Protein kinase inhibitors for the treatment of cancer

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Protein kinases are integrated into nearly every facet of the regulation of mammalian cellular proliferation. The identification of particular kinase isoforms and tissue-specific expression affords the possibility of precise targeting of specific regulatory pathways if potent, highly selective, cell-penetrable inhibitors can be developed. At the very least, such inhibitors would provide opportunities to dissect complex interconnected regulatory pathways in intact cells, and sustain the promise of the development of therapeutic leads. This paper reviews recent developments in the identification of protein kinase targets for the treatment of cancer, and in the design of selective inhibitors for these enzymes.

The phosphorylation state of proteins has emerged as one of the primary regulatory mechanisms in mammalian cells. The balance between protein kinases and phosphatases clearly plays a critical role in the regulation of cellular proliferation, and these enzymes thus represent potential targets to restore controlled growth in cells where these controls are defective. Approximately 1,000 protein kinases are estimated to be present in mammalian cells, so selecting which of these kinases represent legitimate targets is clearly a daunting task. In addition, protein kinase substrates are highly conserved (ATP and serine, threonine or tyrosine), leading to the recently validated hypothesis that the active sites of protein kinases are also highly conserved[1]. This high degree of homology presents great challenges in identifying selective small-molecule inhibitors of protein kinases. Notwithstanding the difficulties of these challenges, much progress has been made in recent years both in identifying critical kinase targets and in designing selective inhibitors for these targets. Advances in selected areas of particular interest are discussed below.

EGFR/*erb*B

There are at least four members of the type 1 growth factor receptor gene family, including the epidermal growth factor receptor (EGFR, *erb*B-1), *erb*B-2 (*neu*), *erb*B-3 and *erb*B-4 (also known as *her*-1 through *her*-4, respectively). Members of this tyrosine kinase receptor family have been implicated in the establishment or progression of human cancer, most noticeably breast cancer. The overexpression of ErbB-2 and EGFR have been found in many breast carcinomas and correlates with an adverse prognosis for survival[2–5]. There are indications that EGFR is frequently overexpressed in breast cancer metastasis relative to the original tumor in the breast, suggesting a possible role of EGFR in facilitating metastasis or progression to metastasis[4,5]. While ErbB-2 is overexpressed in 30% or more of breast cancers, its role in tumor formation appears to be different from that of the EGFR. Indeed, ErbB-2 overexpression is more frequently found in ductile carcinoma *in situ* than in invasive ductal carcinoma, suggesting a role in initiation of carcinoma rather than progression towards metastasis[6]. The involvement of the other members of the type 1 receptors in tumor formation is less clear. While ErbB-3 has been found to be overexpressed in many breast carcinomas, there does not appear to be a strong correlation of ErbB-3 overexpression with an adverse prognosis. The role of ErbB-4 in cancer remains to be delineated. The involvement of these

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tyrosine kinase receptors in other cancer types is less well documented, although members of the family have been implicated in cancers of the bladder, colon, larynx, esophagus, stomach, lung, kidneys and ovaries. While most of these studies report the overexpression of the wild-type receptor, truncation and/or rearrangements of the EGFR gene occur in glioblastomas. The type 1 receptors are clearly involved in many aspects of human oncogenesis.

Much experimental evidence points to the oncogenic potential of the type 1 growth factor receptors, and indicates that disrupting the signals generated by them can inhibit the growth of transformed cells. Originally, *erb*B-2/*neu* was identified as a transforming oncogene in neuroblastomas in carcinogen-treated rats[7], and was shown to be highly homologous to the EGFR, especially in the tyrosine kinase domain. Feline mammary carcinoma cells, A431 and MDA 468 cells each have elevated EGFR levels, which correlates with an increased ability to form tumors in nude mice[8–10]. Furthermore, addition of EGF by osmotic pump implantation increases the tumorigenic potential of EGFR, suggesting that autocrine loop activity enhances growth of these tumor types[11]. Antibodies directed against ErbB-2 inhibit the growth of tumor cells overexpressing the protein. For example, the 4D5 monoclonal anti-ErbB-2 antibody inhibits growth of SKBR3 cells overexpressing ErbB-2 *in vitro* and *in vivo*[12]. Another approach to disrupting signaling via ErbB-2 has been with the use of antisense oligonucleotides directed against *erb*B-2. Cells treated with antisense *erb*B-2 oligonucleotides exhibited reduced ErbB-2 protein expression, DNA synthesis and monolayer growth compared to the ErbB-2-overexpressing parental cell line[13]. These studies indicate that the type 1 receptors are capable of promoting tumor formation, and that disrupting the signals transduced by them can attenuate their oncogenic potential.

Not surprisingly, an enormous amount of effort has been expended in the attempt to identify specific small-molecule inhibitors of type 1 receptors, and of EGFR and ErbB-2 in particular. These efforts have been the subject of numerous reviews (see, for example, Refs 14,15). One of the most daunting tasks associated with developing potent and selective kinase inhibitors is the high degree of conservation in the kinase domain of most protein kinases, especially in the nucleotide binding site[1].

Many of the earliest EGFR inhibitors, such as quercetin and genistein, were identified in fungal extracts[16,17] (Figure 1). In general, these compounds are competitive with ATP, and display relatively poor activity in intact cells. Erbstatin has been reported to be competitive with both ATP and protein substrate[18,19] and, while it is not a potent inhibitor of EGFR kinase ($IC_{50} \approx 14$ μM), it did serve as the template for the tyrphostins. The primary pharmacophore for these compounds is the hydroxy-*cis*-cinnamonitrile moiety[20] (Figure 1). The original tyrphostins were designed as phosphotyrosine mimetics and were based on the benzylidene malonitrile nucleus. As expected, they were competitive with the polymeric peptide substrate, with K_i values approaching 1 μM and significant specificity compared to the insulin receptor kinase. The more potent compounds also inhibited EGF-dependent cell proliferation. Other derivatives with improved pharmacodynamic properties (e.g. RG 13022) also inhibited the growth of EGF-dependent human squamous cell carcinoma xenografts in nude mice[21], although regression of pre-existing tumors was not observed.

A different class of potent, selective EGFR-selective inhibitors is represented by DAPH-1 (Refs 22,23; Figure 1). The synthetic rationale for compounds in this class is based on staurosporine and diindolylmaleimides, both potent inhibitors of the serine/threonine kinase, protein kinase C (PKC, see below). In spite of the obvious structural similarity between these compounds, the dianilinophthalimides are highly selective tyrosine kinase inhibitors. Like staurosporine, DAPH-1 is competitive with ATP, with $K_i \approx 160$ nM. Compared to EGFR, at least 10-fold higher IC_{50} values were observed for other kinases tested (including eight PKC isozymes). Structural motifs that appear to be important for inhibition of EGFR kinase activity include a free phthalimide nitrogen, intact phenyl rings, small substituents on the aniline rings, and free diphenylamine nitrogens. The fundamental shift in kinase specificity of the dianilinophthalimide derivatives compared with staurosporine is hypothesized to derive from a twisted conformation in DAPH-1 compared to a planar conformation in staurosporine. Inhibition of tumor cell xenograft growth *in vivo* was demonstrated by DAPH-1 at doses between 6.3 mg/kg and 50 mg/kg against A431 and SKOV-3 cell lines expressing EGFR and ErbB-2, respectively. As expected based on *in vitro* specificity controls, growth of a control cell line overexpressing the PDGF receptor was not affected by DAPH-1 treatment.

A remarkable advance in the development of potent, selective inhibitors of EGFR was recently reported by scientists at Parke-Davis Pharmaceutical Research[24]. PD 153035, a substituted 4-anilinoquinazoline, exhibits a $K_i \approx 5$ pM for EGFR kinase (Figure 1). Related inhibitors are competitive with ATP. The compound is reported to exhibit extraordinary selectivity,

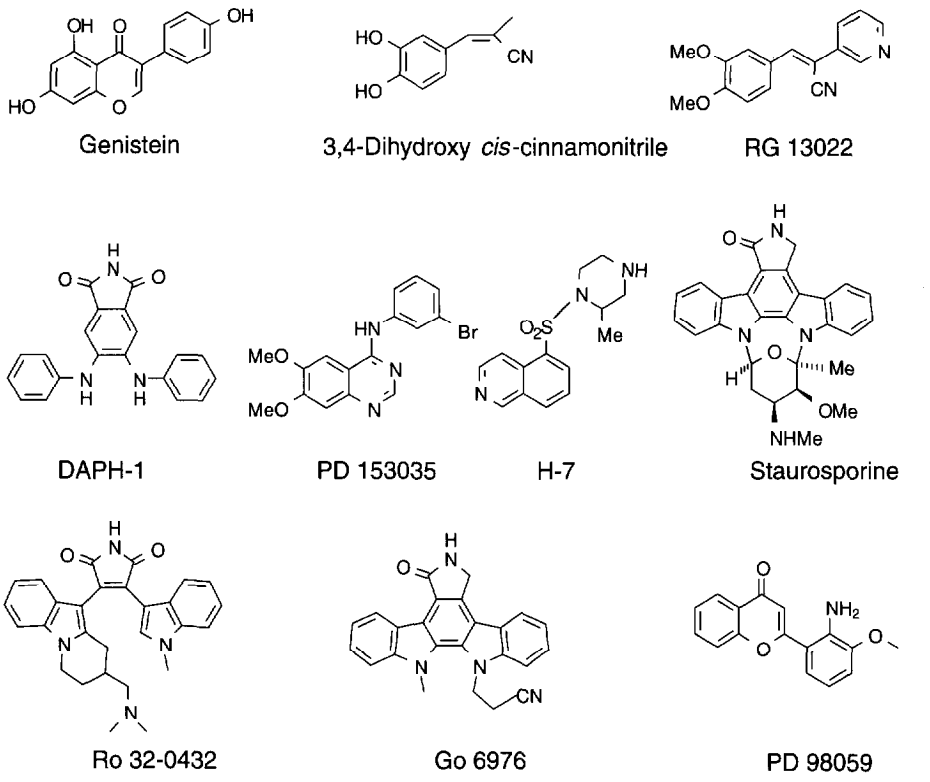

Figure 1. *Protein kinase inhibitors. Structures of key protein kinase inhibitors cited in the text.*

with approximately 10^3-fold higher IC_{50} values when tested against a panel of six other protein tyrosine kinases. Inhibition of EGF-induced mitogenesis was achieved at 80 nM, while stimulation by other growth factor mitogens was not affected at concentrations up to 5 μM. A detailed structure–activity analysis suggests that certain substituent modifications around the 4-anilinoquinazoline nucleus are supra-additive, leading to the hypothesis that PD 153035 and other potent derivatives may be inducing a conformational change in the EGFR active site upon binding[25]. This compound remains among the most potent and selective protein kinase inhibitors reported.

Protein kinase C

The protein kinase C (PKC) family of serine/threonine protein kinases consists of at least 12 isotypes involved in many diverse cellular functions, including development, differen-0tiation and growth control. The various PKC isotypes display heterogeneous tissue distribution, developmental regulation, expression and mechanisms of activation (reviewed in Refs 26,27). Such diversity has complicated the investigation of the role(s) of PKC in oncology, and the use of relatively nonspecific PKC activators and inhibitors further exacerbates the problem (reviewed in Ref. 28). Most forms of PKC are activated by phospholipids and diacylglycerol or fatty acids, which become elevated upon activation of phospholipase enzymes by activated tyrosine kinase or serpentine G-protein-linked receptors. This places PKC in the middle of many signal transduction pathways. PKC is also the major cellular receptor for phorbol esters, which are known to be potent tumor-promoting agents in animal model systems.

The involvement of PKCs in human cancer is complex. PKCs, like the ErbBs described above, are frequently overexpressed in breast cancer, with the more aggressive forms of breast cancer containing higher PKC activity[29]. Tamoxifen inhibits proliferation of breast cancer cells in part by blocking estrogen from binding to the estrogen receptor, but additional growth suppression may be contributed to the ability of tamoxifen to inhibit PKC activity directly[30]. In contrast, PKC may play a growth suppressive role in colon cancer, since both diacylglycerol concentrations and PKC activity are decreased in colon cancer relative to normal surrounding tissue[31,32], and overexpression of PKC-β1 in HT-29 colon cancer cells leads to reduced proliferation *in vitro* and *in vivo*[33]. Finally, the multidrug resistant (MDR) phenotype can be induced by expression of P-glycoprotein alone, but is enhanced by coexpression of PKC-α, which phosphorylates P-glycoprotein. Furthermore, reduction of PKC-α levels with antisense oligonucleotides attenuates the MDR phenotype in this system[34]. Clearly, the development of isotype-specific inhibitors would greatly aid in dissecting the role of PKC in human cancer and other diseases and may lead to beneficial therapeutics.

Protein kinase C inhibitors have been directed against both the regulatory domain and the catalytic domain (reviewed in Refs 35,36). Active-site-directed inhibitors targeting both peptide substrate and nucleotide binding sites have been

reported. Peptides based on the isozyme-specific pseudosubstrate sites of PKC isozymes have been reported to be potent (< 1 μM) inhibitors of PKC, although conversion of these sequences into a low-molecular weight, cell-penetrable inhibitor remains a vexing problem[35,37]. Many examples of nucleotide binding-site-directed PKC inhibitors have been reported, including isoquinoline- (e.g. H-7)[38] and indolecarbazole-containing derivatives (e.g. staurosporine; Figure1)[39]. Extensive modification of the basic staurosporine nucleus has afforded extremely potent inhibitors of PKC with improved selectivity. For example, Ro 32-0432, a bis(indolylmaleimide), is an ATP-competitive inhibitor of rat brain PKC with an IC_{50} of 17 nM, and inhibits bovine heart PKA with an IC_{50} > 20 μM (Figure 1). However, this compound shows only about 10-fold selectivity for Ca^{2+}-dependent versus Ca^{2+}-independent PKC isozymes[36,40]. In the indolocarbazole series, Go 6976 inhibits recombinant PKC-α and -β1 with IC_{50} < 10 nM, but shows no inhibition of the Ca^{2+}-independent δ, ε and ζ isozymes at concentrations up to 1 μM (Ref. 41) (Figure 1). These developments afford hope that potent, isozyme-selective inhibitors of PKC may soon be developed. However, identification of a panel of inhibitors with sufficient *in vivo* potency and selectivity to elucidate the role of different PKC isozymes in signal transduction remains an elusive goal.

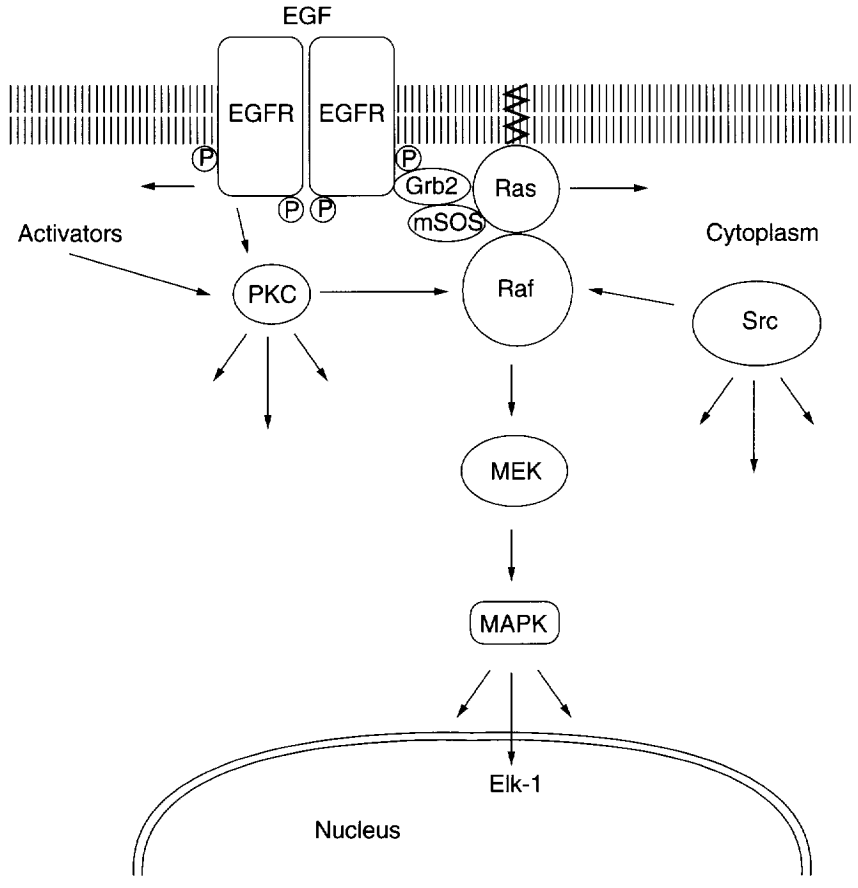

Figure 2. *Simplified schematic diagram of a growth-factor-induced signal transduction pathway in mammalian cells. Activation of EGFR (epidermal growth factor receptor) by ligand binding results in receptor phosphorylation, binding of the Grb2/mSOS complex, and activation of Ras. Activated Ras, in turn, activates components of several signal transduction pathways, including Raf. Raf is also phosphorylated by intracellular kinases, possibly including Src and PKC (protein kinase C). Activated Raf phosphorylates and activates MEK (MAPK/ERK kinase), which phosphorylates and activates MAPK (mitogen-activated protein kinase), which phosphorylates many substrates, including the transcription factor component Elk-1.*

c-Raf-1/MEK

The *raf* serine/threonine protein kinase gene was originally identified when a truncated mutant was discovered to be the transforming element of murine sarcoma virus 3611 (Ref. 42). While direct activation of the endogenous c-*raf*-1 gene in human cancers is rare, overexpression and recombinations have been found in some tumor model systems, although the relevance of these models to human cancers is uncertain[43,44].

The more prominent role for c-Raf-1 in human cancer is via direct interaction with the product of the *ras* oncogene[45,46] (Figure 2). Oncogenic mutations in *ras* are one of the most frequently observed mutations in many human tumor types, especially in the pancreas and colon. Ras transmits signals from receptor tyrosine kinases to the mitogen-activated protein kinase (MAPK) cascade by localizing c-Raf-1 to the cell membrane. Activated c-Raf-1 phosphorylates and activates the threonine/tyrosine kinase, MAPK/ERK kinase (MEK), which phosphorylates and activates MAPK. Since c-Raf-1, MEK and MAPK lie downstream of Ras in the signaling cascade, it is

reasonable to presume that inhibiting any of these kinases would block mitogenic signals from Ras. Indeed, dominant negative forms of either c-*raf*-1 or MEK are sufficient to block transformation of cells when cotransfected with an activated *ras*[47,48]. Furthermore, c-Raf-1 antisense RNA expressed in normal rat kidney cells reduced c-Raf-1 expression and blocked transformation by several oncogenes including v-*erb*B and v-K-*ras* (Refs 47,49). Conversely, constitutively activated forms of c-*raf*-1 (v-*raf*) and *MEK* are sufficient to transform NIH3T3 fibroblasts[48,50], suggesting that constitutive activation of the MAPK cascade by any upstream element may be sufficient for transformation. Multiple isoforms of Raf and MEK are involved in the MAPK cascade, and multiple kinase cascade pathways parallel to the MAPK cascade have been identified in mammalian cells. Development of selective inhibitors of each isoform will aid in unraveling the intertwined signal transduction pathways and may lead to potent anticancer therapies.

Unlike PKC and EGFR, good peptide-based substrates for c-Raf and MEK have not been identified, suggesting that either the sequences surrounding the phosphorylation site are in a highly constrained conformation, which is important for substrate phosphorylation, or that a significant portion of the substrate binding energy is derived from interactions that are distant from the phosphorylation site[51,52]. The first reported small molecule inhibitor of the MAPK pathway was identified in a coupled assay containing unactivated MEK and MAPK in a single reaction mixture[53–55]. PD 98059 inhibits the weak kinase activity of unphosphorylated or mutagenically activated MEK with an IC_{50} of approximately 2 μM, but it is completely inactive when assayed with MEK activated by Raf-catalyzed phosphorylation. The compound inhibits MEK-kinase-dependent or Raf-dependent activation of MEK with an IC_{50} of 4 μM, yet does not inhibit Raf autophosphorylation or Raf-dependent phosphorylation of myelin basic protein. These results suggest that PD 98059 binds unactivated MEK and inhibits its activation by Raf and other MEK kinases. This unusual mechanism of kinase inhibition appears to afford high selectivity, since little inhibition of a panel of 18 other protein kinases is observed at concentrations up to 50 μM. In intact cells PD 98059 inhibits activation of MEK induced by a number of mitogens, including EGF, PDGF (platelet-derived growth factor), TPA (tissue plasminogen activator) and serum. Similarly, this inhibitor reverts the transformed morphology phenotype in *ras*-transformed normal rat kidney cells. This compound appears to be a powerful tool for validating inhibitors of the MAP kinase pathway as potential therapeutic agents for controlling unregulated cellular proliferation.

Kinase targets deserving honorable mention

There are many other protein kinases that are potential cancer therapeutic targets. The Janus protein tyrosine kinases (JAKs) are involved in signal transduction associated with the cytokine receptor superfamily. Amongst other substrates, these kinases phosphorylate and activate STATs (signal transducers and activators of transcription), which translocate to the nucleus and initiate gene transcription (for recent reviews, see Refs 56,57). An inhibitor of JAK2 has recently been reported to inhibit growth of acute lymphoblastic leukemia cells both *in vitro* and *in vivo*[58].

The *src* family of protein kinases constitute the largest group of oncogenes. Although these proteins are not transmembrane receptor kinases, they are frequently associated with the plasma membrane via myristylation and are involved in many signal transduction pathways. Many family members have been identified as constitutively activated viral transforming genes. For example, *abl* was identified as a viral transforming gene originally isolated from the Abelson murine leukemia virus. The human *abl* protein tyrosine kinase gene is frequently rearranged with the *bcr* locus, thus forming the Philadelphia chromosome associated with chronic myelogenous leukemia[59]. These findings suggest that constitutive activation of these kinases may promote uncontrolled growth.

The cyclin-dependent kinases (CDKs) clearly play a central role in the control of progression through the cell cycle. CDK activity is controlled by phosphorylation and by interactions with regulatory proteins (i.e. cyclins and cyclin-dependent kinase inhibitors, CDIs) (for a recent review, see Ref. 60). The significance of appropriate CDK regulation is apparent from the role of the cyclins and CDIs in cell transformation and cancer. Cyclin D1 has been identified as the *prad*1 oncogene and cyclins D1 and E are overexpressed or rearranged in mammary carcinomas, esophageal carcinomas, lymphoid adenomas and other human cancers[61–63]. In contrast, the CDIs have been implicated as growth suppressor genes since some members are deleted in many tumor types[64]. Restoration of cell cycle regulation may be achieved by mimicking the activity of the CDIs, and identifying specific inhibitors of the CDKs is one approach towards that goal.

With apologies to those whose work has not been mentioned, it is clearly impossible to thoroughly discuss the wealth of progress in the kinase field in any detail in a review of this length. We hope that the highlights cited above will provide a feeling for the sense of excitement resulting from the enormous progress in recent years, from both a biologic and a medicinal chemistry perspective, and the anticipation of further advances to come.

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