Dual Inhibition of Both the Epidermal Growth Factor Receptor and erbB2 Effectively Inhibits the Promotion of Skin Tumors during Two-Stage Carcinogenesis

Kaoru Kiguchi1, Takuya Kitamura1, Tricia Moore1, Mohammad Rumi1, Hsiang-Chun Chang1, Devon Treece1, Lynnsie Ruffino1, Kevin Connolly1, and John DiGiovanni1,2

Abstract

The erbB family of receptor tyrosine kinases are known to play important roles in normal epithelial development and epithelial neoplasia. Considerable evidence also suggests that signaling through the epidermal growth factor receptor (EGFR) plays an important role in multistage skin carcinogenesis in mice; however, less is known about the role of erbB2. In this study, to further examine the role of both erbB2 and EGFR in epithelial carcinogenesis, we examined the effect of a dual erbB2/EGFR tyrosine kinase inhibitor, GW2974, given in the diet on skin tumor promotion during two-stage carcinogenesis in wild-type and BK5.erbB2 mice. In BK5.erbB2 mice, erbB2 is overexpressed in the basal layer of epidermis and leads to heightened sensitivity to skin tumor development. GW2974 effectively inhibited skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate in wild-type and BK5.erbB2 mice, although a more marked effect was seen in BK5.erbB2 mice. In addition, this inhibitory effect was reversible when GW2974 treatment was withdrawn. GW2974 inhibited 12-O-tetradecanoylphorbol-13-acetate–induced epidermal hyperproliferation, which correlated with reduced activation of both the EGFR and erbB2. These results support the hypothesis that both the EGFR and erbB2 play an important role in the development of skin tumors during two-stage skin carcinogenesis, especially during the tumor promotion stage. Furthermore, the marked sensitivity of BK5.erbB2 mice to the inhibitory effects of GW2974 during tumor promotion suggest greater efficacy for this compound when erbB2 is overexpressed or amplified as an early event in the carcinogenic process. Cancer Prev Res; 3(8); 940–52. ©2010 AACR.

Introduction

Several receptor tyrosine kinases have been described (1–3). Among them, the erbB family of receptor tyrosine kinases [epidermal growth factor receptor (EGFR), erbB2, erbB3, and erbB4] is important in normal epidermal development, as well as in neoplasia (1, 4). The variety of post-receptor signaling pathways activated by ligand binding, including signaling through the Ras/mitogen-activated protein/extracellular signal-regulated kinase/mitogen-activated protein kinase/extracellular signal-regulated kinase, phospholipase Cγ, signal transducers and activators of transcription, and phosphatidylinositol-3 kinase pathways that are common to nearly all receptor tyrosine kinases, is the result of both the diversity of ligands they bind and the heterodimerization that occurs between the various erbB family members (5, 6). Although all of the erbB family members share similarities in primary structures, receptor activation mechanisms, and signal transduction patterns, they bind to different ligands. Ligand-dependent activation of erbB family receptors can lead to both homodimerization and heterodimerization (7). To date, no ligand has been identified for erbB2 (8–11); it can only act as part of a heterodimer with a ligand-bound receptor, often EGFR or erbB3 (5, 6). In contrast, erbB3 cannot generate signals in isolation because the kinase function of this receptor is impaired, thus relying on interaction with erbB2 for subsequent downstream signaling events (12).

Considerable evidence exists showing that signaling through the EGFR plays an important role during both two-stage and UV light–induced carcinogenesis in mouse skin (13–15). In previous experiments, we found that topical application of diverse tumor promoters elevated the mRNA and protein levels of EGFR ligands including transforming growth factor α (TGF-α), amphiregulin, and heparin-binding EGF-like growth factor (13). The expression levels (both mRNA and protein) of the EGFR and EGFR ligands were also constitutively elevated in primary papillomas and squamous cell carcinomas.
(SCC) generated by an initiation-promotion regimen (13, 16). Studies with transgenic mice have shown that the overexpression of TGF-α in basal or suprabasal epidermal cells can substitute for ras activation and leads to epidermal hyperplasia (17–21). Papillomas develop following initiation with 7,12-dimethylbenz(a)anthracene (DMBA) in TGF-α transgenic mice, indicating that constitutive EGFR signaling can also substitute for the promotion stage of multistage skin carcinogenesis (18). Abrogation of EGFR function in keratinocytes has been shown to inhibit growth and development of skin tumors through reduction in proliferation of basal cells (22, 23). Finally, EGFR activation is critical for UV-induced epidermal proliferation (24, 25) and inhibition of EGFR tyrosine kinase activity with AG1478-inhibited UV promotion of skin tumors in TG:AC mice (14).

In contrast to EGFR, less is known about the role of erbB2 in mouse skin carcinogenesis. We previously reported the activation of both EGFR and erbB2, and the presence of EGFR:erbB2 heterodimers in EGF-stimulated mouse keratinocytes, 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated mouse epidermis, and the epidermis of K14.TGF-α transgenic mice (26). These results suggested the possibility that erbB2 may facilitate signaling through the EGFR during the tumor promotion stage of mouse skin carcinogenesis. To further study the role of erbB2 in skin and skin tumor promotion and skin carcinogenesis, we and others developed transgenic mouse models in which an activated form of erbB2 (rat neu oncogene) was overexpressed in epidermis (27–29). In these transgenic lines, a dramatic phenotype was observed characterized by severe epithelial hyperplasia in multiple organs, including the skin, in which hyperplasia of the hair follicles was particularly striking. To avoid the severity of this phenotype and shortened life span, we generated transgenic mice that overexpress wild-type rat erbB2 under the control of the BK5 promoter (BK5.erbB2 mice; ref. 30). In BK5.erbB2 mice, the overexpression of wild-type erbB2 in the basal layer of the epidermis led to increased proliferation and hyperplasia of both the follicular and interfollicular epidermis as well as sebaceous gland enlargement. In addition, these mice, especially those homozygous for the transgene, displayed significant alopecia as a result of the follicular changes observed. Homozygous BK5.erbB2 transgenic mice also developed spontaneous papillomas, some of which converted to SCCs. Both homozygous and hemizygous BK5.erbB2 transgenic mice were also hypersensitive to the proliferative effects of TPA and were more sensitive to two-stage carcinogenesis. More recently, Hansen and colleagues (31, 32) have shown that erbB2 signaling is also involved in the actions of UV in inducing epidermal proliferation and hyperplasia, and that inhibition of erbB2 using AG825 blocked UV promotion of skin tumors in TG:AC mice (33).

In the current study, to further examine the role of both the EGFR and erbB2 in skin carcinogenesis, we examined the effect of a dual EGFR/erbB2 tyrosine kinase inhibitor (TKI), GW2974, on skin tumor promotion by TPA in wild-type and BK5.erbB2 mice. Treatment with GW2974 throughout the tumor promotion stage resulted in a significant inhibition of tumor formation in wild-type and BK5.erbB2 mice, although the effect was greater in the transgenic mice. Thus, GW2974 has substantial chemopreventive activity through blockade of EGFR/erbB2 signaling during skin tumor promotion. The greater efficacy of GW2974 in BK5.erbB2 mice suggests that this compound may be particularly effective in epithelial cancers in which erbB2 is overexpressed or amplified early in the carcinogenesis process.

Materials and Methods

Tumor induction and treatment

Both wild-type and BK5.erbB2 mice (20–25 mice, 7–8 wk of age, in each group) were shaved 2 days before initiation. Mice were initiated with 50 nmol of DMBA in 0.2 mL of acetone. The mice received either the semisynthetic AIN76A control diet or AIN76A diet containing a supplement of 200 ppm GW2974 supplied by GlaxoSmithKline beginning 2 weeks after tumor initiation. Two weeks later, a promotion regimen consisting of the topical application of 6.8 nmol of TPA in 0.2 mL of acetone twice per week was started. The group of mice receiving the GW2974 was divided into two groups, one of which received GW2974 for the entire experiment, whereas the other was switched to AIN76A control diet after 19 and 9 weeks of TPA treatment in experiment #1 and experiment #2, respectively (Fig. 1). At the end of each experiment, the mice were sacrificed by cervical dislocation, and the tumors and skin were removed and either snap-frozen in liquid nitrogen for Western blot analysis or fixed with formalin for histologic evaluation. All tissues were analyzed through histologic analysis. The mice were examined throughout the experiment, and tumor multiplicity (tumors per mouse) as well as tumor incidence, was recorded weekly.

Thirty minutes before sacrifice in both experiments, mice were injected with bromodeoxyuridine (BrdUrd; 100 μg/g of body weight). To evaluate systemic toxicity, body weight, feed consumption, and neurologic function, the mice were physically assessed twice per week. All experiments were carried out with strict adherence to institutional guidelines for minimizing distress in experimental animals.

Immunofluorescence staining and Western blot analysis

The expression and localization of erbB2 and phospho-erbB2 (p-erbB2) were determined using immunofluorescence techniques on sections of skin as previously described (14). ErbB2 and p-erbB2 antibodies were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. The expression of erbB2, p-erbB2, EGFR, and p-EGFR were also determined by Western blot analysis as previously described (30). All
antibodies used for Western blot analysis were purchased from Cell Signaling Technology.

Histologic evaluation
Dorsal skin samples and tumors were fixed in either formalin or ethanol, and embedded in paraffin before sectioning. Sections of 4 μm were cut and stained with H&E. Mice were injected i.p. with BrdUrd in PBS (100 μg/g body weight) 30 minutes before sacrifice. For the analysis of epidermal labeling index (LI), paraffin sections were stained using anti-BrdUrd antibody as previously described (34). The LI was calculated by determining the percent of BrdUrd-labeled basal cells from observing ∼2,000 interfollicular epidermal basal cells. The determinations of epidermal thickness and LI were done as previously described (35). For the analyses of the expression of keratin 1, keratin 5, keratin 6, and loricrin, tissues were fixed in ethanol and immunostained as previously described (36).

Detection of erbB2 mRNA levels by real-time PCR
ErbB2 mRNA transcript levels were assessed using the fluorescent Taqman method and an ABI Prism 7700 Sequence Detection System to quantify PCR amplification in real-time as previously described (37).

Statistical analysis
All of the data are expressed as the mean ± SD. Significant differences were determined using body weight change and the Student’s t test for analysis of the levels of total and phosphorylated forms of erbB2 and EGFR in the Western blots. The Mann-Whitney U test was used for analysis of epidermal thickness/LI and tumor incidence. P < 0.05 was considered significant. All of the statistical analyses were done using the StatView software (Abacus Concept, Inc.).

Results
The effect of GW2974 on tumor promotion in wild-type and BK5.erbB2 mice during two-stage carcinogenesis
To determine the effects of GW2974 on skin tumor promotion during two-stage skin carcinogenesis, two independent experiments were done as shown in Fig. 1. The first tumor experiment (called experiment #1) was designed to examine the chemopreventive effect of GW2974 on tumor promotion in wild-type and BK5.erbB2 mice, as well as the potential reversibility of this inhibitory effect after the tumor response had reached a plateau (i.e., after 19 wk of promotion in this experiment).
The second tumor experiment (called experiment #2) was again designed to determine the chemopreventive effects of GW2974 on tumor promotion and its reversibility. However, GW2974 treatment was stopped much earlier (i.e., after 9 wk of promotion), before the tumor response had reached a plateau.

In experiment #1, two groups of homozygous BK5.erbB2 transgenic mice and two groups of wild-type mice were initiated with DMBA (50 nmol), followed 2 weeks later by promotion with twice weekly applications of TPA [6.8 nmol]. Diets containing GW2974 were started 2 weeks after initiation with DMBA at the same time that promotion with TPA was begun. In addition, to determine the reversibility of the effects of GW2974 on tumor promotion, the diet in one-half of the group treated with GW2974 was switched to AIN76A control diet after 19 weeks of promotion with TPA. The experiment was continued for an additional 9 weeks after the diets were switched, during which time TPA application was continued and the incidence and multiplicity (average number of tumors per mouse) of tumors were scored in each group. The results of the first 19 weeks of promotion with TPA in experiment #1 are shown in Fig. 2A (tumor multiplicity) and Fig. 2B (tumor incidence). Homozygous BK5.erbB2 mice fed the AIN76A control diet developed tumors faster and in greater number compared with wild-type mice fed the AIN76A control diet (Fig. 2A). These differences in tumor multiplicity were statistically significant (Mann-Whitney U tests, \(P < 0.05\)). In the group of homozygous BK5.erbB2 mice treated with GW2974, tumor promotion was almost completely blocked (5% tumor incidence and an average of 0.2 ± 0.04 tumors per mouse) compared with the 100% tumor incidence (and an average of 14.7 ± 3.0 tumors per mouse) found in the AIN76A control diet group by the end of the 19-week promotion period.

Wild-type mice treated with GW2974 also displayed significantly lower tumor incidence and tumor multiplicity. In this regard, the incidence of skin tumors was 100% and tumor multiplicity was 9.2 ± 2.3 in wild-type mice on the AIN76A diet at 19 weeks of promotion, whereas the incidence and multiplicity were 88% and 3.6 ± 1.2, respectively, in wild-type mice exposed to the GW2974-containing diet. Again, the difference in tumor multiplicity between the wild-type AIN76A control diet group and the GW2974 diet group was significant at all time points as shown in Fig. 2A (Mann-Whitney U tests, \(P < 0.05\)). Thus, both wild-type mice and BK5.erbB2 mice were responsive to inhibition of tumor promotion by dietary administration of GW2974, although it produced a significantly greater inhibitory response in BK5.erbB2 mice.

The BK5.erbB2 mice developed numerous large papillomas. Several of these papillomas upon gross examination seemed to have already undergone malignant conversion to SCCs by the end of the 19-week promotion period (data not shown). Wild-type mice fed the AIN76A control diet developed exclusively papillomas, and the tumors were slightly smaller in size and fewer in number (as noted above) compared with those of the BK5.erbB2 mice. However, GW2974 treatment dramatically inhibited growth of the tumors in wild-type and BK5.erbB2 mice. In this regard, after 19 weeks of promotion, the average tumor sizes (tumor diameters) for the wild-type AIN76A control diet group, BK5.erbB2 AIN76A control diet group, wild-type GW2974 treatment group, and BK5.erbB2 GW2974 treatment group were (in mm) 3.1 ± 0.5, 3.5 ± 0.6, 1.5 ± 0.2, and 0.3 ± 0 (note that only one tumor developed in this latter group), respectively. The differences in tumor sizes between AIN76A and GW2974 diet groups for both genotypes were statistically significant (Mann-Whitney U tests, \(P < 0.05\)). As noted previously, homozygous BK5.erbB2 mice exhibited a gross phenotype characterized by partial alopecia and wrinkled skin (30). The homozygous BK5.erbB2 mice treated with GW2974 also showed reversal of the alopecia with a more typical looking hair coat.

At the end of the 19-week promotion period, the two groups of mice that had been fed AIN76A control diet were sacrificed, and the two groups of mice that had been treated with GW2974 were each divided into two separate groups (see again Fig. 1). One of these groups continued to be treated with GW2974, whereas the other was fed the AIN76A control diet without GW2974 to determine whether the inhibitory effect on tumor promotion was reversible. Four weeks after the diet was switched from GW2974 to AIN76A control diet, the BK5.erbB2 mice started to develop tumors and the average number of tumors per mouse rose quickly (Fig. 2C). In the group of BK5.erbB2 mice that continued to be treated with GW2974, this compound still showed a potent inhibitory effect on tumor development. In wild-type mice, the average number of tumors increased gradually regardless of whether the diet was switched to AIN76A or remained as the GW2974 diet.

At the end of experiment #1 (i.e., 9 wk after the diet was switched), the average number of tumors per mouse in the BK5.erbB2 mice fed with the AIN76A control diet (after being switched from GW2974) and BK5.erbB2 mice treated with GW2974 continuously were 18.2 ± 4.8 and 2.2 ± 0.6, respectively. This difference was highly significant (Mann-Whitney U test, \(P < 0.05\)). Furthermore, at the end of the experiment, the average number of tumors in the wild-type mice fed with the AIN76A control diet after being switched from GW2974, and wild-type mice treated with GW2974 continuously were 7.5 ± 2.0 and 6.1 ± 2.2, respectively. These differences, however, were not statistically significant (\(P > 0.05\)). These results indicate that GW2974 had a potent inhibitory effect on the development of skin tumors when administered during the tumor promotion stage of a two-stage carcinogenesis protocol. Additionally, this inhibitory effect was reversible in the BK5.erbB2 mice, and partially reversible in wild-type mice under the conditions and during the time frame of experiment #1.
In experiment #2, the diets were switched substantially earlier than in experiment #1 to further examine the potential reversibility of the effects of GW2974 on tumor promotion at an earlier stage before the tumor response reached a plateau. The average number of tumors per mouse in this experiment was similar to that of the first experiment at the time of diet switch (i.e., at the 9th wk of TPA promotion; Fig. 3A). Again, BK5.erbB2 mice fed the AIN76A control diet developed tumors faster and in greater number compared with wild-type mice fed the AIN76A control diet. Likewise, GW2974 significantly (P < 0.05) inhibited tumor promotion in BK5.erbB2 and wild-type mice at 9 weeks of promotion. In this regard, the average number of tumors was 19.3 ± 3.9 and 1.0 ± 0.3 in BK5.erbB2 mice on AIN76A and GW2974 diet, respectively, and 2.8 ± 1.0 and 1.5 ± 0.5 in wild-type mice on AIN76A and GW2974 diet, respectively. At the 9th week

Fig. 2. Effects of GW2974 on skin tumor promotion in wild-type and BK5.erbB2 mice. A, number of tumors per mouse in experiment #1. B, tumor incidence in experiment #1. In experiment #1, two groups of homozygous transgenic and two groups of wild-type mice were initiated with DMBA (50 nmol), followed 2 wk later with twice weekly applications of TPA (6.8 nmol). □, BK5.erbB2 mice fed AIN76A control diet; ▪, BK5.erbB2 mice fed AIN76A diet containing 200 ppm GW2974; ○, wild-type mice fed AIN76A control diet; ●, wild-type mice fed AIN76A diet containing 200 ppm GW2974. C, average number of tumors per mouse after diet switch in wild-type mice (Wt) and BK5.erbB2 mice (Tg) in experiment #1. [], BK5.erbB2 mice that were switched from GW2974 to AIN76A control diet; ■, BK5.erbB2 mice that were fed continuously with diet containing GW2974; ●, wild-type mice that were switched from GW2974 to AIN76A control diet; ○, wild-type mice that were fed continuously with diet containing GW2974.

Fig. 3. Effect of GW2974 on skin tumor promotion in wild-type and BK5.erbB2 mice in experiment #2. A, number of tumors per mouse in experiment #2. □, BK5.erbB2 mice fed AIN76A control diet; ■, BK5.erbB2 mice fed AIN76A diet containing GW2974; ○, wild-type mice fed AIN76A control diet; ●, wild-type mice fed AIN76A diet containing GW2974. B, number of tumors per mouse after diet switch in wild-type (Wt) and BK5.erbB2 mice in experiment #2. [], wild-type mice that were switched from GW2974 to AIN76A control diet; ■, wild-type mice that were fed continuously with diet containing GW2974; ●, BK5.erbB2 mice that were switched from GW2974 to AIN76A control diet; ■, BK5.erbB2 mice that were fed continuously with diet containing GW2974.
of promotion, both wild-type and BK5.erbB2 mice exposed to GW2974 were divided into two groups to determine the reversibility of the effects of GW2974 on skin tumor promotion. Thus, as in experiment #1, the wild-type and BK5.erbB2 mice were either maintained on the GW2974 diet or switched to AIN76A. All four groups of mice were then sacrificed 9 weeks after the diet switch.

As shown in Fig. 3B, tumors began to arise within a few weeks following the switch to AIN76A in wild-type and BK5.erbB2 mice. At the end of the diet switch observation period, the average number of tumors per mouse in those two groups was 8.3 ± 1.4 and 10.7 ± 2.8, respectively. In contrast, the number of tumors per mouse in the groups of mice fed the GW2974 continuously remained significantly lower at the end of the additional 9-week observation period. These results from experiment #2 further indicate the reversibility of the inhibitory effect of GW2974 on tumor promotion in BK5.erbB2 mice regardless of the timing, although the effect observed in this experiment was not as great as that seen in experiment #1. The results of this experiment also suggest greater reversibility of the effects of GW2974 in wild-type mice earlier in the tumor promotion process before the papilloma response reaches a plateau.

Figure 4 summarizes the average tumor sizes (i.e., diameters) at the end of experiment #2. As shown, the size of skin tumors was significantly smaller in BK5.erbB2 and wild-type mice treated continuously with GW2974 compared with mice switched to the AIN76A control diet. Throughout the experiment, none of the wild-type or BK5.erbB2 mice treated with GW2974 showed any signs of toxicity or neurologic abnormalities. As shown in Table 1, body weights either remained similar in the different diet groups or increased over the course of the experiment.

**Effect of GW2974 on epidermal hyperplasia and proliferation**

The effect of GW2974 treatment on epidermal hyperplasia and proliferation in experiment #2 is shown in Fig. 5 and Table 2. For the data presented, nontumorous dorsal skin tissue was collected at the end of the experiment. Figure 5 shows representative H&E- and BrdUrd-stained sections from wild-type and BK5.erbB2 mice fed either AIN76A control or GW2974 diet continuously or GW2974 followed by AIN76A. Table 2 summarizes the epidermal thickness determined from H&E-stained sections and LIIs determined from BrdUrd staining in all groups of wild-type and BK5.erbB2 mice from experiment #2 of a similar age. As expected, nontreated BK5.erbB2 mice maintained on the AIN76A diet showed a 3.6- and 5.7-fold increase in epidermal thickness and LI, respectively, compared with that of wild-type mice on the same control diet. In the DMBA/TPA-treated groups continuously fed the AIN76A control diet, there were no significant differences in either epidermal thickness or LI between wild-type and BK5.erbB2 mice. Both wild-type and BK5.erbB2 mice treated with the DMBA/TPA protocol and maintained continuously on GW2974 had significantly reduced epidermal hyperplasia and LI compared with the corresponding AIN76A control diet groups. Finally, in the groups switched from GW2974 to AIN76A diets, the epidermal hyperplasia and LI partially reversed toward the value seen in mice continuously fed the AIN76A diet during the two-stage protocol. Collectively, these data indicate that GW2974 significantly blocked TPA-induced epidermal proliferation in wild-type and BK5.erbB2 mice.
although the magnitude of these inhibitory effects was again greater in the transgenic mice.

**Status of erbB2 and EGFR in skin of GW2974-treated mice**

To further investigate the mechanisms for the effects of GW2974 on skin tumor promotion in wild-type and BK5.erbB2 mice, Western blot analyses were done. For these experiments, mice were maintained on either the AIN76A- or GW2974-supplemented diet for a period of 4 weeks. At this point, mice were treated with TPA (twice weekly for 2 wk), and epidermal protein lysates from wild-type and BK5.erbB2 mice were collected 4 hours after the last treatment with TPA. As previously shown (30), the levels of p-erbB2 and p-EGFR were significantly higher in the epidermis of BK5.erbB2 mice compared with the epidermis of wild-type mice (Fig. 6). Following treatment with TPA, the levels of p-erbB2 and p-EGFR were further increased in epidermis of both wild-type and BK5.erbB2 mice (see again Fig. 6). Treatment with GW2974 significantly reduced the levels of total erbB2 protein and the phosphorylation of both erbB2 and EGFR in the epidermis of nontreated and TPA-treated BK5.erbB2 mice. Treatment with GW2974 also resulted in a significant decrease in the levels of p-erbB2 and p-EGFR in the epidermis of TPA-treated wild-type mice, although there were no significant effects on the levels of total erbB2 and EGFR protein. The reductions in p-erbB2 and total erbB2 protein in BK5.erbB2 mice as a result of treatment with GW2974 were confirmed by immunohistochemical analysis (Fig. 7).

**Status of erbB2 mRNA in skin of GW2974-treated mice**

In light of the data in Fig. 5 showing that GW2974 treatment led to decreased levels of total erbB2 protein, we examined the levels of both mouse (i.e., endogenous) and rat (i.e., transgene) erbB2 mRNA as shown in Table 3. For this experiment, skins from groups of four mice each (both wild-type and BK5.erbB2 mice maintained on control diet and GW2974-containing diet from experiment #1) were collected and total RNA was isolated. Levels of
**Table 2. Comparison of the effects of AIN76 control and GW2974 diets on epidermal hyperplasia and cell proliferation in wild-type and BK5.erbB2 mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Treatment</th>
<th>Diet initial</th>
<th>Final</th>
<th>Epidermal thickness</th>
<th>LI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Acetone/control</td>
<td>AIN76A</td>
<td>AIN76A</td>
<td>7.8 ± 0.4†‡</td>
<td>1.8 ± 0.2†‡</td>
</tr>
<tr>
<td></td>
<td>DMBA/TPA</td>
<td>AIN76A</td>
<td>AIN76A</td>
<td>70.4 ± 3.5†§</td>
<td>74.2 ± 4.8†§</td>
</tr>
<tr>
<td></td>
<td>DMBA/TPA</td>
<td>GW</td>
<td>GW</td>
<td>34.4 ± 3.7†</td>
<td>24.4 ± 3.5†</td>
</tr>
<tr>
<td></td>
<td>DMBA/TPA</td>
<td>GW</td>
<td>AIN76A</td>
<td>40.1 ± 3.8</td>
<td>45.4 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>DMBA/TPA</td>
<td>GW</td>
<td>GW</td>
<td>28.4 ± 2.6†</td>
<td>10.2 ± 2.5†‡</td>
</tr>
<tr>
<td></td>
<td>DMBA/TPA</td>
<td>AIN76A</td>
<td>AIN76A</td>
<td>78.6 ± 3.9†§</td>
<td>68.4 ± 5.1†§</td>
</tr>
<tr>
<td></td>
<td>DMBA/TPA</td>
<td>GW</td>
<td>GW</td>
<td>28.4 ± 3.1†</td>
<td>14.4 ± 2.2†</td>
</tr>
<tr>
<td></td>
<td>DMBA/TPA</td>
<td>GW</td>
<td>AIN76A</td>
<td>56.7 ± 4.4†</td>
<td>47.8 ± 3.3†</td>
</tr>
</tbody>
</table>

**NOTE:** Values in table represent ± SD. The difference in epidermal thickness and LI value were found to be statistically significant (P < 0.01) by the Mann-Whitney U test.

*Significant difference between acetone-treated (AIN76A-AIN76A diet) group and DMBA/TPA-treated (AIN76A-AIN76A diet) group.
†Significant difference between acetone-treated (AIN76A-AIN76A diet) group and DMBA/TPA-treated (GW-GW diet) group.
‡Significant difference between wild-type (acetone-treated, AIN76A-AIN76A) and transgenic (acetone-treated, AIN76A-AIN76A) mice.
§Significant difference between DMBA/TPA-treated (AIN76A-AIN76A) group and DMBA/TPA-treated (GW-GW) group.
∥Significant difference between DMBA/TPA-treated (AIN76A-AIN76A) group and DMBA/TPA-treated (GW-AIN76A) group.

**erbB2 mRNA were determined by quantitative reverse transcription-PCR. Rat erbB2 mRNA was only detected in skin from BK5.erbB2 mice and was not markedly decreased in the GW2974-treated group compared with that of non-treated mice. The relative percentage mRNA level in BK5.erbB2 mice treated with GW2974 was 92 ± 10% compared with that of the nontreated control group (100%; see Table 3). In wild-type mice, treatment with GW2974 resulted in a slight reduction in the level of mouse (endogenous) erbB2 mRNA (90 ± 7%) compared with wild-type on the control diet. This slight reduction also was not statistically significant. In BK5.erbB2 mice, the level of mouse erbB2 mRNA was significantly lower in the control diet (Table 3, P < 0.05). Thus, GW2974 did not appear to alter expression of the rat erbB2 transgene. The reduction in the level of endogenous erbB2 mRNA in BK5.erbB2 mice maintained on the control diet may be due to high levels of transgene mRNA and protein; however, this possibility will require further experimentation to confirm.**

**Discussion**

The present study has investigated the effect of inhibiting both the EGFR and erbB2 during the tumor promotion stage on development of skin tumors in a two-stage skin carcinogenesis protocol. This was accomplished by determining the effects of orally administered GW2974, a dual specific TKI on skin tumor promotion in BK5.erbB2 and wild-type mice. The major findings of this study are as follows: (a) GW2974 showed a potent chemopreventive effect on skin tumor promotion by TPA in BK5.erbB2 and wild-type mice; (b) the inhibitory effect was more reversible early during tumor promotion in wild-type mice when GW2974 treatment was switched to control diet, whereas the inhibitory effects of GW2974 on tumor promotion in BK5.erbB2 mice were highly reversible regardless of the timing; and (c) GW2974 showed a potent inhibitory effect on TPA-induced epidermal proliferation as determined by epidermal thickness and BrdUrd incorporation in BK5.erbB2 and wild-type mice. The magnitude of these effects was again greater in BK5.erbB2 mice, and (d) the chemopreventive effects of GW2974 on skin tumor promotion were associated with a significant reduction in activation of both the EGFR and erbB2. Collectively, these data indicate that targeting both the EGFR and erbB2 may be an effective strategy for prevention of epithelial cancers in which signaling through this pathway is upregulated early in the carcinogenic process. Furthermore, drugs that possess dual specificity such as GW2974 may be more efficacious when erbB2 is overexpressed or amplified early during the carcinogenesis process.

As noted in the Introduction, considerable evidence suggests an important role for the EGFR in the development of skin tumors in mice undergoing two-stage and UV skin carcinogenesis. In normal human skin, immunohistochemical analyses showed that EGFR expression was detected predominantly in the basal layer of epidermis and decreased toward the corneum layer (38, 39). EGFR expression was also found in sebocytes, outer root sheath cells of hair follicles, smooth muscle cells of arrector pili, and dermal arteries (38, 39). Overexpression of EGFR protein has been reported in approximately 50% to 100% of basal cell carcinomas (BCC; ref. 40) and 90% to 100% of SCCs (40, 41), as determined by immunohistochemical analysis. Highly elevated mRNA levels of EGFR were...
reported in 38%, 57%, and 80% in normal epidermis, BCCs, and SCCs, respectively (42). In contrast, other studies have reported that the levels of EGFR protein in BCCs, SCCs, and normal epidermis were all similar (43, 44). It has also been reported that amplification of EGFR is observed in ~20% of SCCs, as determined by Southern blot (45). Elevation of EGFR ligands and EGFR activation (as detected by phosphorylation at Tyr1068) was also reported.
to be elevated in SCCs compared with BCCs and normal skin (43). Finally, UV exposure to both cultured human keratinocytes and human skin leads to the activation of the EGFR (46–49). Thus, considerable evidence exist that activation of the EGFR plays a role in skin tumor development (especially SCCs) in humans. These data also suggest that the EGFR is a potential target for prevention of human skin cancer.

Although there has been intensive research about erbB2 and its role in many cancers, including breast, colorectal, cervical, biliary tract, head and neck SCC, testicular, ovary, stomach, urinary bladder, lung, osteosarcoma, and childhood medulloblastoma (50), relatively little is known about the role of erbB2 in human skin cancer. In normal human epidermis, erbB2 stains in the cytoplasm of cells in the basal layer and in the plasma membrane of cells in the

**Table 3.** Average relative erbB2 mRNA expression in skins from wild-type and BK5.erbB2 mice fed AIN76A control diet or GW2974-containing diet as determined by real-time PCR analysis

<table>
<thead>
<tr>
<th></th>
<th>WT control diet</th>
<th>WT GW diet</th>
<th>TG control diet</th>
<th>TG GW diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat mRNA (%)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>91.8 ± 9.7</td>
</tr>
<tr>
<td>Mouse mRNA (%)</td>
<td>100</td>
<td>89.8 ± 7.0</td>
<td>47.5 ± 4.2</td>
<td>83.25 ± 8.7</td>
</tr>
</tbody>
</table>

NOTE: Four skins from each group (both wild-type and BK5.erbB2 mice maintained on control diet and GW2974-containing diet from experiment #1) were collected and analyzed for erbB2 mRNA transcript levels. Mouse erbB2 mRNA levels are expressed as a percentage relative to WT mice fed with control diet (AIN76A). Rat erbB2 mRNA levels are expressed as a percentage relative to TG mice fed with control diet. Abbreviations: WT, wild-type; TG, BK5.erbB2 mice.
upper suprabasal layers (51–53). ErbB2 is also found in the external root sheath of hair follicles and in eccrine gland secretory cells (54). In terms of cutaneous carcinomas, Lebeau et al. (51) showed that in SCCs, the subcellular distribution of erbB2 remains unchanged (localized primarily in plasma membrane and cytoplasm), but in BCCs, erbB2 is distributed from the plasma membrane into cytosolic aggregates. In another study, it was reported that normal human skin, keratoacanthomas, and actinic keratoses showed no or barely detectable erbB-2 protein expression; however, the outer epidermal layer of SCCs showed a few strongly positive cells (55). This study also showed that 20 of the 24 cases of SCC examined had elevated expression of erbB2, whereas only 5 of the 10 cases of BCC stained for erbB2 and more weakly than seen in SCCs. Thus, the expression patterns and exact role of erbB2 in normal human skin remains somewhat unclear; however, overexpression of erbB2 may play a role in the malignant conversion of keratinocytes. As noted in the Introduction and as shown in the current study, erbB2 seems to play an important role in chemically and UV-mediated skin carcinogenesis in mice (26–33).

To further explore the role of EGFR/erbB2 signaling in the development of skin tumors in BK5.erbB2 and wild-type mice, we used GW2974, a dual EGFR/erbB2 TKI. GW2974 belongs to an orally active quinazoline group of TKIs and inhibits both EGFR and erbB2 by targeting the ATP binding sites of these molecules. GW2974 showed a potent inhibitory effect on cells overexpressing both EGFR and erbB2 in vitro and in a tumor xenograft model (56). In an earlier study, we reported significant chemopreventive and therapeutic efficacy of GW2974 given in the diet on gallbladder carcinoma that developed in BK5.erbB2 mice (57). In the current study, an almost complete chemopreventive efficacy was observed in BK5.erbB2 mice and a potent chemopreventive effect in wild-type mice was observed after treatment with GW2974 during the tumor promotion stage in a two-stage skin carcinogenesis protocol. This inhibition was reversible when GW2974 was withdrawn from the diets early in the process of tumor development of both wild-type and BK5.erbB2 mice. In addition, treatment of both BK5.erbB2 and wild-type mice with GW2974 diet resulted in a significant reduction in activation of both the EGFR and erbB2 during tumor promotion by TPA. These results indicate that the significant inhibitory effect of GW2974 on the development of skin tumors is due primarily to its ability to block the activation of both EGFR and erbB2 and inhibition of epidermal hyperproliferation during tumor promotion. The greater efficacy of GW2974 in BK5.erbB2 mice may be due to its ability to reduce both erbB2 protein levels as well as p-erbB2 levels. The ability of GW2974 to reduce erbB2 and p-erbB2 levels in BK5.erbB2 mice was not due to an effect on transgene expression as shown in Table 3.

In the present study, we chose to analyze the dual specific inhibitor GW2974. This decision was based on our previous findings that both the EGFR and erbB2 are activated in keratinocytes exposed to EGFR ligands or TPA (26, 58) and that exposure to EGFR ligands or TPA increases EGFR/erbB2 heterodimer formation (26). In addition, two different EGFR-selective inhibitors blocked TPA-induced epidermal hyperproliferation (58). Data in the literature support the hypothesis that erbB2 is the preferred partner for activated EGFR (5, 6, 59, 60). This interaction also reduces the rate of EGFR degradation (61). Thus, selectively blocking the EGFR should also be effective at reducing both EGFR activation as well as erbB2 activation and therefore block TPA-induced skin promotion. The data showing inhibition of TPA-induced epidermal hyperproliferation by EGFR-selective tyrosine kinase inhibitors supports this hypothesis (58). In addition, preliminary data from our laboratory shows that mice fed a diet containing a specific inhibitor for the EGFR, gefitinib (400 ppm for 1 mo), significantly reduced levels of both p-EGFR as well as total and p-erbB2 protein in the skins of both wild-type and BK5.erbB2 mice.3 Based on this, it is likely that selective EGFR inhibitors would also be effective at blocking both EGFR and erbB2 activation and skin tumor promotion by TPA. The suppression of UVB-mediated skin tumor promotion in TG:AC mice by either an EGFR or an erbB2 TKI also supports this hypothesis (14, 33).

In conclusion, the current study shows that a dual erbB2/EGFR TKI, GW2974, effectively inhibited skin tumor promotion in BK5.erbB2 and wild-type mice when given orally through the diet. The effects of GW2974 were essentially reversible, although reversibility occurred to a lesser extent in wild-type mice with a longer duration of skin tumor promotion. A more marked effect of GW2974 was seen in BK5.erbB2 mice, suggesting greater efficacy for this compound when erbB2 is overexpressed or amplified as an early event in the carcinogenic process. Targeting the EGFR, or both EGFR and erbB2 may be an effective strategy for prevention of epithelial cancer, including skin cancer, when signaling through the EGFR/erbB2 pathway is upregulated early in the carcinogenic process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Dual Inhibition of Both the Epidermal Growth Factor Receptor and erbB2 Effectively Inhibits the Promotion of Skin Tumors during Two-Stage Carcinogenesis

Kaoru Kiguchi, Takuya Kitamura, Tricia Moore, et al.


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