Genistein and erbstatin inhibition of normal mammary epithelial cell proliferation is associated with EGF-receptor down-regulation

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Abstract. Epidermal growth factor (EGF) is a potent mitogen for normal mouse mammary epithelial cells grown in primary culture. EGF activation of the EGF-receptor (EGF-R) induces intrinsic tyrosine kinase activity which results in EGF-R autophosphorylation and tyrosine phosphorylation of other intracellular substrates involved in EGF-R signal transduction. Genistein and erbstatin are anticancer agents which have been shown to be potent tyrosine kinase inhibitors. However, the effects of these compounds in modulating EGF-dependent normal mammary epithelial cell proliferation is presently unknown. Therefore, studies were conducted to determine the effects of genistein and erbstatin on EGF-dependent proliferation, and EGF-R levels and autophosphorylation in normal mouse mammary epithelial cells grown in primary culture and maintained in serum-free media. Chronic treatment with 6.25–100 μM genistein or 1–16 μM erbstatin significantly decreased EGF-dependent mammary epithelial cell proliferation in a dose-responsive manner. However, the highest doses of genistein (100 μM) and erbstatin (16 μM) were found to be cytotoxic. Additional studies showed that acute treatment with 6.25–400 μM genistein did not affect EGF-R levels or EGF-induced EGF-R autophosphorylation, while acute treatment with 1–64 μM erbstatin caused a slight reduction in EGF-R levels, but had no effect on EGF-dependent EGF-R autophosphorylation in these cells. In contrast, chronic treatment with similar doses of genistein or erbstatin resulted in a large dose-responsive decrease in EGF-R levels, and a corresponding decrease in total cellular EGF-R autophosphorylation intensity. These results demonstrate that the inhibitory effects of chronic genistein and erbstatin treatment on EGF-dependent mammary epithelial cell proliferation is not due to a direct inhibition of EGF-R tyrosine kinase activity, but results primarily from a down-regulation in EGF-R levels and subsequent decrease in mammary epithelial cell mitogenic-responsiveness to EGF stimulation.

Normal mammary gland growth and function is dependent upon a variety of hormones and growth factors (Imagawa, Bandyopadhyay & Nandi 1990). *In vitro* studies have shown that epidermal growth factor (EGF) is a potent mitogen for normal mammary epithelial cells

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grown in primary culture (Imagawa, Tomooka & Nandi 1982, Imagawa *et al.* 1990). The mitogenic actions of EGF are mediated through specific membrane-bound receptors (EGF-R) which contain a cytoplasmic tyrosine kinase domain (Ullrich *et al.* 1984, Carpenter 1987). In normal cells, tyrosine phosphorylation is highly regulated and represents only a small percentage of total intracellular protein phosphorylation (Jove & Hanafusa 1987, Hunter 1989, Ullrich & Schlessinger 1990). However, neoplastic transformation is characterized by large increases in intracellular protein tyrosine phosphorylation (Jove & Hanafusa 1987, Hunter 1989, Ullrich & Schlessinger 1990). Furthermore, a variety of oncogenic products which are associated with mammary epithelial cell neoplastic transformation, such as HER2, are members of the EGF-receptor (EGF-R) superfamily and display constitutively high levels of tyrosine kinase activity (Nicholson *et al.* 1993, Dougall, Qian & Greene 1993).

Genistein (5,7,4'-dihydroxyisoflavone) and erbstatin (methyl 2,5-dihydroxycinnamate) are anticancer agents which have been shown to act as potent inhibitors of tyrosine kinase activity (Akiyama *et al.* 1987, Imoto *et al.* 1987, Toi *et al.* 1990, Umezawa *et al.* 1990, Burke 1992, So *et al.* 1996). Although the antiproliferative effects of these compound in tumour cells has been associated with their actions to inhibit tyrosine kinase activity, the effects of genistein and erbstatin on EGF-R tyrosine kinase mitogenic signalling in normal mammary epithelial cells is presently unknown. Therefore, studies were conducted to investigate the interrelationship between the effects of genistein and erbstatin on EGF-R levels, EGF-induced EGF-R autophosphorylation, and EGF-dependent mitogenesis in normal mouse mammary epithelial cells grown in primary culture and maintained in serum-free media.

MATERIALS AND METHODS

Isolation and culture of mammary epithelial cells

Mammary epithelial cells were isolated and prepared for culture, as previously described (Sylvester *et al.* 1994). All materials were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated. Briefly, mammary glands from midpregnant BALB/c mice were removed, minced, and subjected to collagenase followed by pronase E (Type XIV bacterial from *Streptomyces griseus*) digestion. Mammary epithelial cells isolated from midpregnant mice were selected for experimentation because these cells are highly responsive to EGF-induced mitogenesis (Imagawa *et al.* 1990). Mammary epithelial cell organoids were isolated by a series of filtrations through sterile 250 μm and 48 μm nitex filters (Tetko Inc., Briarcliff Manor, NY), plated within rat tail collagen gels at a density 2.2×10^7 cells/8 ml of collagen in 100 mm plastic plates, fed 10 ml growth media (DMEM/F12 1:1, insulin 10 $\mu\text{g}/\text{ml}$, penicillin 100 U/ml, streptomycin 100 $\mu\text{g}/\text{ml}$, 10% bovine calf serum (Hyclone, Logan, UT), and placed in a humidified incubator at 37°C in an environment of 95% air and 5% CO₂. After a 2-day incubation period, mammary epithelial cell organoids were again isolated by collagenase digestion and filtration. This double isolation procedure provides isolated mammary epithelial cell organoids ready for experimentation, which are devoid of fibroblast and adipocyte contaminants (Jones *et al.* 1983). Mammary epithelial cells were then plated within collagen gels at an initial density 2×10^5 cells/well in 24 well tissue culture plates and maintained on serum-free treatment media consisting of DMEM/F12 containing 5 mg/ml BSA, 10 $\mu\text{g}/\text{ml}$ transferrin, 100 U/ml soybean trypsin inhibitor, 1 $\mu\text{g}/\text{ml}$ d- α -tocopherol, 10 ng/ml EGF, 10 $\mu\text{g}/\text{ml}$ insulin, and either 6.25–100 μM genistein or 1–16 μM erbstatin analogue (methyl 2,5-dihydroxycinnamate, LC Laboratories, Woburn, MA).

Genistein was dissolved in dimethylsulfoxide (DMSO), whereas erbstatin was dissolved in 100% ethanol prior to addition to treatment media. DMSO or ethanol was added to all treatment media so that the final concentrations were the same in all treatment groups within a given experiment, and did not exceed 0.02% (v/v).

Measurement of viable cell number

Mammary epithelial cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colourimetric assay as described previously (Sylvester *et al.* 1994). On the day of assay, treatment media was replaced with fresh growth media containing 0.83 mg/ml MTT and returned to the incubator for 4 h. Afterwards, gels were removed from the culture plates, placed 12 × 75 mm test tubes, and dissolved in 0.5 ml of 25% acetic acid. MTT crystals were pelleted by centrifugation and the MTT crystals dissolved in 1 ml of propan-2-ol. Optical density of each sample was read on a microplate reader (model 7520 Cambridge Technology, Inc., Watertown, MA) at 570 nm against a blank prepared from cell-free collagen gels. The number of cells/well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by haemocytometer, at the start of each experiment (Sylvester *et al.* 1994). In separate control studies, various doses (0–100 μM) of genistein and erbstatin (0–16 μM) were not found to affect the specific activity of the MTT colourimetric assay.

Discontinuous electrophoresis and Western blot analysis

Sample preparation has been previously described in detail (McIntyre, Birkenfeld & Sylvester 1995, Birkenfeld *et al.* 1996). Isolated mammary epithelial cells were plated in 100 mm culture plates (1×10^7 cells/8 ml of collagen), and cultured in defined media (DMEM/F12, 10 $\mu\text{g/ml}$ d- α -tocopherol, 10 $\mu\text{g/ml}$ soybean trypsin inhibitor, 10 $\mu\text{g/ml}$ apotransferrin, 10 $\mu\text{g/ml}$ insulin and 10 ng/ml EGF). To determine the effects of genistein and erbstatin on EGF-induced autophosphorylation, mammary epithelial cells were isolated from their collagen gels with collagenase, washed three times, and resuspended in EGF-free defined media containing various treatment doses of genistein (0–400 μM) or erbstatin (0–64 μM). After a 3 h incubation, cells were washed with ice-cold DMEM/F12 and stimulated with 100 ng/ml EGF for 5 min. Cells were then pelleted, rinsed, and lysed with 100 μl Laemmli sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 100 μM sodium orthovanadate 6.8 pH). For studies characterizing the chronic effects of genistein and erbstatin on EGF-R levels and tyrosine phosphorylation, cells were cultured in defined media containing various doses of either genistein (0–100 μM) or erbstatin (0–16 μM). After 5 days in culture, cells were isolated from collagen gels with collagenase, pelleted, washed and then immediately lysed with Laemmli buffer as described above. Protein concentrations in each sample were determined using a Bio-Rad protein assay kit (Bio-RAD, Hercules, CA) according to the manufacturer's directions. Equal amounts of protein from each sample (20 $\mu\text{g/lane}$) were loaded on polyacrylamide minigels and electrophoresed through a 7.5% resolving gel. Proteins were transblotted (25 V for 12–16 h) to PVDF membranes (Dupont, Boston, MA) according to the method of Towbin (Towbin, Staehelin & Gordon 1979). To determine EGF-induced EGF-R autophosphorylation, membranes were blocked with 2% BSA in 10 mM Tris-HCl containing 50 mM NaCl and 0.1% Tween 20, pH 7.4 (TBST) and then incubated with anti-phosphotyrosine monoclonal antibody (PY20) (Transduction Laboratories, Lexington, KY) diluted 1:5000 in TBST with 2% BSA for 2 h. Membranes were then rinsed four times with TBST and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Transduction Laboratories, Lexington, KY),

diluted 1:5000 in TBST with 2% BSA for 1 h. Afterwards, blots were rinsed with TBST and protein bands were visualized by chemiluminescence according to the manufacture's instructions (Pierce, Rockford, IL). After Western blot analysis for phosphotyrosine was concluded, the same membranes were stripped (Kaufmann, Ewing & Shaper 1987), washed in TBST with 2% BSA, and then re-probed for EGF-R (anti-EGF-R antibody clone F4, Sigma Chemical Co.) diluted 1:2000 (McIntyre *et al.* 1995). In each experiment, blots from each treatment group was exposed on the same piece of film (Kodak X-OMAT AR, Rochester, NY). Images were acquired with a HP Scan Jet scanner (Hewlett Packard, Sunnyvale, CA) and analysed with RFLPscan software (Scanalytics, Billerica, MA). All experiments were repeated at least three times.

Statistical analysis

Differences among the various treatment groups were determined by analysis of variance, followed by Duncan's multiple range test. A difference of $P < 0.05$ was considered to be significant, as compared to controls.

RESULTS

The effects of various doses of genistein and erbstatin on EGF-dependent normal mammary epithelial cell proliferation in primary culture are shown in Figure 1. After 5 days in culture, cells grown in serum-free control media containing 10 ng/ml EGF displayed more than a twofold increase in viable cell number, as compared to day 1 of culture (Figure 1a and b). Treatment with either 6.25–100 μM genistein (Figure 1a) or 2–16 μM erbstatin (Figure 1b) significantly inhibited mammary epithelial cell growth in a dose-responsive manner, as compared to controls. However, the highest doses of genistein (100 μM) and erbstatin (16 μM) were found to be cytotoxic, since viable cell number in these groups were less on day 5 than initially plated on day 1 of culture (Figure 1a and b).

The effects of acute treatment with various doses of genistein on EGF-R levels and EGF-induced EGF-R autophosphorylation are shown in Figure 2. Whole cell lysates obtained from mammary epithelial cells acutely treated for 3 h with 6.25–400 μM genistein showed no differences in relative levels of EGF-R prior to (Figure 2a, EGF-R lanes 2–8) or after (Figure 2b, EGF-R lanes 2–8) exposure to 100 ng/ml EGF, as compared to controls (Figure 2a and b, EGF-R lane 1). When these same membranes were subjected to Western blot analysis for phosphotyrosine intensity, it was found that prior to EGF exposure, a 170 kDa protein band which correlated with the EGF-R displayed a low level of basal tyrosine phosphorylation in all treatment groups (Figure 2a, PY lanes 1–8). After a 5-min exposure to 100 ng/ml EGF, the 170 kDa band EGF-R band displayed an intense increase in tyrosine autophosphorylation in controls (Figure 2b, PY lane 1), and acute treatment with 6.25–100 μM genistein did not alter this response (Figure 2b, PY lanes 2–6). However, acute treatment with extremely high and cytotoxic doses of genistein (200–400 μM) were found to greatly reduce EGF-induced EGF-R autophosphorylation in these cells (Figure 2b, PY lanes 7–8). Scanning densitometric analysis of acute treatment effects of genistein on EGF-R levels and EGF-R phosphotyrosine intensity are shown below their respective Western blots (Figure 2c and d).

The effects of acute treatment with various doses of erbstatin on EGF-R levels and EGF-induced EGF-R autophosphorylation are shown in Figure 3. Whole cell lysates obtained from mammary epithelial cells acutely treated for 3 h with 1–64 μM erbstatin showed a reduction in relative EGF-R levels prior to (Figure 3a, EGF-R lanes 2–8) and after

(Figure 3b, EGF-R lanes 2–8) a 5-min exposure to 100 ng/ml EGF, as compared to their respective controls (Figure 3a and b, EGF-R lane 1). When these same membranes were subjected to Western blot analysis for phosphotyrosine intensity, it was found that prior to EGF exposure, a 170 kDa protein band which correlated with the EGF-R displayed a low level of basal tyrosine phosphorylation in all treatment groups (Figure 3a, PY lanes 1–8). Following a 5 min exposure to 100 ng/ml EGF, the 170 kDa EGF-R band displayed a large increase in phosphotyrosine intensity in controls, and acute treatment with 1–64 μM erbstatin had no effect on this response (Figure 3b, PY lanes 1–8). Scanning densitometric analysis of

Figure 1. Effects of genistein (a) and erbstatin (b) on EGF-dependent mammary epithelial cell growth in primary culture. Viable cell number was determined by the MTT colourimetric assay on days 1 and 5 of culture. Vertical bars represent mean cell number $\pm$ SEM for six wells in each treatment group. * $P < 0.05$, as compared to controls within each respective treatment groups.

Figure 2. Effects of various doses of genistein on EGF-R levels and EGF-induced autophosphorylation of the 170 kDa EGF-R. Mammary epithelial cells were isolated from collagen gels on day 5 of culture and then resuspended in EGF-free media containing 0, 6.25, 12.5, 25, 50, 100, 200, or 400 μM genistein. After a 3 h incubation period, cells were exposed to 0 or 100 ng/ml EGF for 5 min. Whole cell lysates (20 $\mu\text{g}/\text{lane}$) from the different treatment groups were fractionated by SDS-PAGE and then subjected to Western blot analysis for the phosphotyrosine (PY) followed by EGF-R prior to (a) and after (b) exposure to EGF. Scanning densitometric analysis of EGF-R levels and tyrosine phosphorylation intensity visualized in Western blots displayed in (a) and (b) are shown in (c) and (d), respectively.

Erbstatin concentration (μM)

Figure 3. Effects of various doses of erbstatin on EGF-R levels and EGF-induced autophosphorylation of the 170 kDa EGF-R. Mammary epithelial cells were isolated from collagen gels on day 5 of culture and then resuspended in EGF-free media containing 0, 1, 2, 4, 8, 16, 32, or 64 μM erbstatin. After a 3 h incubation period, cells were exposed to 0 or 100 ng/ml EGF for 5 min. Whole cell lysates (20 $\mu\text{g}/\text{lane}$) from the different treatment groups were fractionated by SDS-PAGE and then subjected to Western blot analysis for phosphotyrosine (PY) followed by EGF-R prior to (a) and after (b) exposure to EGF. Scanning densitometric analysis of EGF-R levels and tyrosine phosphorylation intensity visualized in Western blots displayed in (a) and (b) are shown in (c) and (d), respectively.

acute treatment effects of erbstatin on EGF-R levels and EGF-R phosphotyrosine intensity are shown below their respective Western blots (Figure 3c and d).

The effects of chronic genistein and erbstatin treatment on EGF-R levels and EGF-R tyrosine phosphorylation intensity are shown in Figure 4. Western blot analysis for EGF-R in whole cell lysates obtained from control mammary epithelial cells showed an intense 170 kDa protein band (Figure 4a and b, EGF-R lane 1). Chronic treatment for 5 days with 6.25–100 μM genistein resulted in a large decrease in relative EGF-R levels (Figure 4a, EGF-R lanes 2–6). Western blot analysis for phosphotyrosine intensity in these same membranes showed that chronic treatment with 6.25–100 μM genistein caused a dose-responsive decrease in total intracellular EGF-R tyrosine autophosphorylation, as compared to controls (Figure 4a, PY lanes 1–6). Similarly, chronic treatment for 5 days with 2–16 μM erbstatin resulted in a large decrease in relative EGF-R levels in normal mammary epithelial cells, as compared to controls (Figure 4b, EGF-R lanes 1–6). When these same membranes were subjected to Western blot analysis for phosphotyrosine, chronic treatment with 2–16 μM erbstatin resulted in a decrease in total cellular EGF-R tyrosine autophosphorylation intensity (Figure 4b, PY lanes 1–6). Scanning densitometric analysis of chronic treatment effects of genistein and erbstatin on EGF-R levels and EGF-R phosphotyrosine intensity are shown below their respective Western blots (Figure 4c and d).

DISCUSSION

Results in this study demonstrate that treatment with genistein and erbstatin significantly inhibits EGF-dependent mitogenesis in normal mammary epithelial cells grown in primary culture. Although these compounds have been shown to act as potent tyrosine kinase inhibitors in various tumour cell lines, the antiproliferative effects of genistein and erbstatin in normal mammary epithelial cells were not found to be directly associated with a reduction in EGF-R tyrosine kinase activity. Acute treatment with doses of genistein or erbstatin which were effective in suppressing mammary epithelial cell proliferation, had little or no effect on EGF-induced EGF-R tyrosine autophosphorylation. Furthermore, genistein and erbstatin treatment were found to inhibit EGF-R tyrosine kinase activity only when extremely high and cytotoxic doses were used. However, these studies did show that chronic treatment of genistein or erbstatin caused a dose-responsive decrease in EGF-R levels in normal mammary epithelial cells. These findings indicate that the antiproliferative effects of genistein and erbstatin primarily result from a down-regulation in EGF-R and subsequent decrease in normal mammary epithelial cell mitogenic-responsiveness to EGF stimulation.

At present, the exact mechanism mediating genistein and erbstatin-induced decrease in cellular EGF-R levels is unknown. Previous studies have demonstrated that genistein induces the activation of c-Jun N-terminal kinase (JNK), a member in the family of stress-kinases (Croisy-Delcey *et al.* 1997), and JNK activation results in the phosphorylation of various transcription factors, particularly c-jun (Anderson 1997). Since c-jun activation is associated with the down-regulation of the EGF-R in some types of cells (Okimoto *et al.* 1996), genistein and erbstatin might also induce EGF-R down-regulation in normal mammary epithelial cells through JNK activation. In addition, erbstatin has been shown to cross-link irreversibly with the EGF-R to form inactive receptor/drug aggregates (Stanwell, Burke & Yuspa 1995). Studies have shown that EGF-R aggregates are targeted for elimination by endocytosis and enzymatic degradation (Carpenter 1987). Although previous investigations showed that doses greater than 50 μM were required to induce erbstatin/EGF-R cross-linking, it is possible that chronic exposure to low doses of erbstatin may also result in the formation

Figure 4. Effects of chronic genistein (a) and erbstatin (b) treatment on EGF-R levels and tyrosine phosphorylation intensity. Mammary epithelial cells were cultured for 5 days in serum-free media containing 10 ng/ml EGF and 10 $\mu\text{g/ml}$ insulin, and supplemented with various doses of genistein or erbstatin. Whole cell lysates were prepared from mammary epithelial cells in the various treatment groups, fractionated by SDS-PAGE (20 $\mu\text{g/lane}$) and then subjected to Western blot analysis for phosphotyrosine (PY) followed by EGF-R. Scanning densitometric analysis of EGF-R levels and tyrosine phosphorylation intensity visualized in Western blots displayed in (a) and (b) are shown in (c) and (d), respectively.

of erbstatin/EGF-R complexes which could ultimately lead to a down-regulation in cellular EGF-R levels.

Findings in this present study support and extend those of previous investigations which demonstrated that treatment with genistein and erbstatin inhibited growth of various tumour cell lines which constitutively overexpress EGF-R (Akiyama *et al.* 1987, Dean *et al.* 1989, Umenzawa *et al.* 1990, Peterson 1995). However, in contrast to the present findings these previous studies showed that low doses of genistein and erbstatin caused a decrease in EGF-R tyrosine kinase activity and EGF-R autophosphorylation (Akiyama *et al.* 1987, Imoto *et al.* 1987, Umenzawa *et al.* 1990). Since EGF-R autophosphorylation is believed to be a prerequisite for EGF-dependent mitogenesis, it was concluded that the growth inhibitory effects of genistein and erbstatin in these tumour cell lines were directly related to their inhibitory effects on EGF-R tyrosine kinase activity (Akiyama *et al.* 1987, Imoto *et al.* 1987, Dean *et al.* 1989, Umenzawa *et al.* 1990). However, the effect of genistein and erbstatin on cellular EGF-R levels was not investigated in these previous studies.

The possibility also exist that the antiproliferative effects of genistein and erbstatin are mediated through mechanisms independent of receptor tyrosine kinase levels and/or activity. Treatment with very high doses of genistein has been shown to inhibit the activity of enzyme systems other than tyrosine kinases (Akiyama *et al.* 1987, Peterson 1995). Treatment with genistein at doses greater than 100 μM has been shown to inhibit the activity of protein kinase A, protein kinase C, and topoisomerase II (Akiyama *et al.* 1987, Markovits *et al.* 1989, Peterson 1995, Barnes 1995). Similarly, treatment with doses greater than 50 μM erbstatin has been shown to inhibit protein kinase C, and topoisomerase I and II activity (Bishop *et al.* 1990, Markovits *et al.* 1994, Azuma *et al.* 1995, Simizu *et al.* 1996). High doses of erbstatin has also been shown to generate reactive oxygen species which can cause DNA damage and stimulate apoptosis (Simizu *et al.* 1996). However, these effects were observed in other cell types using doses of genistein and erbstatin which were found in this present study to be cytotoxic to normal mammary epithelial cells. Additional studies are needed to determine if the antiproliferative effects induced by treatment with low doses of genistein and erbstatin result from the inhibition of tyrosine kinase-independent enzymes systems which are also associated with EGF-R mitogenic signalling in normal mammary epithelial cells.

In conclusion, genistein and erbstatin inhibition of EGF-dependent mitogenesis in normal mammary epithelial cells is associated with EGF-R down-regulation, and corresponding decrease in mammary epithelial cell mitogenic-responsiveness to EGF stimulation. In contrast to previous reports, the antiproliferative effects of these compounds were not found to be directly related to a reduction in EGF-dependent EGF-R tyrosine-kinase activity.

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REFERENCES

- AKIYAMA T, ISHIDA J, NAKAGAWA S *et al.* (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **262**, 5592.
- ANDERSON P. (1997) Kinase cascades regulating entry into apoptosis. *Microbiol. Mol. Biol. Rev.* **61**, 33.
- AZUMA Y, ONISHI Y, SATO Y, KIZAKI H. (1995) Effects of protein tyrosine kinase inhibitors with different modes of action on topoisomerase and death of IL-2-dependent CTLL-2 cells. *J. Biochem.* **118**, 312.

- BARNES S. (1995) Effects of genistein on *in vitro* and *in vivo* models of cancer. *J. Nutr.* **125**, 777S.
- BISHOP WR, PETRIN J, WANG L, DOLL RJ. (1990) Inhibition of protein kinase C by the tyrosine kinase inhibitor erbstatin. *Biochem. Pharmacol.* **40**, 2129.
- BIRKENFELD HP, MCINTYRE BS, BRISKI KP, SYLVESTER PW. (1996) Role of protein kinase C in modulating epidermal growth factor- and phorbol ester-induced mammary epithelial cell growth *in vitro*. *Exp. Cell Res.* **223**, 183.
- BURKE TR. (1992) Protein-tyrosine kinase inhibitors. *Drugs Fut.* **17**, 119.
- CARPENTER G. (1987) Receptors for the epidermal growth factor and other polypeptide mitogens. *Ann. Rev. Biochem.* **56**, 881.
- CROISY-DELCEY M, CROISY A, MOUSSET S *et al.* (1997) Genistein analogues: effects on epidermal growth factor receptor tyrosine kinase and on stress-activated pathways. *Biomed. Pharmacother.* **51**, 286.
- DEAN NM, KANEMITSU M, BOYNTON AL. (1989) Effects of the tyrosine-kinase inhibitor genistein on DNA synthesis and phospholipid-derived second messenger generation in mouse 10T1/2 fibroblasts and rat liver T51B cells. *Biochem. Biophys. Res. Comm.* **165**, 795.
- DOUGALL WC, QIAN X, GREENE MI. (1993) Interaction of the neu/p185 and EGF receptor tyrosine kinases: implications for cellular transformation and tumor therapy. *J. Cell. Biochem.* **53**, 61.
- HUNTER T. (1989) Protein modification: phosphorylation on tyrosine residues. *Curr. Opin. Cell Biol.* **1**, 1168.
- IMAGAWA W, BANDYOPADHYAY GK, NANDI S. (1990) Regulation of mammary epithelial cell growth in mice and rats. *Endocr. Rev.* **11**, 494.
- IMAGAWA W, TOMOOKA Y, NANDI S. (1982) Serum-free growth of normal and tumor mouse mammary epithelial cells in primary culture. *Proc. Natl. Acad. Sci. (USA)* **79**, 4074.
- IMOTO M, UMEZAWA K, SAWA T, TAKEUCHI, UMEZAWA H. (1987) *In situ* inhibition of protein tyrosine kinase by erbstatin. *Biochem. Int.* **15**, 989.
- JONES W, HALLOWES RC, CHOONGKITTAWORN N, HOSICK HL, DILS R. (1983) Isolation of the epithelial subcomponents of the mouse mammary gland for tissue-level culture studies. *J. Tiss. Cult. Methods* **8**, 17.
- JOVE R, HANAFUSA H. (1987) Cell transformation by the viral src oncogene. *Ann. Rev. Cell Biol.* **3**, 31.
- KAUFMANN SH, EWING CM, SHAPER JH. (1987) The erasable Western blot. *Anal. Biochem.* **161**, 89.
- MARKOVITS J, LINASSIER C, FOSS'E P *et al.* (1989) Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. *Cancer Res.* **49**, 5111.
- MARKOVITS J, LARSEN AK, S'EGAL-BENDIRDJIAN E. (1994) Inhibition of DNA topoisomerase I and II and the induction of apoptosis by erbstatin and tyrophostin derivatives. *Biochem. Pharmacol.* **48**, 549.
- MCINTYRE BS, BIRKENFELD HP, SYLVESTER PW. (1995) Relationship between epidermal growth factor receptor levels, autophosphorylation and mitogenic-responsiveness in normal mouse mammary epithelial cells *in vitro*. *Cell Prolif.* **28**, 45.
- NICHOLSON RI, MCCLELLAND, FINLAY P *et al.* (1993) Relationship between EGF-R, c-erbB-2 protein expression and Ki67 immunostaining in breast cancer and hormone sensitivity. *Eur. J. Cancer* **29**, 1018.
- PETERSON G. (1995) Evaluation of the biochemical targets of genistein in tumor cells. *J. Nutr.* **125**, 784S.
- OKIMOTO T, KOHNO K, KUWANO M, GOPAS J, KUNG HF, ONO M. (1996) Regulation of epidermal growth factor receptor by activated H-ras and V-myc oncogenes in mouse Balb/3T3 cells: possible roles of AP-1. *Oncogene* **12**, 1625.
- SIMIZU S, IMOTO M, MASUDA N, TAKADA M, UMEZAWA K. (1996) Involvement of hydrogen peroxide production in erbstatin-induced apoptosis in human small lung carcinoma cells. *Cancer Res.* **56**, 4978.
- SO FV, GUTHRIE N, CHAMBERS AF, MOUSSA M, CARROLL KK. (1996) Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr. Cancer.* **26**, 167.
- STANWELL C, BURKE TR JR, YUSPA SH. (1995) The erbstatin analogue 2,5-dihydroxycinnamate cross-links proteins and is cytotoxic to normal and neoplastic epithelial cells by a mechanism independent of tyrosine kinase inhibition. *Cancer Res.* **55**, 4950.

- SYLVESTER PW, BIRKENFELD HP, HOSICK HL, BRISKI KP. (1994) Fatty acid modulation of epidermal growth factor-induced mouse mammary epithelial cell proliferation *in vitro*. *Exp. Cell Res.* **214**, 145.
- TOI M, MUKAIDA H, WADA T *et al.* (1990) Antineoplastic effect of erbstatin on human mammary and esophageal tumors in athymic nude mice. *Eur. J. Cancer* **26**, 722.
- TOWBIN H, STAEGELIN T, GORDON J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. (USA)* **76**, 4350.
- ULLRICH A, SCHLESSINGER J. (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203.
- ULLRICH A, COUSSENS L, HAYFLICK JS *et al.* (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* **309**, 418.
- UMEZAWA, HORI T, TAJIMA H, IMOTO M, ISSHIKI K, TAKEUCHI T. (1990) Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. *FEBS Lett.* **260**, 198.