Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors as Potential Cancer Chemopreventives

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Abstract
Among the most important targets for chemopreventive intervention and drug development are deregulated signal transduction pathways, and protein tyrosine kinases are key components of these pathways. Loss of tyrosine kinase regulatory mechanisms has been implicated in neoplastic growth; indeed, many oncogenes code for either receptor or cellular tyrosine kinases. Because of its deregulation in many cancers (bladder, breast, cervix, colon, esophagus, head and neck, lung, and prostate), the epidermal growth factor receptor (EGFR) has been selected as a potential target for chemoprevention. Because growth factor networks are redundant, selective inhibition of signaling pathways activated in precancerous and cancerous cells should be possible. Requirements for specific EGFR inhibitors include specificity for EGFR, high potency, activity in intact cells, and activity in vivo. Inhibition of autophosphorylation is preferred, because it should result in total blockade of the signaling pathway. Inhibitors that compete with substrate rather than at the ATP-binding site are also preferable, because they are not as likely to inhibit other ATP-using cellular enzymes.

Several classes of specific EGFR inhibitors have been synthesized recently, including structures such as benzylidene malononitriles, dianilinophthalimides, quinazolines, pyrimidines, (alkylamino)methylacrylophenones, enollactones, dihydroxybenzylaminosalicylates, 2-thiindoles, aminoflavones, and tyrosine analogue-containing peptides. A possible testing strategy for the development of these and other EGFR inhibitors as chemopreventive agents includes the following steps: (a) determine EGFR tyrosine kinase inhibitory activity in vitro; (b) evaluate EGFR specificity and selectivity (relative to other tyrosine kinases and other protein kinases); (c) determine inhibition of EGFR-mediated effects in intact cells; (d) determine inhibition of EGFR-mediated effects in vivo (e.g., in nude mouse tumor xenografts); and (e) determine chemopreventive efficacy in vivo (e.g., in the hamster buccal pouch or mouse or rat bladder).

Mechanism-based Strategies in the Development of Cancer Chemopreventive Agents: Signal Transduction Pathways

A successful and comprehensive strategy to address the cancer problem encompasses both preventive and therapeutic approaches. Several types of prevention programs can be envisioned; one is chemoprevention, which involves prophylactic intervention with drugs or micronutrients to inhibit, delay, or reverse the carcinogenic process before malignancy. Development of agents that can be used for this type of proscriptive intervention involves numerous research avenues, including identification of chemicals that prevent tumorigenesis in animal models or in relevant in vitro screening assays. Another approach is to target specific mechanisms involved in the carcinogenic process. Such a rational, targeted strategy toward development of chemopreventive drugs has been facilitated by increased understanding of the biochemical and genetic abnormalities involved in carcinogenesis. Discovery of agents with novel mechanisms of action that counter these abnormalities and have chemopreventive potential is a major focus of the National Cancer Institute Chemoprevention Branch drug development program (1-3).

One of the most significant advances in recent years is increased understanding of the biochemical control mechanisms involved in regulating cell growth and development. Cells respond to signals from extracellular stimuli via a complicated network of highly regulated events, collectively referred to as signal transduction pathways. Stimulation of these pathways results in changes in transcriptional activity (reviewed in Refs. 4 and 5). Although normal cells respond appropriately to extracellular stimuli, many precancerous and cancerous cells have lost this ability and display aberrant signaling (reviewed in Ref. 6).

Numerous places to interfere with deregulated signaling pathways can be targeted as potential sites for chemopreventive intervention. Key components of these pathways are the PTKs, which catalyze the transfer of the γ-phosphate of ATP to the hydroxyl group of tyrosine on numerous proteins (7). Loss of PTK regulatory mechanisms has been implicated in neoplastic growth; indeed, many oncogenes code for PTKs (reviewed in Refs. 8 and 9).

The abbreviations used are: PTK, protein tyrosine kinase; AR, amphiregulin; CIN, cervical intraepithelial neoplasia; CIS, carcinoma in situ; CSF-1r, colony-stimulating factor 1 receptor; DMBA, 7,12-dimethylbenz(a)anthracene; EGFR, epidermal growth factor receptor; ER, estrogen receptor; HPV, human papillomavirus; IHC, immunohistochemical; INSr, insulin receptor; PDGFR, platelet-derived growth factor receptor; PK, protein kinase; SCC, squamous cell carcinoma; TGF-α, transforming growth factor α; DAPHL, 4,5-dianilinoephthalimide; DAPH2, 4,5-bis(4-fluorooanilino)phthalimide.

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Two general classes of PTKs are currently recognized: receptor tyrosine kinases, which receive signals directly through their extracellular domains; and cellular tyrosine kinases, which are signal transducers (reviewed in Ref. 10). Specific PTKs activated during the development of many human neoplasias have been identified. Examples of aberrant expression of receptor PTKs are EGFR in head and neck cancers (11), p185$^\text{c-erbB-2}$ in breast and ovarian cancers, and PDGFR in glioblastomas and breast cancers (reviewed in Ref. 12).

Targeting Specific PTKs: EGFR

Based on the redundancy of growth factor networks, selective inhibition of signaling pathways activated in precancerous and cancerous cells should be possible. Proliferation of normal cells is dependent on more than one growth factor, and one growth factor activates multiple intracellular signaling pathways. Numerous genetic knockout experiments have established that if a particular growth factor-signaling pathway is inactivated, an alternative pathway takes over (reviewed in Refs. 8 and 12). Because overexpression or mutation of an oncogene can lead to constitutive activation of a single signaling pathway, inhibition of this specific pathway should not disturb other pathways necessary for normal cell function. Thus, inhibiting specific PTKs activated in target tissues should result in fewer effects on the growth of normal compared with precancerous or cancerous cells (reviewed in Refs. 8 and 12).

As noted above, many specific PTKs associated with cancers of various organs have been identified. One of the most extensively studied is the product of the c-erbB-1 proto-oncogene, EGFR (reviewed in Refs. 13 and 14). Disrupting the EGFR-mediated signaling pathway represents a novel approach for cancer prevention. The rationale for using EGFR antagonists as chemopreventive agents, analysis of potential clinically relevant target organs, and identification of inhibitors with specificity toward intrinsic EGFR tyrosine kinase activity are the subjects of this review.

EGFR and Signal Transduction

EGFR is a 170-kilodalton transmembrane protein that is a member of a family of related receptor PTKs that also includes the proteins encoded by the c-erbB-2 (neu), c-erbB-3, and c-erbB-4 genes (reviewed in Ref. 15). Presently recognized EGFR ligands are EGF, TGF-α, AR, betacellulin, and heparin-binding EGF (reviewed in Ref. 16). Some ligands including AR, EGF, and TGF-α are capable of acting as inhibitors, as well as stimulators, of cell proliferation, depending on the specific circumstances (reviewed in Ref. 17). The importance of EGFR and TGF-α in controlling cell growth has been well established (reviewed in Refs. 14 and 16); the importance of other ligands is only beginning to be explored.

Activation of EGFR can occur via autocrine (ligands secreted by the same cell), paracrine (ligands secreted by other cells), or juxtacrine (membrane-bound forms of growth factors activate the receptor on adjacent cells) mechanisms (reviewed in Ref. 15). On ligand binding, EGFR dimerizes with neighboring receptors and is autophosphorylated at three major tyrosine residues. Subsequently, the receptor interacts with several proteins, which are elements of signal transduction pathways, including phospholipase Cγ (PLCγ), phosphatidylinositol-3′-kinase (PI3-Kinase), growth factor receptor-binding protein 2 (Grb2, Src family kinases, and components of the Jak/STAT pathway; MAPK, mitogen-activated protein kinase.

Association of EGFR with Carcinogenesis

General Considerations

Deregulated expression of EGFR and its ligands has been associated with the development of neoplasia in both animals and humans. Aberrant EGFR signaling can occur with receptor overexpression or with mutated forms of the receptor. Both methods of activation have been associated with human cancers, although overexpression is much more common. The most frequently identified receptor mutant, EGFRVIII, has lost amino acids 6–273. This truncation results in ligand-independent tyrosine kinase activity, altered subcellular location, increased stability (21, 22), and enhanced tumorigenicity (23). Until recently, this mutation had only been identified in a limited number of gliomas. However, using more sensitive methods, it has been detected in up to 57% of high-grade and 86% of low-grade glial tumors, 78% of breast cancers, 73% of ovarian cancers, and 16% of non-small cell lung cancers, but not in any normal tissues examined to date (24–26). EGFR overexpression generally occurs in the absence of detectable alterations in the gene, although some cases of amplification and rearrangement have been observed (reviewed in Ref. 27). Recently, Soler et al. (28) reported that the elements of the signal transduction pathway activated by EGFR are determined by the level of receptor expressed. At physiological receptor levels, EGFR binding results in phosphorylation of Src homology 2 domain-containing proteins, which is sufficient to induce a normal mitogenic response. However, when expression of EGFR is increased, other substrates, such as phospholipase Cγ and Ras-GAP, become phosphorylated. This may explain, at least in part, how EGFR overexpression contributes to carcinogenesis.

Increased expression of the EGFR ligands, particularly TGF-α, has also been linked to tumorigenesis. Transgenic mice overexpressing TGF-α develop benign skin lesions and cancers of the liver and mammary gland (29, 30). TGF-α can cooperate with both viral and cellular oncogenes in the induction of neoplasia in multiple tissues (31, 32), and evidence to support a role for TGF-α in transformation independent of its stimulatory effects on cell growth has been presented (33). EGFR has also been associated with carcinogenesis both in vitro and in vivo (reviewed in Ref. 34). Additionally, the oncogenic action of ras genes may be mediated in part by the EGFR-signaling pathway; both TGF-α (35) and other ligands (36) have been implicated.
Role of Aberrant EGFR Signaling in the Development of Specific Human Cancers

Abnormalities in the EGFR-signaling pathway have been associated with the development of many human cancers, although in most tissues, the precise role remains unclear. Much of the available clinical data have been generated from studies of established cancers. These investigations have shown that in some organs, EGFR is overexpressed in a subset of cancers; overexpression may or may not be associated with poor prognosis (reviewed in Ref. 14).

Targets for chemopreventive intervention are tissues in which aberrant EGFR-mediated signal transduction occurs at a relatively early time point during tumor development: bladder, breast, cervix, colon, esophagus, head and neck, lung, and prostate. Examination of changes in EGFR signaling during premalignant stages of carcinogenesis has been less extensively studied than in fully transformed tissues. However, two general patterns have emerged (discussed in detail below). The first pattern is a trend toward increased expression of EGFR during the early stages of carcinogenesis, followed by receptor down-regulation, often subsequent to increased ligand production. This pattern has been observed, for example, in the lung, cervix, and prostate.

The second recurring pattern observed is expansion of receptor and/or ligand expression from a subset of cells in normal tissue (usually in the basal layer) to extended cellular layers during neoplastic progression (e.g., head and neck, bladder, and cervix). These changes in cellular distribution are usually detected using IHC techniques, which allow the maintenance of tissue architecture. However, it should be noted that changes in receptor distribution can be obscured by the use of ligand-binding assays, which, despite their increased sensitivity relative to immunohistochemistry, generally necessitate the use of tissue homogenates.

Furthermore, interpretation of clinical studies has been clouded by the use of "normal-appearing" tissue from cancer patients to determine normal receptor and ligand expression patterns and levels. It is well known that field-wide damage occurs in many tissues exposed to a common carcinogen or harboring a genetic defect. This helps explain the increased risk that persons who have been treated for a primary tumor exhibit for the development of second primary tumors (37); indeed, such "cured" cancer patients are important cohorts for chemoprevention clinical trials. Because changes in expression may occur during the very early stages of tumorigenesis, they would likely be overlooked if histologically normal tissue from cancer patients were used to assess normal tissue levels. For example, such early changes have been noted in expression of EGFR during the development of oral cancers (see below).

Head and Neck. Increased expression of EGFR protein has been found in histologically normal tissue adjacent to head and neck SCC compared with cancer-free, non-smoking controls (11). Levels of EGFR remained elevated but did not increase in hyperplastic and dysplastic tissue; a second large increase in receptor levels was noted in carcinomas. Additionally, although only the basal layer expressed EGFR in the normal epithelium, expression expanded to all layers, including the superficial layer, during neoplastic progression. Increased levels of EGFR and TGF-α mRNA have also been found in histologically normal mucosa isolated from head and neck SCC patients compared with noncancer controls (38).

Deregulated signaling through the EGFR pathway has also been examined at specific head and neck cancer sites. Tumor and normal-appearing mucosal samples from patients with oral SCC, as well as the normal-appearing oral mucosa of heavy drinkers and smokers, displayed increased EGFR expression compared with levels detected in healthy, non-drinking, non-smoking controls (39). Shirasuna et al. (40) reported EGFR expression limited to the basal cells in oral mucosa from healthy normal controls but extending to other cell layers in oral leukoplakia. Connective tissue from normal controls stained weakly for EGF, and the intensity increased as lesions progressed to higher degrees of dysplasia, with the strongest staining in carcinomas. Taken together, these observations suggest that deregulated expression of EGFR and ligands is an early event during the development of head and neck cancers.

Retinoids prevent the development of second primary tumors in cured head and neck cancer patients (41), and these effects have been suggested to result from down-regulation of EGFR and TGF-α (38). Some (42) but not all evidence supports a role for modulation of the EGFR-signaling pathway in the antiproliferative effects of retinoids in head and neck cancers (43).

Lung. In biopsy specimens from heavy smokers, the percentage of cells expressing EGFR increased as the degree of bronchial epithelial changes progressed from normal appearing to squamous metaplasia to dysplastic squamous metaplasia to CIS (44). However, the percentage of positively staining cells was decreased in SCC relative to CIS. A shift in the distribution of cells expressing EGFR was also noted. In the normal epithelium, EGFR was only expressed in the basal layer, but as neoplasia progressed, EGFR extended upward through the epithelial layer to occupy the full thickness of the epithelium.

It should be noted that abnormal EGFR distribution has been observed in reactive (squamous metaplasia and inflammatory atypia) as well as precancerous (dysplasia and CIS) bronchial lesions associated with both non-small cell lung cancer adenocarcinomas and SCC; however, staining was more intense and involved more of the superficial layer of the epithelium in precancerous compared with reactive lesions. Simultaneous aberrant expression of EGFR and p53 occurred in SCC and associated preinvasive lesions but not adenocarcinomas (45).

Esophagus. Deregulated EGFR-mediated signaling has been linked to the development of both esophageal adenocarcinomas and SCC. In Barrett’s mucosa, a premalignant lesion associated with the development of esophageal adenocarcinoma, EGFR and TGF-α expression increased with higher degrees of dysplasia. Both were also increased in high-risk intestinal metaplasia compared with lower-risk gastric cardiac metaplasia (46, 47).

In squamous esophageal lesions, stronger IHC staining for EGFR was observed in dysplasias and CIS than in surrounding normal mucosa; in invasive cancers, variations in intensity and homogeneity of receptor expression were reported (48).

Bladder. The distribution of EGFR differs between normal and neoplastic urothelia. In the normal bladder, EGFR is only expressed on cells of the basal layer, whereas in malignant, dysplastic, and histologically normal tissue from bladder cancer patients, EGFR is equally expressed on cells of the urothelial layers, including those directly in contact with urine (49). Rao et al. (50) observed a progressive increase in the percentage of EGFR-expressing cells obtained from tissue distant from the carcinoma, to tissue adjacent to the tumor, to tumor tissue. Furthermore, patients whose non-muscle-invasive tumors (pTa and pT1) expressed EGFR were significantly more likely to progress toward invasive disease (51). Because high levels of EGF are excreted in the urine, expression of EGFR in the
premalignant and malignant bladder transitional epithelium could have a significant impact on proliferation of these cells (49). Messing and Resnikoff (49) is currently evaluating EGFR as a biomarker for bladder neoplasia in a Phase II clinical chemoprevention trial of the antiproliferative 2-difluoromethylornithine.

**Prostate.** Strong positive IHC staining for EGFR has consistently been reported in all basal cells from normal and benign prostate glands. Although reports of EGFR-positive prostate cancers vary, results of seven different studies found diminished EGFR expression in basal cells of tumors when compared with benign glands (reviewed in Ref. 52). Both high-grade prostatic intraepithelial neoplasia and malignant lesions express significantly less EGFR protein compared with normal, atrophic, hyperplastic, and low-grade prostatic intraepithelial neoplasia (53). In one study in which reduced EGFR levels were noted in malignant compared with normal or benign prostatic hyperplastic cells, expression of the EGFR ligand TGF-α was only observed in malignant cells (54).

**Colon.** Salomon et al. (17) collated data on 559 colon tumors from 12 studies and concluded that 25–77% expressed EGFR. In general, no significant differences between EGFR levels in adjacent, noninvolved colonic mucosa and tumor tissue were observed. Koenders et al. (55) reported higher EGFR expression in the proximal compared with the distal colon in normal-appearing, but not carcinomatous, tissue. They suggested that these regional differences might account, at least in part, for the lack of quantitative differences in EGFR levels between normal-appearing and cancerous tissue observed in other studies. Indeed, this group also reported significantly higher levels of EGFR in normal-appearing tissue than in carcinomas, suggesting that aberrant EGFR expression may be an early event during colon cancer development.

A number of observations suggest that EGFR ligands can influence the growth and development of precancerous and cancerous colon cells. TGF-α levels were higher in colon carcinomas compared with normal-appearing mucosa (56), and a progressive increase in the percentage of lesions expressing TGF-α was observed during the progression from colonic adenomas to CIS to invasive cancers (57). TGF-α enhanced the growth of an adenoma cell line, and growth was completely inhibited by anti-EGFR antibodies (58). Deoxycholate, an endogenous colon tumor promoter, induces TGF-α mRNA in vitro, further supporting a role for this growth factor in the development of colorectal cancer (59). Additionally, studies suggest that TGF-α expression can contribute to the transformation of colon cells independent of proliferative effects (33).

Other EGFR ligands have also been implicated in colorectal cancer development. Saeki et al. (60) detected AR immunoreactivity in approximately 100% of normal colon samples, 15% of noninvolved adjacent mucosal samples, 64% of adenomas, and 50% of colon carcinomas. These results suggest that changes in AR expression occur in normal-appearing, high-risk colorectal mucosa relative to normal tissue.

**Cervix.** Most studies agree that in the normal cervical epithelium, expression of EGFR is limited to the basal and parabasal cells, whereas in CIN, expression expands throughout the full thickness of the epithelium (61–64). Most (61, 62, 65) but not all (66) studies have also shown that expression of EGFR in cancerous tissue decreases relative to intraepithelial lesions. Indeed, based on preliminary findings that EGFR levels increased in low- and medium-grade CIN but decreased in high-grade CIN and carcinoma, Mitchell and coworkers (65) are currently evaluating EGFR as a biomarker in chemopreventive efficacy trials of 2-difluoromethylornithine and the retinoid all-trans-N-(4-hydroxyphenyl)retinamide.

In support of the importance of deregulated signaling via the EGFR pathway during the development of cervical cancer, immortalization of normal human ectocervical epithelial cells with HPV-16, a known risk factor for cervical cancer, results in increased EGFR levels. Retinoic acid reduces EGFR protein levels and EGF binding in HPV-16-immortalized human ectocervical cells but not in normal cells (67). This is interesting in light of the ability of retinoic acid to cause regression of CIN lesions (68).

However, other groups have found no correlation between expression of EGFR and the presence of HPV or between EGFR and low- versus high-risk HPV types when stratified by grade of CIN (63, 64). Because the malignant potential of CIN is highly correlated to HPV type, these results suggest that EGFR expression may be a marker of proliferation rather than an integral component of the cervical transformation process.

**Breast.** The results of studies examining expression of EGFR and its ligands in breast cancers vary significantly. Klijn et al. (69) compiled data from 40 different series of patients (n = 5232) and calculated that, on average, 45% (range, 14–91%) of human breast cancers express EGFR. A much higher percentage (about 80%) express one or more EGFR ligands. Cancer cells lacking EGFR that continue to express EGFR-activating ligands may do so to stimulate stromal cells needed to support tumor growth (70).

Although the actual percentages of tumors expressing EGFR differ among studies, a consistent inverse correlation between expression of EGFR and ER in breast cancers has been reported (69, 70). Some cancers express both receptors; however, evidence suggests that even when both receptors are present, they are not expressed in the same cancer cells. Co-expression in tumor tissue was only observed in a subset of cells from a mixed invasive ductal CIS. In contrast, EGFR and ER are frequently coexpressed in normal-appearing cells obtained from breast cancer patients (71). One explanation for these changes in expression patterns is that malignant conversion may be associated with attainment of independence for at least one of these growth factors. Furthermore, this suggests that premalignant cells unresponsive to antiestrogens may still be responsive to growth inhibition via the EGFR-signaling pathway.

Particularly relevant for the purposes of chemoprevention, expression of EGFR in ductal cells of needle aspirates from women at high risk for breast cancer development (first-degree relative with breast cancer, prior breast cancer, or precancerous mastopathy) was significantly higher than in low-risk controls (72).

Taken together, these observations suggest that normal breast cells are dependent on both EGFR and ER for growth. Cells at high risk for tumor development may overexpress EGFR, leading to increased proliferative activity. As they progress toward malignancy, these cells may lose their dependence on one or both of these growth factors. However, the subset of transformed cells that no longer express cellular receptors may continue to express EGFR ligands to stimulate the growth of stromal cells needed to support tumor cell growth.

Thus, it is clear that EGFR signaling is deregulated during the carcinogenic process in a number of tissues, making selective inhibition of this pathway a viable chemopreventive approach.
Inhibiting the EGFR-signaling Pathway

Although the effects of retinoids on EGFR function, such as those described above for head and neck and cervix, may be involved in its observed chemopreventive action, these compounds are well known to have numerous other biological activities (reviewed in Ref. 73). More definitive conclusions regarding the effects of modulation of EGFR function can be drawn by examining the consequences of specifically blocking EGFR activity. Antitumor activity against xenografts of EGFR-dependent cell lines has been demonstrated with EGFR-specific monoclonal antibodies (e.g., Ref. 74). Another approach is to block EGFR function by inhibiting the receptor's intrinsic tyrosine kinase activity.

Numerous naturally occurring compounds inhibit PTK activity. Quercetin, a flavonoid and well-known chemopreventive agent (reviewed in Ref. 75), was the first PTK inhibitor discovered. However, quercetin also inhibits several other enzymes including cAMP-dependent PKA, PKC, and other ATP-using enzymes (reviewed in Ref. 76). The related isoflavone genistein is more selective toward PTKs than quercetin, but it too inhibits other enzymes, such as histidine protein kinases (reviewed in Ref. 77) and topoisomerases (reviewed in Ref. 78). Other natural products, such as erastin, herbimycin A, favadustin A, and (+)-aeropylinin-1, are also potent PTK inhibitors with increased selectivity toward PTKs and are thus potential lead compounds for the design of novel antisignaling drugs. Numerous synthetic tyrosine phosphorylation inhibitors, collectively known as tyrphostins, have been developed (reviewed in Ref. 76); some specifically inhibit EGFR tyrosine kinase activity and are discussed below.

Requirements for Specific PTK Inhibitors

Several authors have elaborated the requirements for development of specific PTK inhibitors (e.g., 10, 12, 76, 77, 79). Requirements include: specificity among the kinases [e.g., serine and threonine protein kinases (such as PKC) and histidine protein kinases]; specificity toward the target PTK, in this case, EGFR (numerous other PTKs, such as PDGFR, CSF-1R, and INSR, may also be inhibited); high potency; activity in intact cells; and activity in vivo. Inhibitors can act at the level of autophosphorylation or on downstream targets; autophosphorylation is the preferred site, because it should result in total blockade of the signaling pathway. Inhibitors that compete with substrate rather than at the ATP-binding site are also preferable, because they are not as likely to inhibit other ATP-using cellular enzymes. Additionally, the high ATP concentrations in the cell may render inhibitors that are quite effective against the isolated enzymes inactive in intact cells (80).

EGFR Kinase Inhibitors

The following discussion presents an overview of the structural classes of drugs that have been developed as EGFR inhibitors (see Fig. 2). Based on their activity in in vitro and in vivo screening assays, some of these drugs may have chemopreventive potential. It should be noted that PTK inhibitors that do not meet all the requirements set forth above may still be viable chemopreventive drugs under appropriate circumstances. Also, most currently available animal data have been obtained in models relevant for establishing chemotherapeutic effectiveness, i.e., inhibition of growth of transplantable tumors or tumor cells in vivo. Because changes in EGFR-mediated signaling occur during the process of carcinogenesis, effectiveness or ineffectiveness toward established tumors may not

### EGFR Inhibitors

- **Benzyldened Malononitriles**
- **Diaminophthalimides**
- **Quinazolines**
- **[O(Allylamo)imethyl]acrylphophenes**
- **Enollactones**
- **Dihydrobenzylaminosalicylates**
- **Tyrosine Analog Containing Peptides**

**Fig. 2.** Structures of EGFR-specific tyrosine kinase inhibitors.
accurately predict activity or inactivity toward precancerous lesion growth and development. This differential effectiveness has been demonstrated, for example, with retinoids, which are well-established chemopreventive agents but have generally been ineffectual against established tumors in animal models (reviewed in Ref. 81).

**Benzylidene Malononitriles**

Extensive work on structures based on erbsatin and tyrosine incorporating the benzylidene malononitrile nucleus has been carried out by Levitzki (12, 76) and Gazit et al. (82). Some of these compounds can discriminate between PTKs; some can even discriminate between EGFR and the closely related p185-

\[ \text{ERBB2} \], which have 80% homologous kinase domains (12, 80). Based on its inhibitory activity toward EGFR kinase activity, Rhône-Poulenc Rorer is developing the tyrphostin RG 14620 as a topical antiangiogenic. Abnormalities in EGFR regulation are implicated in the etiology of this disease (83). RG 14620 inhibits EGFR-stimulated cancer cell proliferation in vitro and EGFR-dependent tumor growth in nude mice (84). Topical and i.p. pharmacokinetic data on RG 14620 have been published (85). RG-13022 may also be undergoing development for treatment of EGFR-associated cancers (10, 84); however, studies have shown that this drug shows limited specificity for EGFR (86).

**Dianilinophthalimides**

The class of DAPH1 compounds differs by only two carbon-carbon bonds from the PKC inhibitor staurosporine aglycone; however, they have very different physicochemical and biochemical profiles. DAPH1 (CGP 52411) is specific for EGFR relative to PKC, casein kinases, and several other PTKs. In cells, DAPH1 inhibited autophosphorylation of EGFR and the closely related p185-

\[ \text{ERBB2} \] but not PDGFR. Most importantly, DAPH1 displayed antitumor activity in a nude mouse xenograft model against tumors overexpressing EGFR or p185-

\[ \text{ERBB2} \] but had no effect on PDGFR-dependent tumors. The compound did not cause gross toxicity after 15 days of treatment, although it did not have absolute specificity for EGFR (87).

To enhance the stability of DAPH1 in vitro, blockade of metabolic hydroxylation was achieved by synthesis of a fluorine-substituted derivative, DAPH2. In cells, DAPH2 selectively inhibited signals mediated via the EGF and p185-

\[ \text{ERBB2} \] pathways, but not other tyrosine kinases; selective antitumor activity was also demonstrated for EGFR-dependent relative to PDGFR-dependent xenografts in mice. However, DAPH2 was not as selective toward specific PKC isoforms as the parent compound; in particular, it inhibited the PKC-\[ \beta \]2 isoyme with potency equal to that of EGFR. Pharmacokinetics of DAPH2 in rats and mice has been determined (88).

**Quinazolines**

Fry et al. (89) have developed quinazoline derivatives, as exemplified by the highly potent and specific EGFR kinase inhibitor PD 153035 [4-(3-bromoanilino)-6,7-dimethoxyquinazoline]. It is competitive with respect to ATP, rapidly suppresses autophosphorylation of EGFR, with a low micromolar inhibition constant, and selectively blocks EGF-mediated cellular processes, including mitogenesis, early gene expression, and oncogenic transformation. However, PD 153035 was recently reported to be inactive in vitro against the growth of EGFR-dependent tumors (90). As noted above, this result does not necessarily preclude its having chemopreventive activity. Further structure-activity studies on quinazoline and related heterocyclic derivatives have been carried out by this Parke-Davis research group (91); however, PD 153035 remains the most potent compound identified.

The closely related quinazolines CAQ (92) and AG 1478 (93) have also been synthesized. AG 1478 has been reported to be selective for EGFR and to block autophosphorylation in intact cells. It is being evaluated in vivo (cited in Ref. 12).

**Pyrimidines**

A study of 7-aminopyrido[4,3-d]pyrimidines containing aromatic side chains revealed that this series displayed EGF inhibitory activity similar to the corresponding quinazolines (94); however, as for the related quinazolines, none was as potent as PD 153035.

\[(\text{Alkylamino})\text{methyl}]\text{acrylophenones}\]

This group of compounds was designed to act as multistate complex inhibitors based on the transition state proposed for tyrosine kinases. In vitro, they selectively inhibited EGFR relative to v-abl, c-src, or PKC. i.p. administration of the EGFR inhibitor 18 (1-[4-(benzyloxypyphenyl]-2-[(dimethylamino)methyl]-3-[3,4-dimethylphenyl]-thio)propan-1-one at 0.1 \(
\times \) the maximum tolerated dose significantly inhibited the growth of human breast carcinoma cells in nude mice (95). However, based on the differences between inhibition of EGFR-dependent tyrosine phosphorylation and antiproliferative activity, the authors suggested that other mechanisms of action are also likely (95).

**Enolactones**

Tanaka et al. (96) isolated and synthesized the fungal metabolite BE-23372M, which consists of an enolactone ring and two catechol rings. It appears to be specific for EGFR and is competitive with respect to substrate peptide and to ATP. Autophosphorylation of solubilized EGFR and of EGFR in intact cells was also inhibited.

**Dihydroxybenzylaminosalicylates**

A series of 5-[2,5-dihydroxybenzyl]amino]salicylate compounds have been tested against EGFR kinase activity (97). The 5-[2,5-dihydroxybenzyl]-amino]salicylate moiety was chosen as a simplified model for lavendustin A, one of the most potent in vitro natural product PTK inhibitors identified to date. Some of these compounds were competitive with respect to ATP; others were noncompetitive. All the inhibitors were noncompetitive with respect to the peptide substrate. Based on selectivity toward EGFR relative to PKC and PAK and the ability to inhibit EGFR autophosphorylation, promising compounds were identified (e.g., 2-hydroxy-5-[N-(2,5-dihydroxyphenyl)methyl]amino]benzoic acid 3-phenylpropanoate). No results from studies of the inhibitory activity of these compounds toward the growth of tumors bearing amplified EGFR are yet available.

**2-Thioindoles**

A group at Parke-Davis (98), in collaboration with researchers from the University of Auckland School of Medicine (99, 100), screened approximately 100,000 compounds from their chemical library for inhibitory activity against a variety of tyrosine kinases. Using this approach, 2-thioindoles were found...
Aminoflavones

Determine EGFR Tyrosine Kinase Inhibitory Activity

Evaluate EGFR Specificity and Selectivity

Approach to Developing EGFR Kinase Inhibitors as Chemopreventive Agents

To be inhibitors of EGFR. Some compounds in this series, such as PD 146568, have shown selectivity toward EGFR.

Aminoflavones

Some members of a series of synthetic aminoflavones (e.g., 6-hydroxy-3',5,7-triaminoflavone) were effective in vitro against EGFR kinase (101).

Tyrosine Analogue-containing Peptides

Two decapeptides containing the fluorenylethoxycarbonyl derivatives of two homochiral tyrosine analogues, a pyridine N-oxide and a pyridone, were synthesized by Andrews et al. (102). Both peptides inhibited autophosphorylation in vitro, but relatively high concentrations were required. Although the inhibitory mechanism was not determined, the authors suggested that these peptides have the potential to serve as mechanism-based (irreversible) inhibitors based on their similarity to the enzyme substrate.

Approach to Developing EGFR Kinase Inhibitors as Chemopreventive Agents

A number of in vitro and in vivo tests may be used in the development of EGFR kinase inhibitors as potential chemopreventive agents. A possible testing strategy is outlined below (Table 1). Priorities for further development would be based on results at each of the following steps (in order).

Determine EGFR Tyrosine Kinase Inhibitory Activity

Inhibition of EGFR-catalyzed incorporation of [32P]ATP into various substrate peptides can be assessed (e.g., Ref. 89). Sources of EGFR include A431 human epidermoid carcinoma cells (e.g., Refs. 89 and 92) and the purified recombinant EGFR intracellular domain (e.g., Ref. 87). It is important to demonstrate inhibition of EGFR autophosphorylation.

Evaluate EGFR Specificity and Selectivity

The specificity (e.g., serine and threonine protein kinase versus PTK) and selectivity (various PTKs versus EGFR) should be determined. Fry et al. (98) have described an extensive screening battery examining the effects of potential inhibitors on several PTKs in addition to EGFR, including p185"c-src", p60"c-src", PDGFR, CSF-1r, and INS-r. Barret et al. (79) have recently described a simple method to ascertain the ability of a compound to inhibit the activity of a panel of protein kinases using a single substrate to facilitate targeting of specific PTKs and cell types.

Determine Inhibition of EGFR-mediated Effects in Intact Cells

Selective inhibition of autophosphorylation and effects on EGFR-mediated proliferation and transformation can be examined in whole cells. A431 carcinomas, which express high levels of EGFR, have been extensively used for this purpose (e.g., Refs. 89 and 98). Swiss 3T3 fibroblasts stimulated with various growth factors can be used to determine inhibitor selectivity on phosphorylation reactions (e.g., Refs. 87, 89, and 98).

Determine Inhibition of EGFR-mediated Effects in Vivo

Several authors (e.g., Refs. 8 and 98) have discussed requirements for testing PTK inhibitors in vivo. Minimal requirements include deregulation of the specific PTK in the target tissue and demonstration that tumor growth depends on the targeted signaling pathway. In this case, EGFR should be known to be overexpressed in the tumor, and tumor growth should be dependent on its expression. Xenografts of A431 cells in nude mice overexpress EGFR, and their growth can be inhibited by treatment with anti-EGFR monoclonal antibodies (reviewed in Refs. 87 and 98). This animal model has been used to demonstrate activity of EGFR kinase inhibitors in a chemotherapeutic setting (e.g., Ref. 87).

Determine Chemopreventive Efficacy in Vivo

DMBA-induced cancer in the hamster buccal pouch has been suggested as a model for human head and neck cancers (103). Overexpression of EGFR in this model has been observed in both dysplastic lesions and SCCs (104). Pending validation of the dependence of these lesions on EGFR for tumor growth, this model may be especially useful to assess the chemopreventive effects of EGFR kinase inhibitors. Tests for chemopreventive efficacy in other established animal tumor models in which high levels of proliferation are particularly important to carcinogenesis (e.g., rat bladder and colon and mammary and mouse bladder) may logically follow. See the article by Steele et al. (105) for a description of the animal models currently used in the National Cancer Institute Chemoprevention Branch drug development program.

Discussion and Conclusions

The use of specific and selective PTK inhibitors represents a rational, targeted approach toward development of chemopreventive drugs. Because of the redundancy of cellular signaling pathways, inhibition of a specific deregulated pathway in cancerous or precancerous lesions offers the possibility of minimal effects on normal cell function. Deregulated signaling via the EGFR-mediated pathway has been linked to carcinogenesis in several target organs of high interest to the Chemoprevention Branch, as reviewed above. However, the precise role of aberrant EGFR signaling during neoplastic development in most tissues remains unclear. Interpretation of results has been further obfuscated by the use of normal-appearing tissue from cancer patients to determine receptor and ligand expression in normal tissue, which could obscure potential field effects. However, in many tissues, increased expression of EGFR occurs early during neoplastic progression, followed by down-regulation, often subsequent to...
increased ligand production. This suggests that inhibition of EGFR tyrosine kinase activity may be a viable chemopreventive strategy.

Targeting EGFR activity may afford some unique chemopreventive opportunities. Based on the inverse correlation between expression of EGFR and ER, EGFR observed during breast cancer development, premalignant cells unresponsive to antigens may still be responsive to growth inhibition via the EGFR signaling pathway. In the bladder, expression of EGFR in a normal-appearing, high-risk urothelium but not in normal tissue exposed to urine may provide a means to selectively target precancerous cells.

Several authors have elaborated on problems with the current methods available for establishing the effectiveness of specific PTK inhibitors (e.g., Refs. 7–9, 77, 79, and 98). These range from appropriate methods for in vitro testing to identification of relevant animal models. Establishing selectivity and specificity toward PTKs may be quite difficult because of the numerous protein kinases currently known. Additionally, many of the compounds specific for a particular PTK inhibit other protein kinases at higher doses, thus, high doses could lead to lack of selectivity and specificity accompanied by toxicity. Careful dose-response studies will be necessary to establish therapeutic windows.

Furthermore, effects on unknown enzymes may occur. For example, Osherov et al. (80) developed two tyrphostins, both of which are competitive with respect to ATP but specific in vitro for EGFR or p185Erbb-2, despite the 80% homology between the kinase domains of the two receptors. In intact NIH/3T3 cells transfected with either EGFR or p185Erbb-2 (possessing the p185Erbb-2 kinase domain), autophosphorylation and endogenous substrate phosphorylation were not inhibited by either compound, despite the observation that mitogenic signaling induced by EGF in both transfected cells was blocked by these two agents. The authors suggested that the high intracellular ATP levels prevented binding of the inhibitors to their receptors and that the antiproliferative effects of the compounds were mediated by an element downstream, presumably a tyrosine kinase.

Unfortunately, the design of EGFR inhibitors directed at the catalytic site is hindered by the lack of a three-dimensional structure for the enzyme (106). Many EGFR kinase inhibitors identified to date are competitive with ATP, increasing the likelihood of interacting with other ATP-using enzymes. As discussed above, such compounds may be inactive or active only at much higher concentrations in intact cells compared with the isolated enzyme because of high intracellular ATP levels. Despite these hurdles, progress has clearly been made in developing candidate drugs with apparent specificity toward EGFR kinase activity, some of which are active in vivo. Recent publication of the structure of the INSr kinase domain (107), which shares extensive homology to other PTKs (106), as well molecular modeling of the EGFR catalytic site (108), should enhance drug discovery efforts (10).

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References


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