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Chemoprevention of 7, 12-dimethylbenz[*a*]anthracene (DMBA)-induced oral carcinogenesis in hamster cheek pouch by topical application of a dual inhibitor of epidermal growth factor receptor (EGFR) and ErbB2 tyrosine kinases

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Abstract

Oral cancer is a common neoplasm worldwide with tobacco and alcohol being the major etiological factors contributing to its pathogenesis. Epidermal growth factor receptor (EGFR) and ErbB2 are known to be involved in the development of oral cancer with the former up-regulated in up to 90% human cases. The goal of this study was to evaluate the chemopreventive effects of a dual inhibitor of EGFR and ErbB2 tyrosine kinases, GW2974, in the 7, 12-dimethylbenz[*a*]anthracene (DMBA)-induced hamster cheek pouch model. A short-term experiment (3-week topical DMBA followed by 1-week topical GW2974) was conducted to examine the effects of GW2974 on aberrant arachidonic acid (AA) metabolism and cell proliferation in the hamster oral epithelium. Topical application of 0.1ml GW2974 (160 μM, three times a week) significantly reduced the levels of prostaglandin E2 (PGE2), leukotriene B4 (LTB4), 5-, 12-, 15-hydroxyeicosatetraenoic acid (HETE), and cell proliferation (BrdU-labeling index). In a long-term post-initiation experiment (6-week topical DMBA followed by 18-week topical GW2974), GW2974 (4mM and 8mM) significantly inhibited the incidence, number and size of visible tumors. Under microscope, the numbers of oral lesions (hyperplasia, dysplasia, carcinoma) and the incidence of squamous cell carcinoma (SCC) were also significantly suppressed by GW2974. In summary, our study indicated that dual inhibition of EGFR and ErbB2 tyrosine kinases by GW2974 was effective in preventing oral carcinogenesis in DMBA-

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induced hamster cheek pouch model. GW2974 exerted its chemopreventive effects in part by suppressing aberrant AA metabolism.

Keywords

Oral cancer; EGFR; DMBA; GW2974

Introduction

Oral cancer mainly represents the cancers of the tongue, cheek and palate. It occurs predominantly in developing countries where it constitutes up to 25% of all kinds of cancers [1]. In the United States about 34,360 new cases and 7,550 deaths are expected in 2007 [2]. Despite significant advances in various forms of treatment, the overall five-year survival rate for oral cancer patients has not improved over the past couple of decades and remains around 50% [3]. Therefore, it is important to understand the pathogenesis of this disease and to design novel and efficacious chemopreventive strategies.

Epidermal growth factor receptor (EGFR) family, which includes EGFR/ErbB1, ErbB2, ErbB3 and ErbB4, are known to regulate cell proliferation, differentiation, migration, angiogenesis and apoptosis [4–6]. When activated by various ligands, these receptors homo- or hetro-dimerize with each other to form active tyrosine kinases, and activate various downstream signaling molecules, such as MAPKs, PI3K and STATs [7]. EGFR is expressed in normal oral squamous epithelial cells of humans and hamsters [8, 9]. Amplification and overexpression of EGFR were shown to be early events during oral carcinogenesis, and paralleled progression of the disease [10]. Expression levels of EGFR, ErbB2 and ErbB3 together have stronger prognostic value than either one of them alone [11–13]. Among the four EGFR family members, EGFR and ErbB2 are most frequently co-expressed in the same cell layer of neoplastic epithelium [14]. Patients who exhibit overexpression of both EGFR and ErbB2 tend to have more aggressive tumors and poorer clinical prognosis when compared to those expressing either one of them [13], suggesting an important role of EGFR/ErbB2 heterodimer in oral carcinogenesis. In addition, it was shown recently that hamsters vaccinated in the buccal pouch with a DNA coding for the ErbB2 receptor elicited an antibody response that suppressed the development and progression of SCC [15]. Both *in vitro* and *in vivo* data have suggested that the dual inhibition of both EGFR and ErbB2 is more effective in cancer therapy than agents targeting individual receptors [16].

Aberrant arachidonic acid (AA) metabolism has been suggested to play an important role in the development of human oral cancer, with the involvement of both cyclooxygenase (Cox) and lipoxygenase (Lox) pathways [17, 18]. The major metabolite of the Cox pathway is prostaglandin E2 (PGE2), while the Lox enzymes can give rise to a number of metabolites such as leukotriene B4 (LTB4) and 5-, 12-, 15-hydroxyeicosatetraenoic acid (HETE) depending upon the position of oxygen insertion in AA [19]. These metabolites are known to be potent mediators of inflammation as they recruit and activate inflammatory cells, increase vascular permeability and induce smooth muscle contraction [20]. We have shown previously that Cox-2 and 5-Lox were overexpressed in hamster and human oral cancer, and specific inhibitors of Cox-2 and 5-Lox were able to suppress the development of hamster cheek pouch carcinogenesis [18]. We further demonstrated that LTB4 promoted oral carcinogenesis, while multiple inhibitors of 5-Lox pathway had chemopreventive effects on cancer development, in the same model system [21].

There is ample evidence in the literature to suggest a cross talk between the EGFR signaling cascade and AA metabolism. EGFR mediated activation of MAPK has been shown to upregulate Cox-2 gene transcription, while Cox-2 derived PGE2 can stimulate cell proliferation

through EGFR transactivation [22]. Activation of EGFR and ErbB2 has been shown to stimulate Cox-2 expression and translocation as well as PGE2 synthesis and mitogenesis [23, 24]. It has been reported earlier that AA metabolites act as secondary messengers during EGF-induced cytoskeletal changes in rat fibroblasts and HeLa cells [25]. Also in another study, an anti-EGF monoclonal antibody was able to reduce the levels of PGE2 in human amnion cells [26]. Moreover, combined use of both EGFR and Cox-2 inhibitors has shown synergistic effects in cancer therapy.

The major goal of this study was to investigate the chemopreventive activity of GW2974, a dual inhibitor of EGFR and ErbB2 tyrosine kinases, in oral carcinogenesis. GW2974 was first tested in a short-term experiment for its effects on 7, 12-dimethylbenz[*a*]anthracene (DMBA)-induced aberrant AA metabolism and cell proliferation in hamster oral epithelium. A long-term experiment was then conducted to evaluate its chemopreventive efficacy in oral carcinogenesis.

Materials and Methods

Chemicals

DMBA and GW2974 were purchased from Sigma Chemical Company (St. Louis, MO). They were dissolved in mineral oil at appropriate concentrations for topical application.

Short-term effects of topical GW2974 on aberrant AA metabolism and cell proliferation in DMBA-treated hamster cheek pouch

This study was conducted at Rutgers University under Protocol number 91-024. Male Syrian golden hamsters (6 weeks old) weighing 60–80 g were purchased from Harlan (Indianapolis, IN) and housed 4 per cage in a room with controlled temperature and humidity with 12 h light: dark cycles. All animals were given AIN-93M diet (Research Diets, New Brunswick, NJ) and water *ad libitum*. After 1 week of acclimatization, the animals were divided into 2 groups, with Group 1A serving as the negative control (3 animals). The left cheek pouch of the remaining 12 hamsters was topically treated with 0.5% DMBA in 100 μl mineral oil using a paintbrush 3 times a week for 3 weeks. They were then randomly divided into 2 groups with Group 1B (6 animals) serving as the positive control and receiving no further treatment. Group 1C (6 animals) was treated topically with 160 μM GW2974 in 100 μl mineral oil 3 times per week for 1 week. The animals were sacrificed at the end of the experiment (Week 4), 6 hours after the last treatment. The animals were injected with bromodeoxyuridine (BrdU) *i.p.* at 50 mg/kg body weight 2 hours prior to sacrificing. The cheek pouch was harvested, one half being snap frozen in liquid nitrogen for analysis of AA metabolites, and the other half being fixed in 10% PBS-buffered formalin for histopathology.

Long-term effects of topical GW2974 on DMBA-induced carcinogenesis in hamster cheek pouch

The animals were housed under the same conditions as mentioned above. Group 2A (10 animals) served as the negative control. Other groups were topically treated with 100 μl of 0.5% DMBA in mineral oil using a paintbrush 3 times per week for 6 weeks. The animals were then randomly divided into 3 groups (30 animals each), with Group 2B receiving no further treatment. Groups 2C and 2D were treated with 4 mM and 8 mM of GW2974 respectively, 3 times per week for another 18 weeks. At the end of Week 24, all animals were sacrificed and the left hamster cheek pouch was harvested and fixed in 10% buffered formalin for histopathology.

Histopathological analyses

The whole cheek pouch was excised and flattened on a transparency plate for counting the number of visible tumors. The length, width and height of each tumor was measured with a caliper and the tumor volume calculated using the formula: volume=$4/3\pi r^3$ (where r was the average radius of the three diameter measurements in mm). Formalin-fixed pouches were cut into 4–6 pieces of approximately equal width, Swiss-rolled, processed and embedded in paraffin. Thirty sections (5 μm) of each sample were cut and the 1st, 15th and 30th slides were stained with hematoxylin and eosin (H&E) for histopathological analysis. Basal cell hyperplasia, dysplasia, squamous cell carcinoma and papillomas were diagnosed using established criteria [27, 28].

Cell proliferation analysis

To assess cell proliferation in the squamous epithelium of hamster oral mucosa, bromodeoxyuridine (BrdU) immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections. The avidin-biotin peroxidase method was used with a rat monoclonal antibody (Serotec, Raleigh, NC) at a concentration of 5 μg/ml. Three noncontiguous, randomly selected fields under 400x were photographed per sample, and the sum of all positive cells was divided by total number of cells to obtain the cell proliferation index. Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD) was used for cell counting.

Profiling of AA metabolites with LC/MS/MS

After homogenization in a buffer containing 10 μM zileuton and indomethacin, tissues were extracted with hexane:ethyl acetate under reduced light conditions after the addition of an internal standard (PGE2-d4). The samples were dried under nitrogen, reconstituted in methanol:2mM ammonium acetate, and analyzed using an established method [29]. The levels of AA metabolites (PGE2, LTB4, 5-HETE, 12-HETE, and 15-HETE) were determined and expressed as nanograms per milligram protein.

Statistical analysis

The tumor incidence was analyzed with χ^2 test. One-way ANOVA test was used to compare body weight, the number of visible tumors, and the numbers of oral lesions. Tumor volume was analyzed using the Wilcoxon signed rank test. Statistical significance between the levels of AA metabolites among the various groups was analyzed using the Student's *t* test.

Results

Overall the animals appeared healthy throughout the two experiments. However, DMBA-treated animals weighed less than untreated animals by 4–8% probably due to metabolic stress caused by inflammation or irritation in the cheek pouch. Body weight of the animals was monitored once a week and increased steadily with less than 10% variation among different groups (data not shown).

Inhibition of DMBA-induced aberrant AA metabolism and cell proliferation in hamster cheek pouch by short-term treatment of topical GW2974

At the end of the short-term study (Week 4), DMBA-treated hamster cheek pouch appeared reddish, hyperkeratotic and inflamed. The levels of AA metabolites were found to be elevated in the positive control group (Group 1B), when compared to the negative control group (Group 1A). Topical application of 160 μM GW2974 (Group 1C) significantly reduced the levels of these metabolites (Figure 1a–e).

BrdU immunohistochemical analysis showed that Group 1B had marked increase in the rate of cell proliferation when compared to Group 1A. This increase was significantly inhibited by treatment with GW2974 ($p<0.05$) (Figure 1f).

Chemopreventive effects of topical GW2974 on DMBA-induced oral carcinogenesis in hamster cheek pouch

Consistent with our previous studies [18, 21, 30], topical application of DMBA (100 μl, 0.5% in mineral oil, 3/week for 6 weeks) led to the development of oral lesions in the hamster cheek pouch at the end of 24 weeks. Topical application of GW2974 (4 mM and 8 mM, 100 μl, 3/week for 18 weeks) at the post-initiation stage significantly inhibited the incidence, number and size of DMBA-induced visible tumors. Under the microscope, GW2974 led to a significant decrease in the numbers of oral lesions (hyperplasia, dysplasia, and SCC) (Table 1).

Discussion

The role of ErbB family of receptors in oral carcinogenesis has been well established. Dual inhibition of EGFR/ErbB2 tyrosine kinases could be an effective approach for chemoprevention and therapy of oral cancer. In this study, we demonstrated the chemopreventive effects of GW2974, a dual inhibitor of both EGFR and ErbB2 tyrosine kinases, on DMBA-induced oral carcinogenesis in hamster cheek pouch.

As a quinazoline derivative, GW2974 has been shown previously to be highly potent in suppressing the growth of cancer cells *in vitro* and *in vivo* [31]. It was effective in selectively inhibiting the growth of tumor cells in cancer cell lines overexpressing both EGFR and ErbB2. In addition, GW2974 also suppressed the growth of xenograft tumors in a dose-dependent manner due to selective inhibition of EGFR and ErbB2 receptor phosphorylation [31]. A synergistic effect was observed when GW2974 was treated in combination with Bcl-2 inhibitors in suppressing the growth of various human breast cancer cell lines [32]. In a separate *in vivo* study, GW2974 was more effective than gefitinib in significantly inhibiting the development of gallbladder carcinoma [33].

In our study, topical application of GW2974 thrice a week for 18 weeks to the hamster oral epithelium inhibited not only the number, size, and incidence of visible tumors, but also the numbers of oral lesions and the incidence of SCC. Higher dose of GW2974 (8 mM) was more effective in our study (Table 1). To our knowledge, this is the first study to evaluate the effect of dual inhibition of EGFR and ErbB2 tyrosine kinases for oral cancer chemoprevention in the DMBA-induced hamster cheek pouch model. However, this study has its limitations because of the hamster cheek pouch model although it mimics many aspects of human oral cancer development. The human mouth does not have a similar pouch in structure and histology, and DMBA is not a natural carcinogen related to human oral cancer development [34].

GW2974 may inhibit DMBA-induced oral carcinogenesis through modulation of aberrant AA metabolism and cell proliferation. It was reported by our group that aberrant AA metabolism is involved in the pathogenesis of oral cancer and can be targeted for its chemoprevention [18]. In the present study, GW2974 led to a significant reduction in the levels of pro-inflammatory mediators such as PGE2, LTB4, and 5-HETE which were elevated due to aberrant AA metabolism induced by DMBA treatment (Figure 1a–e). These data are consistent with the observation that EGFR and AA metabolites interact with each other at multiple levels.

In summary, we show here that a dual inhibitor of EGFR/ErbB2 tyrosine kinases prevents oral carcinogenesis in the DMBA induced hamster cheek pouch model at the post-initiation stage. Its chemopreventive activity is in part due to modulation of AA metabolism and inhibition of cell proliferation, in addition to its inhibitory effects on EGFR/ErbB2.

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Abbreviations

AA, arachidonic acid; BrdU, bromodeoxyuridine; Cox, cyclooxygenase; DMBA, 7,12-dimethylbenz[*a*]anthracene; EGFR, epidermal growth factor receptor; LC/MS/MS, high performance liquid chromatography/electrospray ionization tandem mass spectrometry; LTB4, leukotriene B4; 5-Lox, 5-lipoxygenase; PGE2, prostaglandin E2; SCC, squamous cell carcinoma.

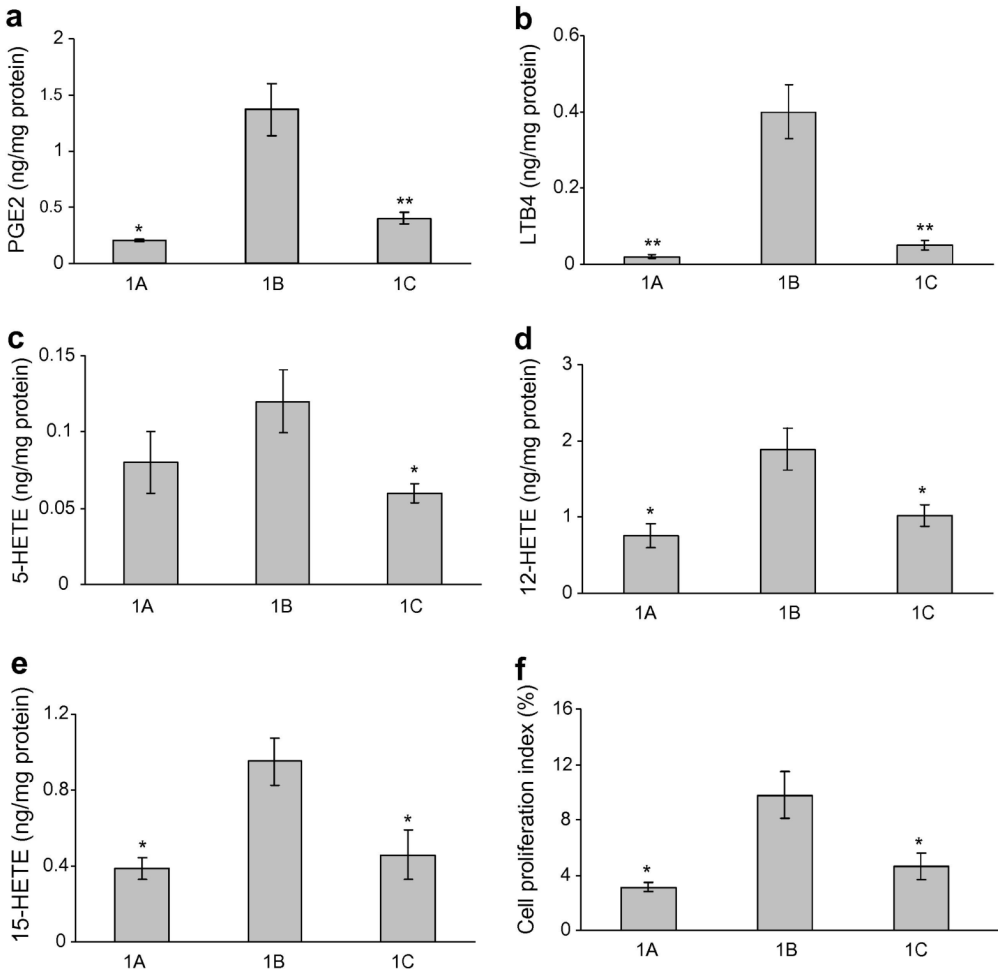

Figure 1.

Effects of topical GW2974 on AA metabolism and cell proliferation in DMBA-treated hamster cheek pouch. The values of AA metabolites are expressed as mean ± SD. Group 1A (negative control) and Group 1C (GW2974) are compared with Group 1B (positive control). $^{*}p<0.05$ and $^{**}p<0.01$.

Table 1

Effects of topical GW2974 on DMBA-induced oral carcinogenesis in hamster cheek pouch [a]

Group	Treatment	No. of animals	Visible Tumors			Microscopic Observations			
			Incidence % (No.)	No.	Size (mm^3)	No. of hyperplasia	No. of dysplasia	No. of SCC	Incidence of SCC % (No.)
2A	Negative control	10	-	-	-	-	-	-	-
2B	Positive control	30	80% (24)	1.00±0.88	256.84±442.53	8.07±4.39	7.57±3.37	1.83±1.91	70% (21)
2C	GW2974 (4 mM)	30	43.3% (13)**	0.57±0.82*	62.08±58.99	5.73±3.09**	6.80±3.74	0.67±0.99*	40% (12)*
2D	GW2974 (8 mM)	30	36.7% (11)**	0.43±0.63**	50.50±54.88	5.30±2.89**	5.97±2.62*	0.43±0.68**	33% (10)**

[a]The number and size of visible tumors, and the numbers of oral lesions (hyperplasia, dysplasia, and SCC) were expressed as mean ± SD. χ^2 test was used for comparison of incidences of visible tumor and SCC, signed rank test for tumor size, and one-way ANOVA for the numbers of visible tumors, hyperplasia, dysplasia and SCC. Values of Groups 2C and 2D were compared with that of Group 2B.

*p<0.05

**p<0.01.