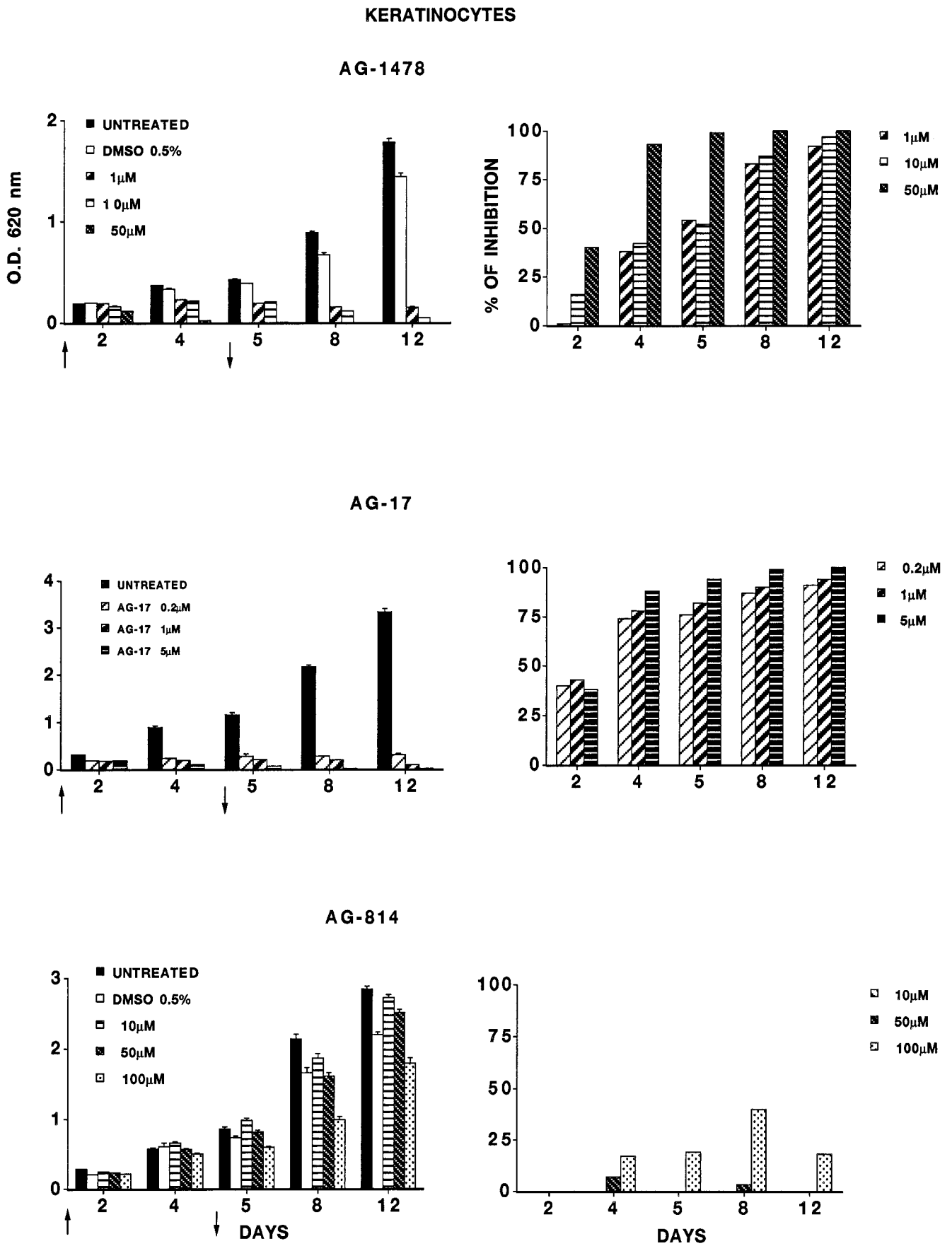

AG-17

AG-814

Fig. 5. The inhibitory effect of tyrphostins groups 2 to 4 on the proliferation of normal keratinocytes. Experimental details are given in the text.
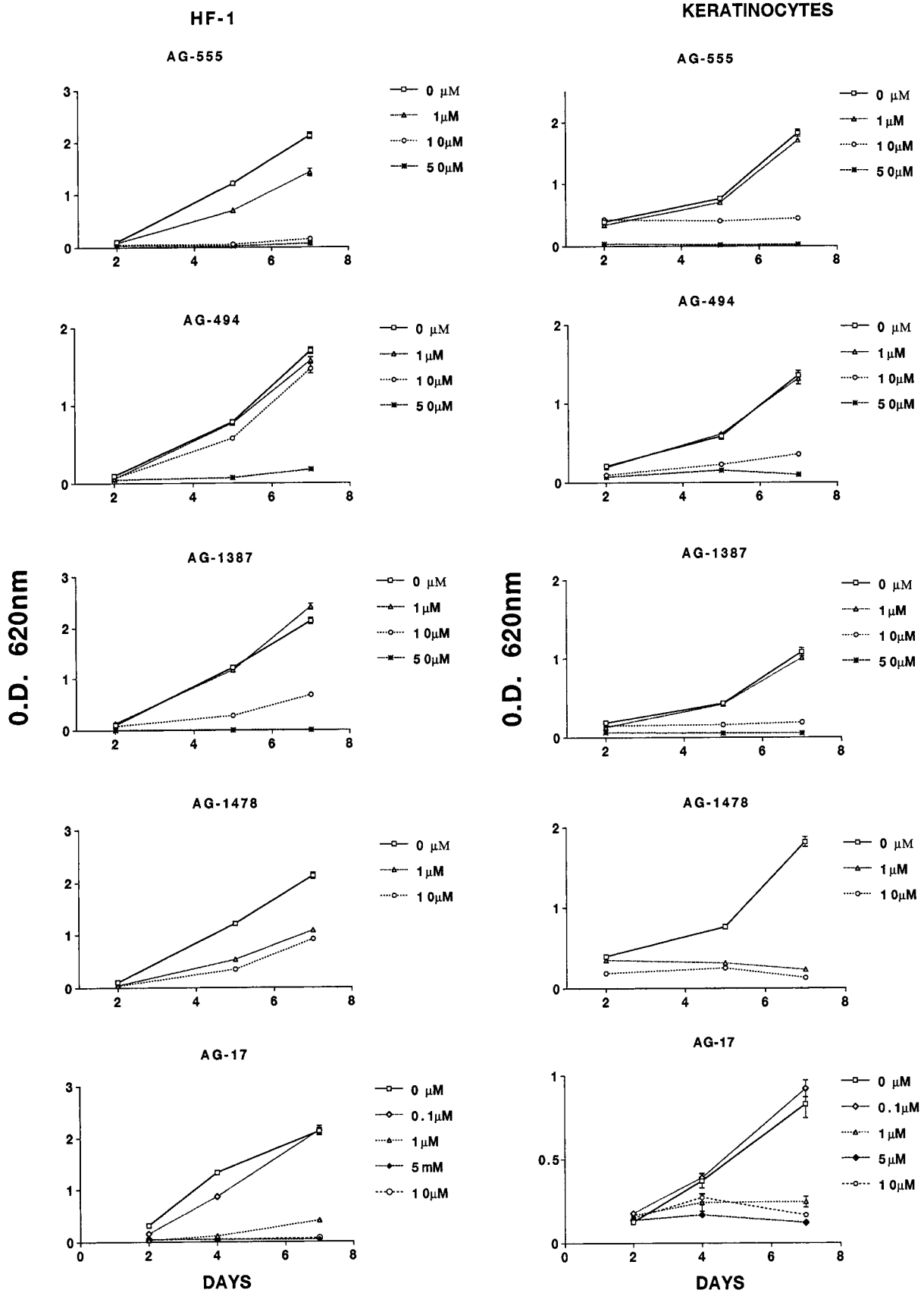
RESCUE

Fig. 6. Rescue after treatment with tyrphostins AG555, AG494, AG1387, AG1478, and AG 17. HF-1 cells (left columns) and human normal keratinocytes (right columns) were treated with tyrphostins for 5 days. Thereafter, the cells were washed, desegregated, and replated. Their self-renewal capacity was measured by the automated microculture methylene blue assay.
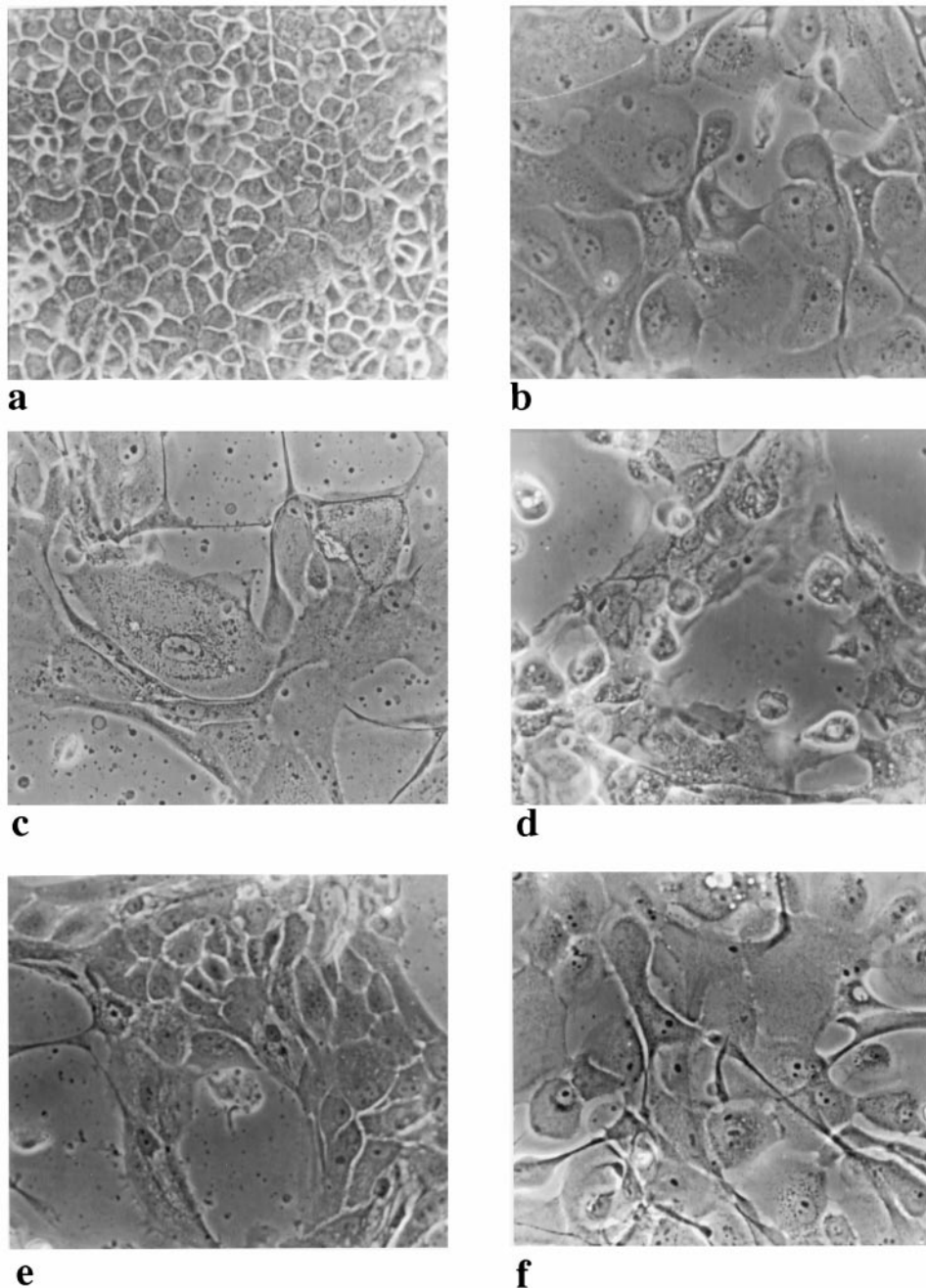
Fig. 7. Photomicrographs of HF-1 cells treated with tyrphostins for 5 days. a, untreated control cells; b, AG555, 50 μM; c, AG494, 50 μM; d, AG1387, 50 μM; e, AG1478, 10 μM; and f, AG17, 1 μM. Areas portraying representative morphologic changes of the cells in the culture dish are presented. Original magnification, 200$\times$.

sume growth at lower tyrphostin concentrations compared with HF-1 cells (Fig. 6). It should be emphasized that the saturation density of normal keratinocytes is lower and at confluence they stop dividing, whereas HF-1 cells are less sensitive to density limitation of growth and reach higher cell densities (Fig. 7).

Tyrphostins Alter Cell Morphology and Culture Organization

Normally, HF-1 cells form compact sheets of typical cobblestone columnar epitheloid cells of homogenous morphology similar to keratinocytes. However, they establish a higher saturation density at confluence, are more regularly

organized, and lack stratification compared with normal keratinocytes (Figs. 7a and 8a, respectively). Treatment of tyrphostins produces distinctive morphological changes in HF-1 cells. These changes are already apparent within 3 days after application and are maximal at 5 days as illustrated in Fig. 7. The cell population is relatively heterogeneous. Some areas contain flat coboidal cells (Fig. 7b), large elongated cells (Fig. 7c) with spindle-like morphology, and giant cells with a marked increase in the cytoplasm (Fig. 7c). Frequently, cells extend cytoplasmic processes traversing several cells in the culture (Fig. 7, e and f). In addition, treated cells also develop small round, highly refractile intracellular bodies distributed through the cytoplasm (Fig. 7).
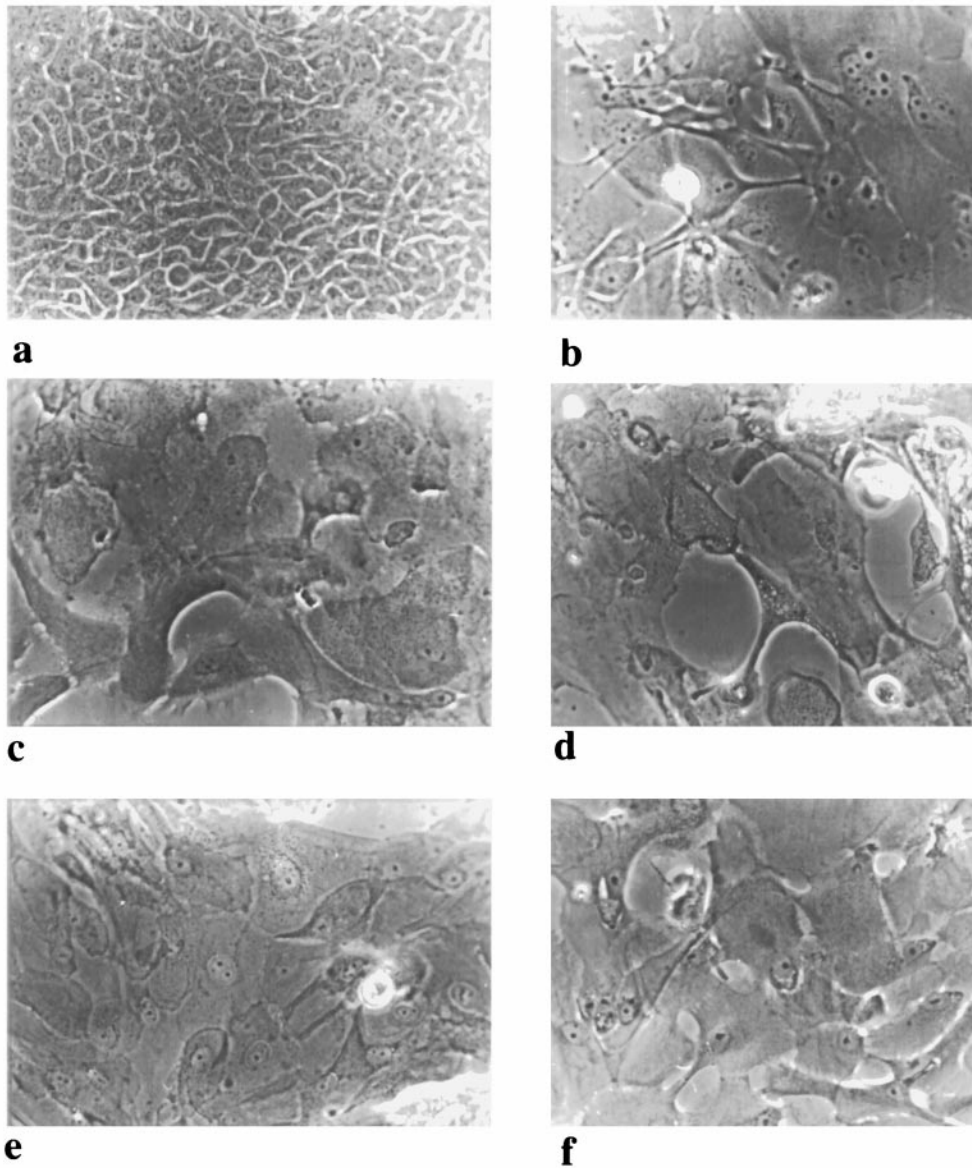
Fig. 8. Photomicrographs of human normal keratinocyte cultures treated with tyrphostins for 5 days. a, untreated control cells; b, AG555, 50 μM; c, AG494, 50 μM; d, AG1387, 50 μM; e, AG1478, 10 μM; and f, AG17, 1 μM. Areas portraying representative morphologic changes of the cells in the culture dish are presented. Original magnification, 200$\times$.

Tyrphostin treatment of normal keratinocytes produces morphological changes similar to those seen in HF-1 cells after treatment and over a similar time course (Fig. 8,). The major difference, however, is that culture organization of normal keratinocytes is less disrupted (Fig. 8, c, e, and f).

Points of Growth Arrest in the Cell Cycle and Apoptosis

To explore more directly the mechanism for growth suppression by the tyrphostins, cell cycle analysis was performed on HF-1 cells and normal keratinocytes treated with the most effective tyrphostins, namely, AG555 (group 1), AG1478 (group 2), and AG17 (group 3). Cells exposed to these compounds were assessed by FACS analysis on day 2 (24 h after initiating treatment), day 5 (cessation of treatment), and day 8 (3 days after withdrawal of the compound).

AG555. This tyrphostin alters the cell cycle distribution of HF-1 cells (Fig. 9A) and of normal keratinocytes (Fig. 9B) in a concentration- and time-dependent manner. After 24 h of treatment (day 2), an alteration in the cell cycle phase distribution was observed, with a significant effect noted only at the highest concentration of tyrphostin. At 50 μM, the proportion of cells in G_1 is increased and the proportion of cells in $G_2 + M$ is decreased, with no effect on the fraction of apoptotic cells. On day 5, the increase in the proportion of cells in G_1 was still evident, but the decrease in $G_2 + M$ was more pronounced, with a significant increase in the apoptotic cell fraction. The decrease in $G_2 + M$ fraction and the sharp increase in the proportion of apoptotic cells up to 30%, persisted and was still evident on day 8 (3 days after cessation of treatment).

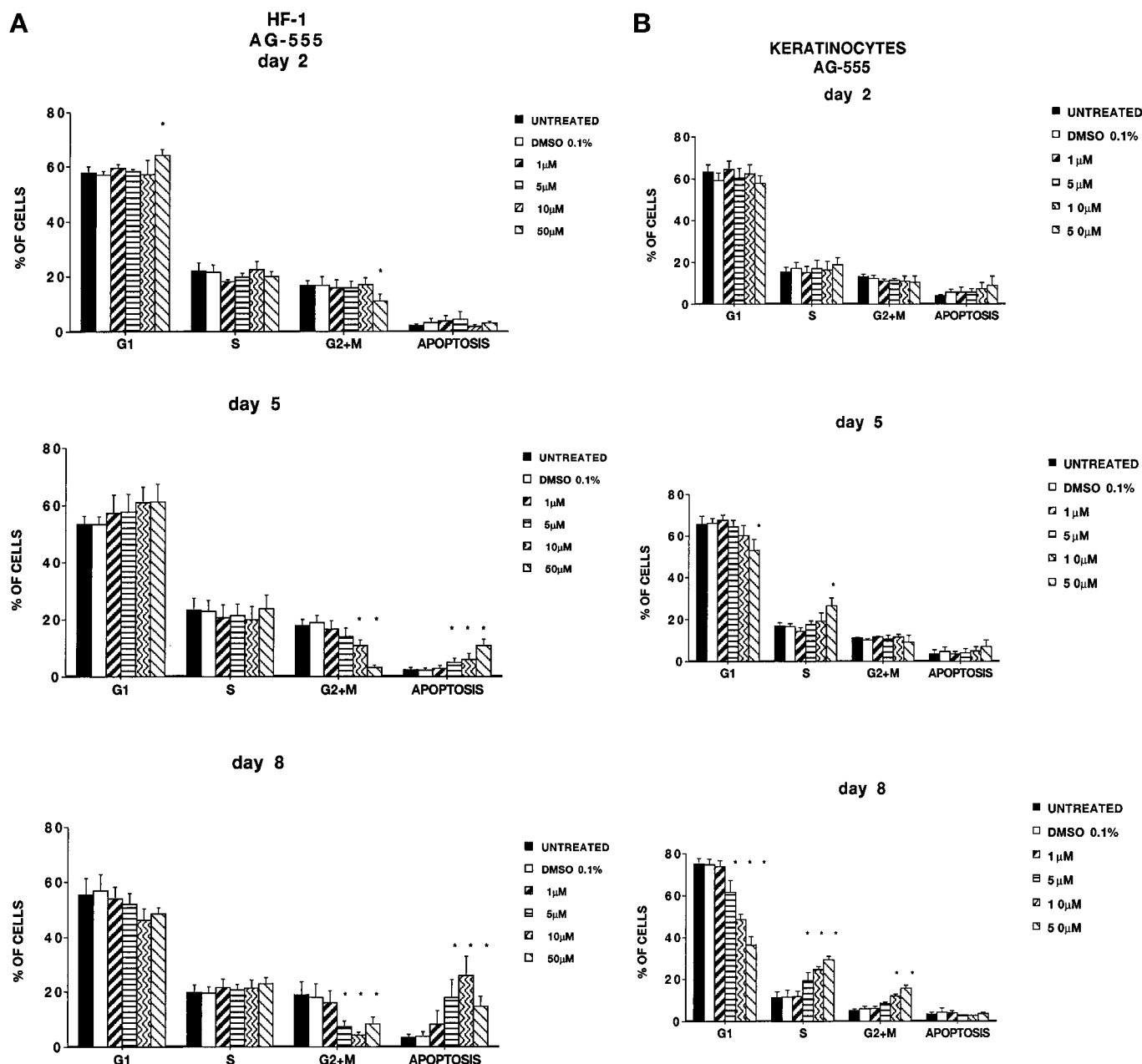AG555 alters the cell cycle distribution also in normal

A

HF-1
AG-555
day 2

B

KERATINOCYTES
AG-555

day 2

day 5

day 5

day 8

day 8

Fig. 9. The effect of AG555 on the cell cycle phase distribution and apoptosis of A, HF-1 cells. B, human normal keratinocytes. The cells were treated with various concentrations of the tyrphostin and harvested on day 2, 5, and 8. Their cell cycle distribution was determined by FACS analysis as described in *Materials and Methods*. Day 2 represents 24 h of treatment; day 5, cessation of treatment, and day 8, the tyrphostin-supplemented medium was replaced by control medium for an additional 3 days.

keratinocytes, producing a dose- and time-dependent effect, but the effect was very different from that on HF-1 cells (Fig. 9B). Cells accumulated in S and G_2 + M, with a marked decrease in the proportion of cells in G_1, with no effect on the fraction of apoptotic cells. The changes in the cell cycle phase distribution in response to AG555 were clearly visible on day 5 (cessation of treatment) and were irreversible after withdrawal of the compound (day 8).

AG1478. Like AG555, this tyrphostin markedly alters the distribution of HF-1 cells in the various phases of the cell cycle (Fig. 10A). AG1478 causes a concentration-dependent increase in the G_1 fraction and a decrease in the S fraction, already evident on day 2. This effect persists through the 5 days of treatment, with a significant de-

crease in the G_2 + M proportion of cells and a significant increase in the fraction of apoptotic cells. The effect of AG1478 on the cell cycle phase distribution of HF-1 is reversible with concentrations below ≥ 10 μM. AG1478 also markedly alters the cell cycle distribution of normal keratinocytes (Fig. 10B). It produces a concentration- and time-dependent increase in the G_1 fraction of cells, with a decrease in S and G_2 + M fraction, evident as early as 24 h after treatment (day 2) and persisting for the entire 5-day treatment period. After removal of the compound (day 8), there is a significant decrease in the proportion of cells in G_1, but there is no significant change in the S and G_2 + M cell fractions. There is little or no effect on the fraction of apoptotic cells in any of the phases of the experiment. This
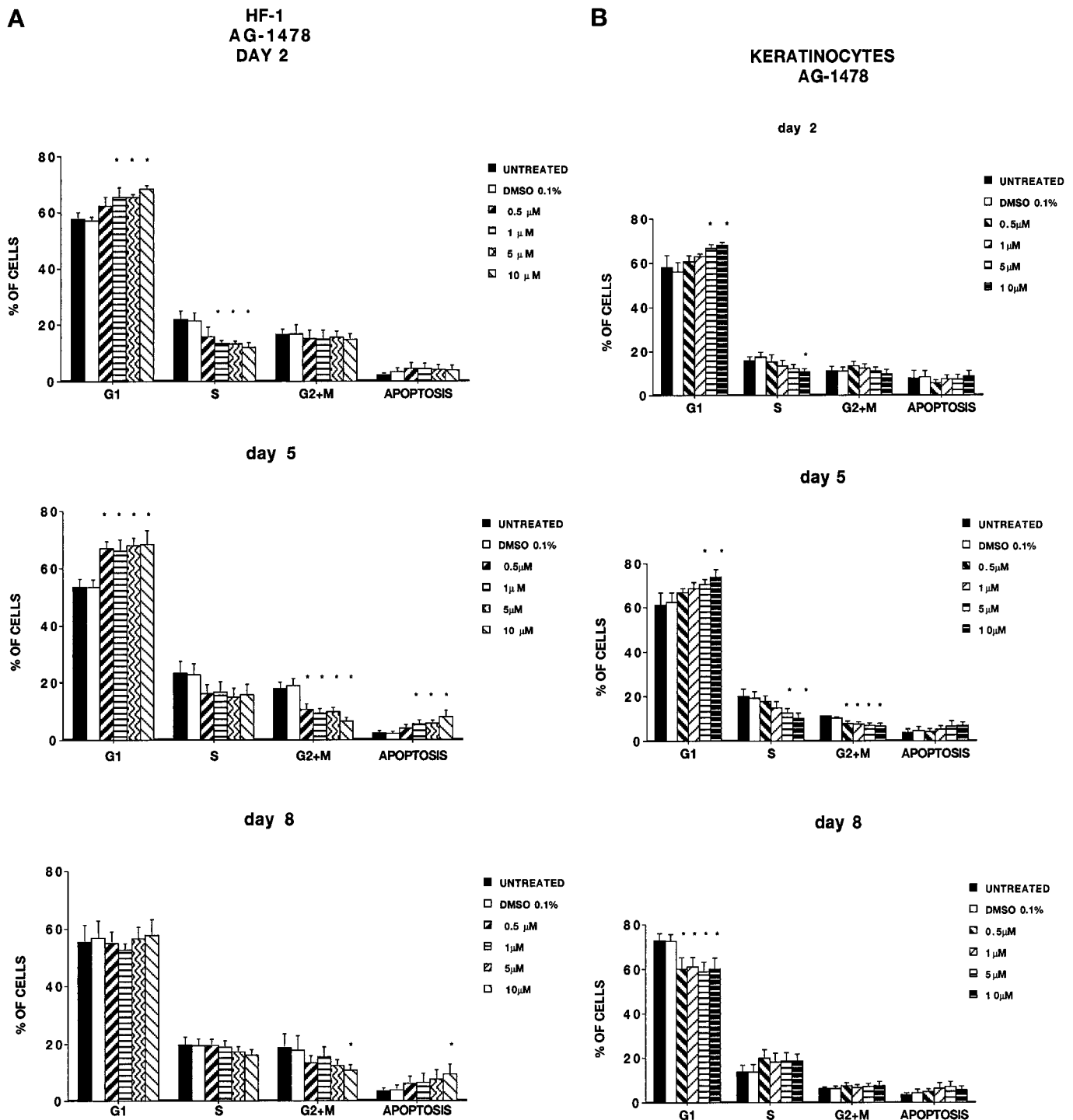
Fig. 10. Effect of AG1478 on the cell cycle phase distribution and apoptpsis of HF-1 cells (A) and human normal keratinocytes (B). The cells were treated with various concentrations of the tyrphostin and harvested on day 2, 5, and 8. Their cell cycle distribution was determined by FACS, as described in *Materials and Methods*. Day 2 represents 24 h of treatment; day 5, cessation of treatment; and day 8, the tyrphostin-supplemented medium was replaced by control medium for an additional 3 days.

is in sharp contrast to the significant increase in the fraction of apoptotic cells in the HF-1 cells.

AG17. This tyrphostin also markedly alters the cell cycle distribution of HF-1 cells (Fig. 11A) and, like AG1478, increases the G_1 fraction and decreases the S fraction on day 2. This effect persists through the 5 days of treatment with a significant decrease also in the G_2 + M proportion of cells.

The effect of AG17 on the cell cycle phase distribution of HF-1 is reversible with a concentration <10 μM (day 8). However, unlike AG1478, AG17 does not affect the fraction of apoptotic cells in HF-1 (Figs. 10A and 11A, respectively). AG17 alters also the cell cycle distribution of normal keratinocytes (Fig. 11B). It produces a concentration-dependent increase in G_1 and decrease in S and G_2 + M evident already 24 h after
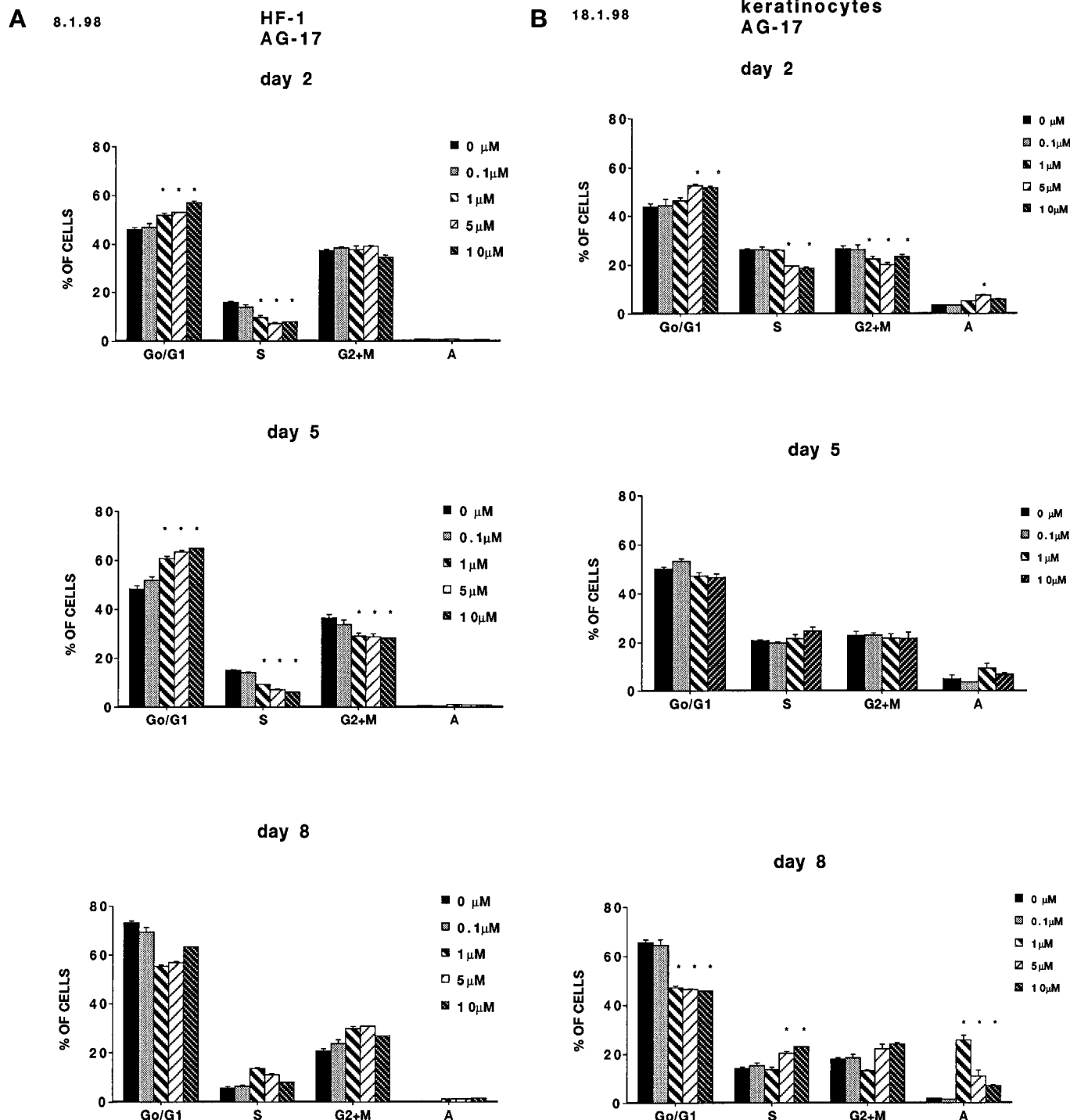
Fig. 11. The effect of AG17 on the cell cycle distribution and apoptosis of HF-1 cells (A) and human normal keratinocytes (B). The cells were treated with various concentrations.

treatment (day 2). After cessation of treatment (day 5) and withdrawal of the compound (day 8), there was a significant decrease in G_1, with a significant increase in S and the apoptotic cell fractions.

Effect of Tyrphostins on EGFR Phosphorylation and EGFR Level

Treatment of HF1 cells with tyrphostins from group 1 (AG555, AG974, AG1387, or AG494) has no significant effect

on EGFR autophosphorylation (Fig. 12), whereas treatment of HF-1 cells with AG1478 (group 2) results in a dose-dependent inhibition of EGFR autophosphorylation (Fig. 12). AG17 (group 3) has no effect on the EGFR autophosphorylation (Fig. 13). Similar experiments were performed with normal keratinocytes (Fig. 13). It can be seen that the tyrphostins from group 1 exhibit no effect on the state of EGFR phosphorylation (Fig. 13), whereas treatment of these cells with AG1478 resulted in a dose-dependent inhibition of EGFR
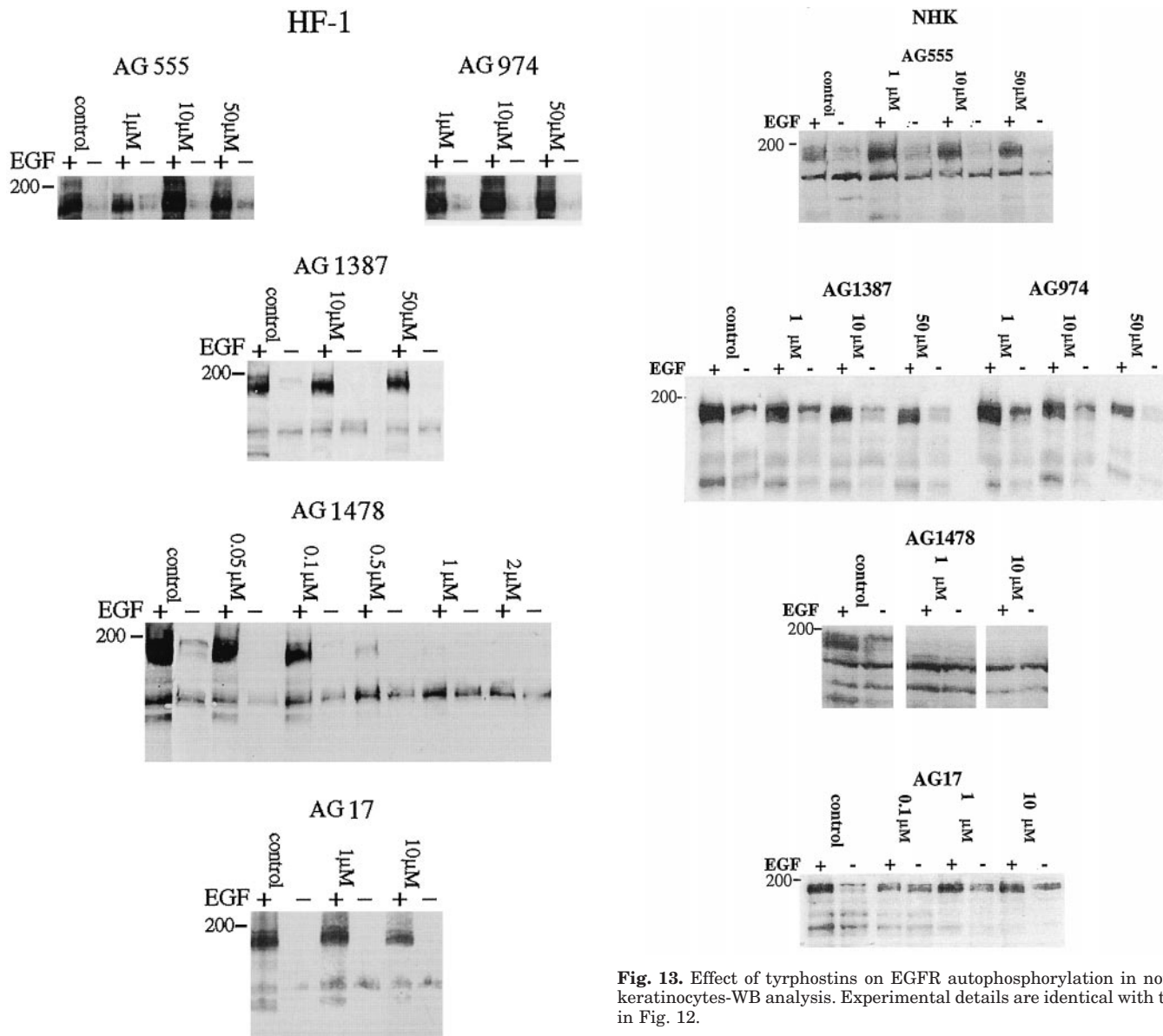
Fig. 12. The effect of the selected tyrphostins on EGFR autophosphory-lation-WB analysis. HF-1 cells were seeded in KGM; after 2 to 3 days the cells were starved (in sKGM) for 48 h. Tyrphostin was added at the appropriate concentration for 4 h and then the cells were stimulated with EGF for 10 min and analyzed as detailed in *Materials and Methods*. Treatment of HF-1 cells with tyrphostins from group 1: AG555, AG974, and AG1378 had no effect on EGFR autophosphorylation; AG1478 (group 2) inhibited EGFR autophosphorylation in a dose-dependent manner, and AG17 (group 3) had no effect on EGFR autophosphorylation.

autophosphorylation. Treatment with tyrphostins does not alter the level of EGFR expressed under the conditions used to examine the inhibition of its phosphorylation.

The results also demonstrate that HF-1 cells overexpress EGFR compared with normal keratinocytes (Fig. 14), and that the EGFR in the HF-1 cells exhibits detectable levels of tyrosine phosphorylation in the absence of EGF (Fig. 12) as previously reported.

Discussion

Characterization of the Tyrphostins. The present results identify tyrphostins that suppress the growth of HF-1,

Fig. 13. Effect of tyrphostins on EGFR autophosphorylation in normal keratinocytes-WB analysis. Experimental details are identical with those in Fig. 12.
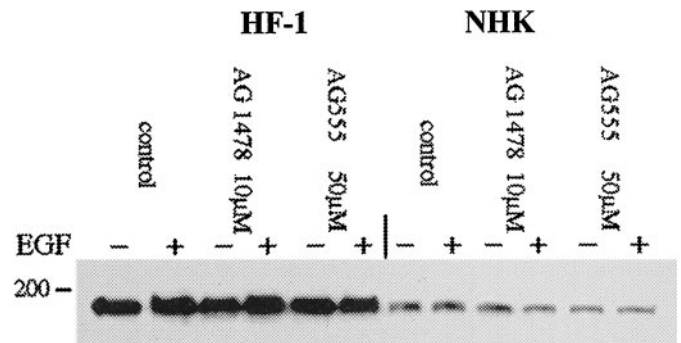
Fig. 14. Effect of AG555 and AG1478 on EGFR expression in HF-1 cells and normal keratinocytes. Experimental details are identical with those in Fig. 12, with the exception of the detecting antibody, which is a monoclonal anti-EGFR antibody. The number of cells per line is 4×10^5 for normal keratinocytes and 2.5×10^4 for HF-1 cells. HF-1 cells overexpress EGFR compared with normal keratinocytes.

cause changes in their cell cycle distribution, alter their morphology, and induce cell death by apoptosis. In general, these cellular responses are dose- and time-dependent. The

compounds chosen represent four different families of tyrphostins (groups 1–4), and were all found to be effective except for AG814 (group 3). Members of groups 1 (AG555 and analogs) and 2 (AG1478) have already been shown as potent inhibitors of cell proliferation (Ben-Bassat et al., 1997; Osherov et al., 1997). AG555 and its analogs, like AG494, block Cdk2 activation (Osherov et al., 1997; Kleinberger-Doron et al., 1998) and arresting cells in G_1 and at the G_1-S boundary (Kleinberger-Doron et al., 1997). AG1478 and its analogs such as AG1517 (SU 5271) selectively block EGFR kinase and cause a G_1 arrest. The bromo analog of AG1478-AG1517, which is identical with PD 153035 (Fry et al., 1994), was also shown to effectively block psoriatic keratinocytes with no adverse effects on normal keratinocytes (Powell et al., 1999) and has currently entered clinical trials for psoriasis. AG17, which is a universal cell proliferation blocker shown to be active in a number of cellular systems (Bilder et al., 1991) is effective in vivo as an antirestenosis agent in the rat (Golomb et al., 1996). AG17 and its analogs induce apoptosis in lymphoma cells with little effect on protein tyrosine kinase activity (Palumbo et al., 1997). We show here that AG17 is effective in arresting HF-1 cell growth (Fig. 4). Rescue experiments (Fig. 6), in which we measured the self-renewal capacity of the cells after removal of the tyrphostins on day 5, confirm the growth inhibition experiments. It seems that the mechanism of action of AG17 is not confined to its potency to inhibit protein tyrosine kinases. In a recent publication (Burger et al., 1995), it was pointed out that AG17 exerts its growth inhibitory effects by targeting the mitochondria.

Cellular and Biochemical Effects. The antiproliferative effect of the different types of tyrphostins was accompanied by profound changes in the morphology of the cells and culture organization. Subsequent to treatment, the HF-1 cell population becomes heterogeneous with some areas containing large and giant cells and cells with long cytoplasmic extensions (Fig. 7). Morphological changes are not restricted to HF-1 cells and are also observed in the normal keratinocyte cultures but with different characteristics (Fig. 8 and see below). The morphological changes induced by these tyrphostins occur at the same concentration range that also suppressed cell proliferation.

Biochemical analysis of the effect of these tyrphostins show that AG1478 inhibits EGF receptor autophosphorylation in a dose-dependent manner, without an effect on the level of EGFR expression (Figs. 12 and 14, respectively). It can also be seen that HF-1 cells overexpress EGF receptors compared with normal keratinocytes (Fig. 14). Increased number of EGFR in HPV16-transformed keratinocytes is attributed to the E5 papilloma protein (Woodworth et al., 1993). AG555 and AG494, as well as the other tyrphostins examined in this study, in contrast to AG1478, have no effect on EGFR receptor autophosphorylation (Fig. 12). AG494, like AG555, is an inhibitor of Cdk2 activation (Kleinberger-Doron et al., 1998; Osherov et al., 1997). None of the tyrphostins examined have any effect on the level of expression of the receptors.

AG555 and AG1478 affected two critical parameters that determine the final number of HPV16-immortalized foreskin keratinocytes: cell cycle progression and apoptosis. AG555 and its analogs, such as AG494, induced cell cycle arrest at G_1 and S, a decrease in the number of cells in G_2 + M, and an increase in the proportion of apoptotic cells to 30%. The effects of the AG555 and its analogs persist after drug removal (Figs. 7, 8 and 9). Thus, apoptosis could be a second mechanism that contributes to the observed antiproliferative effect of tyrphostins of the AG494/AG555 family. AG555 has similar effects on the cell cycle of psoriatic keratinocytes (Ben-Bassat et al., 1995).

AG1478 increases the proportion of cells in G_1 at the expense of cells in S or the G_2 + M phases of the cell cycle. This effect was evident already 24 h after treatment and persisted for the duration of the experiment. AG1478 also induces apoptosis in the HF-1 cells, but to a much lesser extent than AG555: 10% with 10 μM AG1478 compared with >30% with 10 μM AG555 on day 8.

Because HPV16-immortalized cells lack active p53 (Werness et al., 1990) and overexpress bcl-2 (Lang et al., 1995), the balance between apoptosis, differentiation, and proliferation is impaired. The interesting finding that EGFR blockers and Cdk2 activation blocks, represented by AG1478 and AG555/AG494, respectively, can suppress the proliferation of HPV16-immortalized cells suggests that they override the strong antiapoptotic signals in these cells.

The antiproliferative effect of AG17 on HF-1 cells might be attributed mainly to growth arrest, because it increases the proportion of cells in G_1 and decreases the S and G_2 + M fraction, but does not induce apoptosis. The effect of AG17 on the cell cycle of normal keratinocytes is similar, but after cessation of treatment there is a significant decrease in G_1 with a significant increase in S and <30% apoptosis.

Effect of Tyrphostins on HF-1 Cells Compared with Normal Keratinocytes. The effect of tyrphostins on normal keratinocytes is different from that on HF-1 cells. The main effect they exert on normal keratinocytes is inhibition of proliferation. It is noteworthy that AG555 and AG494 are more potent inhibitors of HF-1 cells than of normal keratinocytes: IC_{50} of AG555 for HF-1 is 6.4 μM compared with 9.4 μM for normal keratinocytes, and for AG494, IC_{50} is 5.2 μM for HF-1 cells compared with 9.8 μM for normal keratinocytes. AG555 and AG1478 arrest growth of normal keratinocytes, but in contrast to HF-1 cells do not induce differentiation or apoptosis. It seems that in the case of normal keratinocytes simple cell cycle arrest takes place. It is likely, therefore, that tyrphostin treatment will not affect the normal course of development of normal keratinocytes. Normal keratinocyte cultures generally reach a plateau after 10 to 14 days, and, after several passages, senesce and cannot be cultured further (Rheinwald and Green, 1975, 1988). The HF-1 cells differ from normal keratinocytes in their basic characteristics and in their response to tyrphostins. HF-1 cells resemble simple epithelium; they proliferate faster than normal keratinocytes, establish a higher cell density at confluence, and can be passaged indefinitely (Ben-Bassat et al., 1997). After in vitro propagation (>180 passages), numerous chromosomal aberrations are detected that might indicate changes in the normal pattern of chromosome division. Tyrphostin treatment induces prominent changes in HF-1 cells: inhibition of proliferation, induction of apoptosis, and differentiation characterized by increased expression of filaggrin, a structural protein product in the terminal differentiation pathway and in the reduction of expression of cytokeratin 14, an early cellular marker for proliferating cells (Ben-Bassat et al., 1997). This significant difference in the effect of tyrphostins on HPV16-immortalized keratinocytes and on normal keratinocytes make these compounds attractive candi-

dates for the treatment of papilloma. Because HPV-16-immortalized cells lack active p53 (Werness et al., 1990), the balance between apoptosis, differentiation, and proliferation is impaired. This imbalance is most probably responsible for the different type of response of HPV16- immortalized cells to the tyrphostins. This differential response make tyrphostins, especially those that inhibit EGFR kinase, and Cdk2 activation attractive candidates for the treatment of papilloma.

References

Ben-Bassat H, Eldad A, Chaouat M, Livoff A, Ron N, Neeman Z and Wexler MR (1992) Structural and functional evaluation of modifications in the composite plain graft; cryopreserved dermis and cultured keratinocytes. *Plast Reconstr Surg* **89:**510–520.

Ben-Bassat H, Rosenbaum-Mitrani S, Hartzstark Z, Shlomai Z, Kleinberger-Doron N, Gazit A, Plowman R, Levitzki R, Tsvieli R and Levitzki A (1997) Inhibitors of the EGF receptor and of Cdk2 activation induce growth arrest, differentiation, and apoptosis of HPV16 immortalized human keratinocytes. *Cancer Res* **57:**3741–3750.

Ben-Bassat H, Vardi DV, Gazit A, Klaus SN, Chaouat M, Hartzstark Z and Levitzki A (1995) Tyrphostins suppress the growth of psoriatic keratinocytes. *Exp Dermatol* **4:**82–88.

Bilder G, Krawiec JA, Gazit, A, Gilon C, McVety K, Lyall R, Zilberstein A, Levitzki A, Perrone M and Schreiber AB (1991) Tyrphostins inhibit PDGF induced DNA synthesis, tyrosine specific protein kinase activity and c-fos mRNA expression in vascular smooth muscle cells. *Am J Physiol* **260:**C721–C730.

Braly P (1996) Preventing cervical cancer. *Nat Med* **2:**749–751.

Burger AM, Kaur G, Alley M, Supko JG, Malspeis L, Grever MR and Sausville EA (1995) Tyrphostin AG17, [(3,5-di-tert-butyl-4-hydroxybenzylidene)-malononitrile], inhibits cell growth by disrupting mitochondria. *Cancer Res* **55:**2794–2799.

Dyson N and Howley PM (1989) The HPV 16 E7 oncoprotein is able to bind the Rb gene product. *Science (Wash DC)* **243:**934–937.

Fry DW, Kracker AJ, McMichael A, Ambroso LA, Nelson JM, Leopold WR, Connors RW and Bridges AJ (1994) A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. *Science (Wash DC)* **215:**1093–1095.

Gazit A, App H, McMahon G, Chen J, Levitzki A and Bohmer FD (1996a) Tyrphostin 5. Potent inhibitors of PDGF receptor tyrosine kinase: SAR in quinixalines, quinolines and indole tyrphostins. *J Med Chem* **39:**2170–2177.

Gazit A, Chen J, App H, McMahon G, Hirth P, Chen I and Levitzki A (1996b) Tyrphostin 4. Highly potent inhibitors of EGF receptor kinase: Structure activity relationship study of 4-anilido quinazolines. *Biorg Med Chem* **8:**1203–1207.

Gazit A, Osherov N, Gilon C and Levitzki A (1996c) Tyrphostin 6. Dimeric benzylidene malono nitriles tyrphostins: Potent inhibitors of EGF receptor tyrosine kinase *in vitro*. *J Med Chem* **39:**4905–4911.

Gazit A, Osherov N, Posner I, Bar-Sinai A, Gilon C, and Levitzki A (1994) Benzylidenemalononitrile tyrphostins. *J Med Chem* **36:**3556–3564.

Gazit A, Osherov N, Posner I, Yaish E, Poradosu C and Levitzki A (1991) Tyrphostins II: Heterocyclic and a-substituted benzonemaloronitrile tyrphostins as potent inhibitors of EGF receptor and ErbB2/neu tyrosine kinases. *J Med Chem* **34:**1897–1907.

Gazit A, Yaish P, Gilon C and Levitzki A (1989) Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors. *J Med Chem* **32:**2344–2352.

Goldman R and Bar-Shavit Z (1979) Dual effect of normal stimulated macrophages and their conditioned media on target cell proliferation. *Cancer Inst* **63:**1004–1016.

Golomb G, Fishbein I, Banai S, Mishaly D, Moscowitz D, Gertz A, Gazit A and Levitzki A (1996) Controlled delivery of a tyrphostin inhibits initial hyperplasia in rat carotid artery injury model. *Atherosclerosis* **125:**171–182.

Greenhalgh DA, Wang KJ, Donehower LA and Roop DR (1996) Paradoxical tumor inhibitory effect of p53 loss in transgenic mice expressing epidermal-targeted v-rasHa, v-fos or human transformic growth factor α. *Cancer Res* **56:**4413–4423.

Howley PM (1991) Role of the human papilloma viruses in human cancers. *Cancer Res* **51:**5019s–5022s.

Kleinberger-Doron N, Shelah N, Capone R, Gazit A and Levitzki A (1998) Inhibition of Cdk2 by selected tyrphostins causes cell cycle arrest at late G_1 and S phase. *Exp Cell Res* **241:**340–351.

Lang HH, Mungal S, Ayscue A, Weissner JD, Wodmicki P, Hockenbery D, Lockett S and Herman B (1995) Bcl-2 protooncogene expression in cervical carcinoma cell lines containing inactive p53. *J Cell Biochem* **57:**509–521.

Malejczyk J, Malejczyk M, Majewski S, Breitburd F, Luger TA, Jablonska S and Orth G (1994) Increased tumorigenicity of human keratinocytes harboring HPV16 is associated with resistance to endogenous TNFα mediated growth inhibition. *Int J Cancer* **56:**593–598.

Mitrani-Rosenbaum S and Tsvieli R (1992) Differential cooperation of a carcinogen with human papilloma virus type 6 &16 DNAs in vitro oncogenic transformation. *Intervirology* **33:**76–85.

Nigro JM, Aldape KD, Hess SW and Testy TD (1997) Cellular adhesion regulates p53 protein levels in primary human keratinocytes. *Cancer Res* **57:**3635–3639.

Osherov N and Levitzki A (1997) Tyrphostin AG 494 blocks Cdk2 activation. *FEBS Lett* **410:**187–190.

Palumbo A, Yaron N, Gazit A, Sandalon Z, Baniyash H, Kleinberger-Doron N, Levitzki A and Ben-Yehuda D (1997) The tyrphostin AG 17 induces apoptosis and inhibition of Cdk-2 activity in lymphoma cell line that overexpresses bcl-2 . *Cancer Res* **57:**2434–2439.

Parker SH, Tong T, Bolden S and Winko PA (1996) Cancer statistics. *Cancer J Clin* **46:**5–27.

Pecoraro G, Worgan D and Defendi V (1989) Differential effects of HPV 6, 16 and 18 DNAs on immortalization and transformation of human cervical epithelial cells. *Proc Natl Acad Sci USA* **86:**563–567.

Powell TJ, Chen H, Shenoy N, Gazit A, Chen I, Ben-Bassat H, Hartzstark Z, Chauat M, Shawver L, and Levitzki A (1999) Inhibition of EGF receptor signal transducction and psoriatic keratinocyte growth by 4-(3-bromophenylamino)-6,7-dimethoxyquinazoline (SU5271). *Br J Dermatol*, in press.

Rheinwald JG and Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinazing colonies from single cells. *Cell* **6:**331–344.

Rheinwald JG and Green H (1988) Epidermal growth factor and the multiplication of cultured human epidermal kertatinocytes. *Nature (Lond)* **265:**76–85.

Scheffner M, Munger K, Byrine J and Housley P (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA* **88:**5523–5527.

Shah KV and Howley PM (1999) Papillomaviruses, in *Virology* (Fields BN ed), Raven Press, New York.

Stopler MC, Straight SW, Tsao G, Schlegel R and McCance DJ (1996) The E5 gene of HPV16 enhances keratinocyte immortalization by full length DNA. *Virology* **223:**251–254.

Straight SW, Humble PW, Jewers RJ and McCance DJ (1993) The E5 oncoprotein of HPV16 transforms fibroblasts and effects the downregulation of the EGFR in keratinocytes. *J Virol* **67:**4521–4532.

Testy TD, White A, Livcuos E, Sage W, Realof H, Briot A and Poulose B (1997) Genomic integrity and the genetics of cancer. *Cold Spring Harbor Symp Quant Biol* **59:**265–267.

Werness BA, Levine AJ and Howley PM (1990) Association of HPV 16 and 18 E6 protein with p53. *Science (Wash DC)* **248:**76–79.

Woodworth CD, Bronden PE, Doniger J, Pirise I, Bornes W, Lancaster WD and DiPaolo JA (1988) Characterization of normal human exocervical epithelial cells immortalized in-vitro by HPV 16 and 18 DNA. *Cancer Res* **48:**4620–4628.

Woodworth CD, McMullin E, Iglesias M and Plowman GD (1995) Interleukin 1a and TNFα stimulate autocrine amphiregulin expression and proliferation of human papilloma-immortalized and carcinoma derived cervical epithelium CELls. *Proc Natl Acad Sci USA* **92:**2840–2844.

Woodworth CD, Wang H, Simpson S, Alvarez-Salas LW and Notorio V (1993) Overexpression of wild-type p53 alters growth and differentiation of normal human keratinocytes but not human papillomavirus-expressing cell lines. *Cell Growth Differ* **4:**367–376.

Zur Hausen H (1991) Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184:**9–13.

Zur Hausen H (1994) Viruses in human cancers. *Science (Wash DC)* **254:**1167–1173.

Send reprint requests to: Hannah Ben-Bassat, Laboratory of Experimental Surgery, Hadassah University Hospital, Jerusalem 91120, Israel.