REVIEW ARTICLE

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Tyrosine Kinase Inhibitors Targeted to the Epidermal Growth Factor Receptor Subfamily

Role as Anticancer Agents

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Abstract

Abnormal cell signal transduction arising from protein tyrosine kinases has been implicated in the initiation and progression of a variety of human cancers. Over the past 2 decades pharmaceutical and university laboratories have been involved in a tremendous effort to develop compounds that can selectively modulate these abnormal signalling pathways. Targeting receptor tyrosine kinases, especially the epidermal growth factor receptor subfamily, has been at the forefront of this effort as a result of strong clinical data correlating over-expression of these receptors with more aggressive cancers.

There are a variety of strategies under development for inhibiting the kinase activity of these receptors, targeting both the extracellular and intracellular domains. Antibody-based approaches, immunotoxins and ligand-binding cytotoxic agents use the extracellular domain for targeted tumour therapy. Small molecule inhibitors target the intracellular catalytic region by interfering with ATP binding, while nonphosphorylatable peptides are aimed at the intracellular substrate binding region. Compounds that inhibit subsequent downstream signals from the

receptor by interrupting intracellular protein recognition sequences are also being investigated.

In the past 5 years enormous progress has been made in developing tyrosine kinase inhibitor compounds with sufficient potency, bioavailability and selectivity against this subfamily of receptor tyrosine kinases. The anti-HER2 monoclonal antibody, trastuzumab, for patients with metastatic breast cancer is the first of these inhibitor compounds to gain FDA approval. However, preclinical and clinical trials are ongoing with a variety of other monoclonal antibodies, immunotoxins, and small molecule quinazoline and pyrimidine-based inhibitors. Although their cytotoxic and cytostatic potential has been proven, they are not likely to replace standard chemotherapy regimens as single-agent, first-line therapeutics. Instead, their promising additive and synergistic antitumour effects in combination with standard chemotherapeutics suggest that these novel agents will find their greatest utility and efficacy in conjunction with existing anticancer agents.

Kinases are enzymes that phosphorylate specific protein, carbohydrate or lipid residues. Of these, protein kinases are best known as the regulatory signals for a wide variety of cellular processes such as growth and differentiation. The protein kinase superfamily is among the largest known and is divided into groups depending on the amino acid acting as the substrate for phosphorylation: serine, threonine, or tyrosine. Serine protein kinases were first described in 1955 as enzymatic participants in glycogen metabolism,[1,2] followed later by serine/threonine kinases in the 1960's. Tyrosine kinases were not recognised until 1980 with the discovery of the Rous sarcoma virus *src* gene.[3] Since that time, hundreds of protein kinases have been identified which are intimately involved in both normal cell growth and malignant transformation. One report estimates that the human genome may contain up to 2000 protein kinases.[4,5] Although the functions of individual protein kinases are varied, x-ray crystallographic studies demonstrate that the 3-dimensional structure of the core kinase region is highly conserved, consisting of 2 lobes connected by a hinge region. The smaller N-terminal lobe is involved with ensuring the correct orientation of ATP, which binds in the cleft between the 2 lobes. The larger C-terminal lobe is involved with substrate recognition and catalysis.[6-11]

Protein tyrosine kinases are thought to be younger on an evolutionary scale than their serine and threonine counterparts, and have not been identified in prokaryotes or the yeast genome.[6] Thus, they have been postulated to have evolved out of a need for cell-to-cell signalling via extracellular factors. Consistent with this theory, many of the known tyrosine kinases are integral transmembrane receptors that act to transduce extracellular signals to intracellular responses. These are referred to as receptor tyrosine kinases. The remaining kinases are intracellular and referred to as nonreceptor tyrosine kinases. Table 1 lists most of the currently known families of receptor and nonreceptor kinases as categorised by the Protein Kinase Resource.[12,13] Structural data from known tyrosine kinases in both active and inactive states is emerging at a rapid rate, and newly identified tyrosine kinases continue to be reported.[14-19]

1. Tyrosine Kinases and Cancer

The case for the involvement of tyrosine kinases in cancer is strong. Tyrosine kinase activity has been detected in a significant fraction of oncoproteins,[4,20] and increased kinase activity is a hallmark of neoplastic cells. In the laboratory, normal cells can be transformed to a neoplastic phenotype by overexpressing or mutating various receptor tyrosine kinases to increase kinase activity. This transformation can be reversed by antibodies to the receptor, specific kinase inhibitors, or mutations in the receptor which lead to decreased kinase activity.

Moreover, the degree of malignant transformation correlates with the degree of protein kinase activity. In the clinic, overexpression of c-erbB2/HER2/neu (hereafter referred to as HER2) and/or the epidermal growth factor receptor erbB1/HER1/EGFR (hereafter referred to as EGFR) in tumour isolates correlates with poorer prognosis and shorter survival times for patients with breast and ovarian cancers.[21-23] Additionally, the activity of c-src is known to be increased in a variety of cancers, and in certain colon cancers, c-src kinase activity increases as disease progresses from a localised tumour to the metastatic stage.[24] Mutational activation of c-src has also been recently reported in a subset of colon cancers.[25]

Thus, substantial evidence points to an intimate relationship between the signalling ability of tyrosine kinases and the initiation, growth and metastases of many human tumours. The kinases which mediate the tightly regulated signal transduction

Table I. Tyrosine kinase families[12,13][a]

Membrane spanning tyrosine kinases				**Non-membrane spanning tyrosine kinases**			
EGFR family				*Src family*			
EGFR[b]	HER	DER	let-23	Src[b]	Yes	Fyn	Yrk
HER2[b]	HER4			Fgr	Lyn	Hck	Blk
Eph/Elk/Eck orphan receptor family				Dsrc64	Stk	SRK1	SRK2
Eck	Eek	Hek	Ehk-1	SRK3	SRK4	TorFYK	
Ehk-2	Sek	Elk	Cek10	*Tec/Atk family*			
Cek9	Hek2	Nuk	Eph	Tec	Itk/Tsk	Btk[b]	Dsrc28
Ehk-3				DtSpk-1	Etk/Bmx	Tsk/Rlk	
Axl family				*Csk family*			
Axl	Ark	c-Eyk	Brt/Sky	Csk	Matk		
Tie/Tek family				*Fes (Fps) family*			
Tie	Tek			Fes	Fef	Dfps	
PDGFR family				*Abl family*			
PDGFR-β[b]		PDGFR-α[b]		Abl	Arg	Dabl	Nabl
CSF-IR	c-kit	Flk2	Flt1[b]	*Syk/ZAP70 family*			
Flt4	Fik1[b]	RET		Syk2	XAP70	Htk16	
FGFR family				*Tyk2/JAK1 family*			
FGFR-1[b]	FGFR-2[b]	FGFR-3[b]	FGFR-4[b]	TYK2	JAK1[b]	JAK2[b]	JAK3[b]
Insulin receptor family				*Ack family*			
INS.R	ERR	IGF-IR		Ack-1 Ack-2			
LTK/ALK family				*Fak family*			
Ltk	Alk			Fak	FakB	Pyk2	
Ros/Sevenless family							
c-ros	7LESS						
Trk family							
TrkA[b]	TrkB	TrkC					
Ror family							
Ror1	Ror2	Dror					
DDR/TKT family							
DDR	TKT						
Hepatocyte growth factor receptor family							
MET	c-Sea	RON					

a Other classifications exist and new tyrosine kinases continue to be identified.

b Indicates tyrosine kinases with inhibitors in development for anticancer therapeutics.

EGFR = epidermal growth factor receptor; **Fak** = focal adhesion kinase; **FGFR** = fibroblast growth factor receptor; **JAK** = Janus kinase; **PDGFR** = platelet-derived growth factor receptor.

pathways in normal cells can be mutated or overexpressed leading to constitutive signalling and abnormal cell growth, morphology and the malignant phenotype.

2. Strategies for Tyrosine Kinase Inhibition

In the nearly 20 years since tyrosine kinases were first identified, there has been an enormous effort to develop compounds which can inhibit tyrosine kinase activity. Both pharmaceutical companies and university laboratories have been active in this effort with the expectation that a potent and selective tyrosine kinase inhibitor would represent a new class of therapeutics for cancer as well as other proliferative diseases. Although the majority of compounds described have been small molecule inhibitors of the kinase itself, there are many points at which intervention can take place. Figure 1 illustrates the many possible strategies, from upstream interaction with an extracellular ligand, to downstream interference with kinase signalling. Although only few of these strategies have advanced beyond the pre-clinical stage, the concept of selectively interrupting the signal transduction pathway has attracted increasing biotechnology resources.

3. Targeting the Epidermal Growth Factor Receptor Tyrosine Kinase Subfamily

Several extensive reviews document the enormous number of tyrosine kinase inhibitor compounds that have emerged in the past few years.[26-30] We have chosen to highlight advances in the development of compounds active against the EGFR tyrosine kinase subfamily, in particular, HER2. These receptors and their agonists are the most studied members of the receptor tyrosine kinase family and evidence implicating a direct link between these receptors and human cancers is the most convincing. This receptor subfamily has been targeted by nearly all the strategies shown in figure 1, and several of these compounds are currently in clinical trials. In fact, a recent report estimates that of all the tyrosine kinase inhibitors described to date, >50% have been characterised with respect to the EGFR subfamily.[28] Finally, several major advances have recently taken place including FDA approval of the anti-HER2 monoclonal antibody, trastuzumab, for metastatic breast cancer and the synthesis of highly potent and specific irreversible EGFR kinase inhibitors. Therefore, in this review, we are narrowing the discussion of specific tyrosine kinases, while broadening the traditional definition of a tyrosine kinase inhibitor.

3.1 Monoclonal Antibodies

Directing monoclonal antibodies (mAbs) toward either the receptor kinase or its corresponding ligand, represents a rational and promising anticancer strategy. By selectively targeting the receptor in the extracellular space, the resulting intracellular signal cascade can be modulated. Moreover, these antibodies may help to augment a host antitumour immune response. Antibodies have been raised against several epitopes on EGFR and HER2, as well as against the EGFR ligands EGF and transforming growth factor (TGF)α. Although these antibodies are far more effective *in vitro* than *in vivo*, the field of targeted immunotherapy is advancing rapidly.

Trastuzumab, formerly known as rhumAb4D5, is the first of these tyrosine kinase-targeted immunotherapeutics to gain FDA approval and binds the extracellular domain of membrane-anchored HER2 with nanomolar affinity. *In vitro*, trastuzumab-like antibodies on continuous exposure have been shown to downregulate the kinase signalling of this receptor, decrease cell proliferation, and increase cell sensitivity to both tumour necrosis factor α as well as cisplatin, paclitaxel and doxorubicin in HER2 overexpressing cell lines.[31-33] Animal models substantiated these results, demonstrating tumourstatic and tumouricidal responses to HER2 overexpressing tumour xenografts.[34] As recently reviewed,[35] a multinational phase II clinical trial showed that approximately 17% of patients with metastatic breast cancer expressing the highest levels of HER2 achieved >50% reduction in tumour size when given trastuzumab as a single agent. In phase III testing and when given in combination

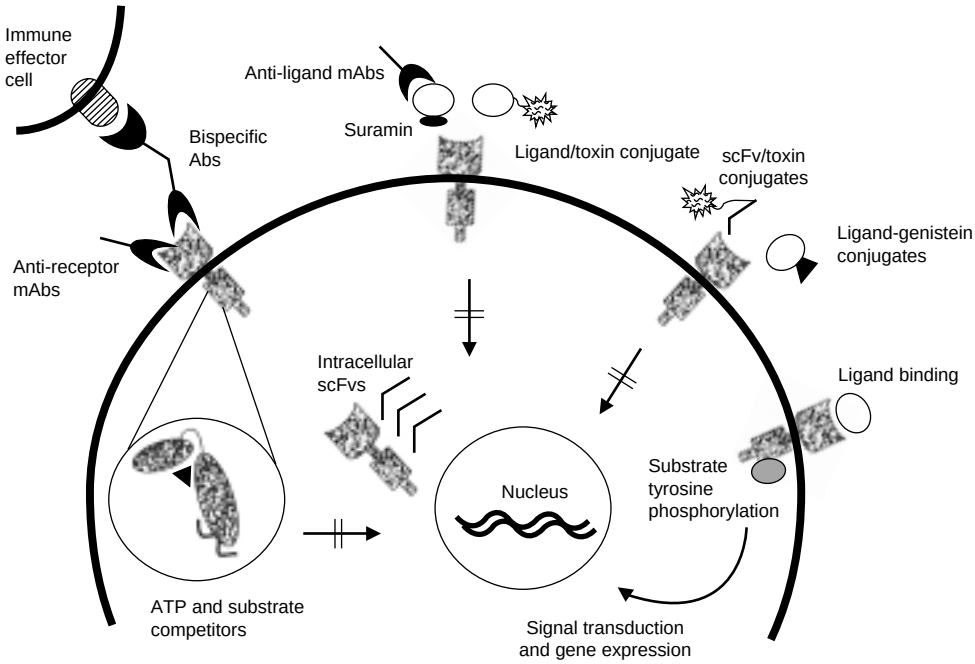

Fig. 1. Potential strategies for inhibiting receptor tyrosine kinase activity for anticancer purposes. The enlarged insert is a schematic representation of the conserved catalytic domain of both receptor and nonreceptor protein tyrosine kinases derived from 3 dimensional structure analysis. **Abs** = antibodies; **ATP** = adenosine triphosphate; **mAbs** = monoclonal antibodies; **scFv** = single chain Fv (variable region-containing fragment) antibodies.

with paclitaxel or doxorubicin/cyclophosphamide, objective responses increased to 44% and 53%, respectively. Surprisingly, trastuzumab-induced cardiotoxicity was seen in these trials, despite the minimal level of HER2 expression on mature cardiac myocytes.[35-37]

HER2 overexpression is seen in approximately 30% of all breast cancers,[22] yet only a small fraction of these cancers are responsive to trastuzumab as single agent therapy.[35] Both alternatively-spliced/secretable forms of the extracellular domain (ECD) of HER2, and proteolytic shedding of the ECD have been described in cell culture and animal models.[38-41] Serum-shed ECD may form serum immunoprecipitation complexes with trastuzumab, leading to a shortened serum half-life and less than growth inhibiting serum concentration of trastuzumab. Proteolytic shedding may also leave an inaccessible membrane bound cytoplasmic kinase with unregulated activity, or may interfere with HER2 assays for overexpression.[35] Clearly further studies are needed to address the molecular mechanisms of trastuzumab sensitivity and host toxicity, and more specifically identify the subset of patients who benefit from this antibody.

Monoclonal antibodies to EGFR have also been generated[42-44] and a human/mouse chimeric antibody [anti-EGFR-mAb-225 (C225)] is currently in clinical trials.[45] This antibody has been shown to bind to the EGFR and effectively compete with ligand binding, resulting in decreased receptor kinase activity, decreased cell proliferation, and cell cycle arrest.[46] The mechanism for this growth arrest has been shown to involve upregulation of cyclin-dependent kinase-2 (cdk-2) inhibitors and decreased cdk-2 activity (a serine/threonine kinase) leading to cell cycle arrest in the G_1 phase.[47] A decrease in angiogenesis may also contribute to

the observed antiproliferative effects.[48] As with trastuzumab, this antibody exerts a strong synergistic effect when given in combination with chemotherapy in EGFR receptor-overexpressing tumour xenografts.[49] Early reports on toxicity demonstrate that this antibody is well tolerated at receptor saturating doses. Results from phase II and III clinical trials are expected to shed new perspective on the spectrum of malignancies treatable by mAb immunotherapy.

Tyrosine kinase activity can be blocked *in vitro* by producing mAbs to some of the known ligands of EGFR, such as EGF and TGFα. However, these ligand-neutralising mAbs have not yet been shown to be effective as anticancer agents *in vivo*.[50,51]

3.2 Bispecific and Single Chain Antibodies

Bispecific antibodies (BsAbs) are another approach to targeting receptor tyrosine kinases.[52-54] These antibodies are engineered to contain one bivalent Fv (variable region-containing fragment) antibody arm targeting an epitope on a tumour surface, and another targeting an immune effector cell in order to recruit immune effector cells to the tumour target and produce an increased host-generated cytotoxic effect. Early phase clinical trials with bispecific antibodies targeting HER2 and EGFR (e.g. MDX-H210 and MDX-447, respectively) are ongoing.[53,55-57] One immunological epitope targeted by BsAbs is the type 1 receptor for immunoglobulin G. This receptor directs cytotoxic macrophages, monocytes, and cytokine-activated polymorphonuclear cells to the HER2 or EFGR overexpressing tumour. *In vitro*, these BsAbs are specific and demonstrate dose-dependent activity. *In vivo*, objective responses have been noted in several patients with advanced renal cell and prostate carcinomas.[53] Large scale manufacturing and purification problems are significant, but recent improvements have been reported.[58] As with all of these immunotherapies, their specific role in the treatment of human cancers has yet to be determined.

Single chain Fv antibodies (scFvs, the smallest high-affinity monovalent binding fragment of an antibody) generated against the EGFR and HER2 family have also been reported, both alone and conjugated to toxins.[59-61] These recombinant proteins can be exogenously administered or intracellularly generated when provided with either secretory or retention signal peptides. When expressed in HER2 positive cells, these proteins can either inhibit ligand-mediated receptor kinase activity or prevent receptor maturation and cell surface expression, thus acting in either an autocrine or intracrine fashion.[62,63] Development of scFv-based therapeutic agents is still immature and obstacles in delivery, stability and gene transfer must be overcome.

3.3 Immunotoxin Conjugates

Advances in recombinant technology have led to the development of a large number of immunotoxin conjugates specific for the EFGR family. These bifunctional fusion proteins consist of a tumour-targeting segment conjugated to a potent cellular toxin. The tumour-targeting segment of the fusion protein can be either a scFv directed to the receptor or ligand, or the recombinant ligand itself. The toxin component most commonly used is a truncated form of Pseudomonas exotoxin A (ETA), but fungal and plant-derived toxins have also been shown to be effective cytotoxic agents. The truncated forms of a toxin lack their endogenous targeting sequence, and upon fusion to an ectopic sequence result in better tumour accessibility and penetration.

ScFv/ETA conjugates have been generated against epitopes on EGFR and HER2,[64-67] and potent cytotoxicity has been noted both in cell culture and in nude mouse xenograft systems. Among the more promising of these compounds is scFv-14e1-ETA-fusion-toxin, which binds to both the EGFR and a constitutively active tumour-associated variant EGFRvIII with equal affinity, yet shows a 100-fold increased cytotoxicity against tumour expressing variant EGFRvIII.[64,65] Another compound, scFv-FRP5-ETA-fusion-toxin targets HER2, and has been shown to detect and eliminate the formation of new metastatic lesions and to decrease the size of established lesions when given systemically to nude mice transplanted with HER2-overexpressing tumour xenografts *in vivo*.[66] A commercial

form, AR-209 (OLX-209), with favourable *in vivo* pharmacodynamics and efficacy in the picomolar range has entered phase I clinical testing against HER2-overexpressing breast, prostate, ovarian and nonsmall cell lung cancers.[68-70]

Bispecific scFv/ETAs have also been developed by combining the receptor-targeting ScFv(FRP5)-ETA with a TGFα ligand-targeting scFv. These conjugates have resulted in regressions of established squamous cell A431 tumour xenografts.[71] In addition, scFv/ETAs containing both HER2 and EGFR binding sites have been reported.[72] This latter strategy addresses the synergy seen in transforming ability and tumour aggressiveness when these two receptors are co-overexpressed. It is currently unclear whether these bispecific compounds confer an added advantage over monospecific scFvs given alone or in combination. Increased tumour specificity may be gained at the price of decreased tumour accessibility.

3.4 Ligand-Binding Cytotoxic Agents

Ligand/toxins represent another targeted anticancer therapy under active investigation and several compounds have been generated using heregulin/neu differentiation factor, EGF and TGFα conjugated to various truncated forms of ETA and other less immunogenic cellular toxins.[73-77] Although these agents are cytotoxic *in vitro* and *in vivo*, they require internalisation of the receptor/ligand/toxin complex in order to be effective. Since this internalisation requires an activated receptor kinase, these compounds are agonistic and result in increased tyrosine phosphorylation. Thus, they will not be discussed further. For a recent review highlighting advances made targeting cancer cells with ligand/toxin conjugates, see Fitzgerald.[78]

While ligand/toxin conjugates lead to receptor tyrosine kinase activation, several other ligand-associated compounds have been described that either directly or indirectly inhibit tyrosine kinase activity. The most advanced of these compounds is an EGF-genistein fusion protein. Genistein, a natural isoflavone derived from soybeans, has long been known as a tyrosine kinase inhibitor.[79] However, insufficient specificity and potency, coupled with unacceptable toxicity, has limited its development as an independent anticancer agent. When genistein is photochemically conjugated to EGF, potency is increased from the micromolar to the nanomolar range, and toxicity is markedly reduced. These EGF-genistein conjugates have been shown to be more effective than standard chemotherapy agents at producing long-term survival in mice when given 24 hours after an inoculation with EGFR-positive breast cancer cells. However, in established MDA-231 xenografts, tumour shrinkage only occurred with small (<0.5cm) tumours and not with larger (≥1.0cm) tumours. No antitumour activity was seen when genistein was given alone or in combination with EGF, suggesting that the conjugate indeed led to EGFR targeting of the tyrosine kinase inhibitor.[80,81] Although unlikely to be first-line agents, this class of compounds may be useful as adjuvant therapy of EGFR-positive cancers.

Suramin sodium (and related derivatives) have been tested for anticancer potential because of their ability to bind a variety of growth factors, including EGF, and to inhibit these factors from activating their receptors.[82-84] Although they were initially developed to treat trypanosomal diseases, it was noted early on that they also inhibited cancer cell growth *in vitro*. Several studies have tested these compounds in EGFR overexpressing oesophageal and lung cancer cell lines with inconsistent results. In one study, suramin reportedly increased EGFR autophosphorylation and stimulated tumour cell growth at subtoxic concentrations.[85] In a second study, a slight inhibition of cell growth was noted.[86] However, unless more specific and less toxic derivatives are synthesised, suramin is not likely to gain acceptance as an anticancer agent.

3.5 Small Molecule Kinase Inhibitors

Small molecule inhibitors directly inhibit tyrosine phosphorylation by physical interactions with the highly conserved kinase domain shared by both receptor and nonreceptor tyrosine kinases. These compounds are what are typically referred to as tyrosine kinase inhibitors (TKIs) in the literature and

hundreds have been patented to date. The first 15 years of drug discovery in this arena did not produce inhibitors that were sufficiently potent, specific, or bioavailable to be clinically useful. However, the past 5 years have produced compounds with increased specificity, >4 orders of magnitude greater potency than their precursors, and with tumour bioavailability on *in vivo* administration, thus renewing optimism that these compounds may become clinically useful anticancer agents.

Several different classes of TKIs have emerged as a result of 3 different strategies: mimicking the structure of known natural kinase inhibitors, molecular modelling of the kinase domain, and large scale screening methods. Genistein, herbimycin-A, quercetin, lavendustin-A and erbstatin, are all naturally derived compounds which have demonstrated broad inhibition of tyrosine kinase activity in cell-free systems with IC_{50} values in the micromolar range.[28] The last compound, erbstatin,[87] is a competitive bisubstrate inhibitor with respect to both ATP and protein substrates. Erbstatin was noted to contain a single phenolic ring similar to tyrosine itself and was initially reported to be a pure substrate competitor. Believing that the key to achieving specificity was to target the substrate rather than the highly conserved ATP binding site, erbstatin served as the starting point for an array of compounds with inhibitory activity. These inhibitors, subsequently referred to as tyrphostins (for *tyr*osine *phos*phorylat*i*on *in*hibitors) share a common benzylidenemalononitrile (BMN) core and make up the majority of early TKIs.[26] Other natural inhibitors also served to generate early pharmacophores. Potencies were generally in the micromolar range and correlation between kinase inhibition in cell-free systems and in intact cells was poor.

After x-ray crystallography revealed the highly conserved structure of the kinase domain in several protein kinases, molecular modelling was employed to develop new compounds that could selectively and tightly bind to various kinase targets.[88-90] The EGFR was the focus of many of these modelling efforts and in combination with large scale drug screening programmes, several classes of compounds emerged as effective inhibitors in the picomolar to nanomolar range with improved specificity and pharmacokinetics. Although many other classes of compounds have been reported,[27,28] the quinazolines and the pyrazolo/pyrrolo/pyridopyrimidines seem the most promising and are furthest along in their development. In contrast to earlier belief, these compounds are competitive at the conserved ATP binding site yet achieve a high degree of specificity. Figure 2 shows some examples of investigational compounds from these classes of TKIs.

3.5.1 Quinazoline Inhibitors

In the mid 1990s, a major advance was made with the discovery and disclosure of the quinazoline class of inhibitors. The potency of these inhibitors surpassed that of previously described EGFR TKIs in cell-free systems by 3-5 orders of magnitude, while displaying specificity against other subfamilies of receptor and nonreceptor tyrosine kinases. Even within the EGFR subclass, only HER2 was shown to be inhibited at micromolar concentrations versus picamolar concentrations for EGFR. At least two investigational compounds are currently in clinical trials after demonstrating *in vivo* bioavailability with favourable pharmacodynamics and pharmacokinetics. The first is ZD-1839, an orally bioavailable nontoxic compound developed by AstraZeneca (Wilmington, Delaware, USA). In the EGFR overexpressing A431 tumour xenograft mouse model, this compound was able to eradicate 1.5g subcutaneous tumours within 2 weeks and achieve tumouristasis for up to 4 months. Similar results were obtained in a variety of other EGFR-positive solid tumour xenografts. Tumouristatic (versus tumouricidal) efficacy was evident from the observation that termination of ZD-1839 therapy resulted in the regrowth of these tumours.[94] Clinical testing with ZD-1839 is currently underway in patients with metastatic breast cancer. The second compound, CP-358774, developed by Pfizer (New York, New York, USA), showed similar anticancer activity both *in vitro* and *in vivo*. In a head and neck squamous cell carcinoma model, EGF-dependent cell proliferation was inhibited by 50% with drug con-

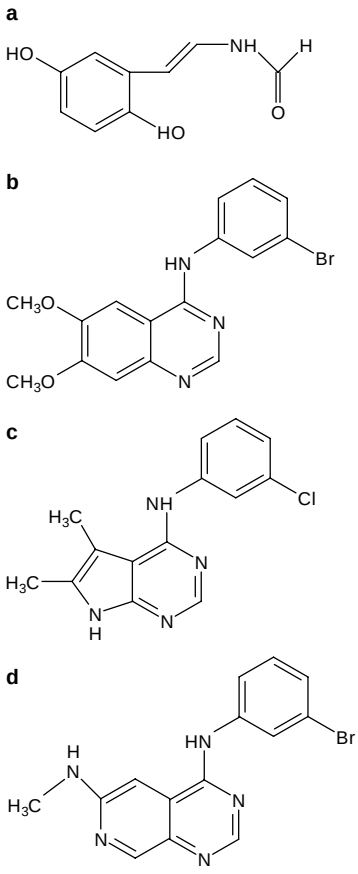

Fig. 2. Representative structures of small molecule inhibitors of the EGFR tyrosine kinase subfamily. (**a**) Erbstatin, a natural inhibitor used as a model for the first generation of benzylidenemalononitrile (BMN) inhibitors[26] (**b**) PD-153035, a quinazoline inhibitor[91] (**c**) CPG-59321, a pyrrolopyrimidine inhibitor[92] (**d**) PD-158780, a pyridopyrimidine inhibitor.[93]

centrations in the nanomolar range, and pre-treatment with this compound at a dose of 100 mg/kg completely eliminated *in vivo* EGF-induced autophosphorylation of tumour.[95]

In 1994, Parke-Davis (Ann Arbor, Michigan, USA) disclosed a class of brominated quinazolines, exemplified by PD-153035, which set new standards for potency and specificity in TKI development. Reversibly competitive with respect to ATP, the inhibition constant of purified receptor activity was estimated at 5 pmol/L and EGF-mediated mitogenesis and cell proliferation were inhibited by 50% in Swiss 3T3 fibroblasts at 15 nmol/L.[91] Despite profound *in vitro* potency and selectivity, these compounds lacked significant *in vivo* efficacy. However, this same group recently described a novel class of EGFR and HER2 irreversible inhibitors that outperform their reversible TKIs.[96] Based on molecular modelling studies which revealed a prominent cysteine residue in the ATP binding site of each receptor, quinazolines were developed that incorporated an electrophilic acrylimide positioned to bind and covalently alkylate these cysteines (PD-168393, PD-160678). Surprisingly, these compounds were nonreactive in solution and reacted only when bound to the receptor in the precise orientation. In vitro assays demonstrated that the receptors were irreversibly inhibited, and *in vivo* studies using A431 xenografts demonstrated prolonged tyrosine kinase inhibition and tumourstasis without detectable host toxicity. The therapeutic effect appeared to be determined by the receptor turnover rate, suggesting that irreversible inhibition is associated with more favourable pharmacokinetics since only brief receptor exposure is needed to achieve receptor kinase inactivation.[96] Although the ultimate clinical utility of these agents is not yet established, this progress illustrates the advantages of molecular modelling to rational drug design.

3.5.2 Pyrazolo/pyrrolo/pyridopyrimidine Inhibitors

Another group, in collaboration with Novartis (East Hannover, New Jersey, USA), has identified active compounds with potencies approaching those of the quinazolines. By a large scale screening approach, a series of phenylamino- and pyrazolopyrimidine compounds were discovered with selectivity for Bec-Abl and EGFR tyrosine kinases, respectively.[29,97] The resulting compounds demonstrated potencies in the nanomolar range and high selectivity for receptor and nonreceptor tyrosine kinases. Orally bioavailable STI-571 is effective at producing growth delays in xenograft models and showing antileukaemic activity in early clinical trials.

This same group modeled a dianilinophthalimide pharmacore and by iteratively optimising its proposed binding, developed a series of pyrrolopyrimidine EGFR/HER2 TKIs. Binding of the most promising compound, PKI-166, was 0.7 nmol/L [dissociation constant (Kd)] against the pure EGFR kinase with submicromolar potency against EGF-stimulated cellular phosphorylation.[92] Daily oral doses of 100 mg/kg resulted in regression of A431 tumour xenografts. PKI-166 has now entered clinical trials for the treatment of human tumours stimulated by either EGFR or HER2 TKIs.

In parallel with development of quinazoline inhibitors, the Parke-Davis group has also been investigating the structure-activity relationships of a series of compounds based on a pyridopyrimidine pharmacore.[93] As with all the TKIs mentioned, slight structural modifications have resulted in drastic changes in activity, leading to a range of potencies from low picamolar to mid-micromolar against the isolated EGFR enzyme. All appear to competitively and reversibly inhibit at the ATP binding site. At least one compound, PD-158780, was selected for further development. Since this quinazoline is active against all members of the EGFR subfamily, while retaining its selectivity against other families of tyrosine kinases. This reduction in subfamily specificity may translate into clinical benefit as overexpression of multiple members of this receptor subfamily can be observed in very aggressive tumours. Intraperitoneal administration was able to produce growth delays in both low EGFR expressing MCF-7 breast cancer xenografts and high EGFR expressing A431 xenografts.[98] Analogues of PD-158780 showing improved solubility and oral bioavailability have since been described.[99,100]

3.5.3 Other Small Molecule Inhibitors

Numerous other classes of compounds with activity against the EGFR subfamily of receptor kinases have been developed at both university and pharmaceutical laboratories. Examples of such pharmacores include the thioindoles,[101] dianilinopthalimides,[102,103] and anthraquinones.[104] In addition, other natural products have been isolated that display tyrosine kinase inhibitory activity.[105,106] However these compounds have not shown the *in vitro* potency or specificity of the compounds described above and have generally not advanced into *in vivo* studies. A comprehensive review of all such compounds has recently been published.[29]

3.6 Substrate Inhibitors

Historically, the first small molecule TKIs were designed to compete with the target protein for substrate binding as it was believed that targeting the highly homologous ATP binding site would not confer adequate specificity. However, the quinazolines, with their steep structure activity relationship, have demonstrated that high degrees of family and subfamily specificity can be achieved by targeting the ATP binding site. In fact, nearly all the synthetic TKIs discussed are thought to be competitive against the ATP binding site, despite the mmol/L concentrations of intracellular ATP. This incongruity leaves unanswered the question of how these compounds produce *in vivo* tyrosine kinase inhibition. In contrast, substrate inhibitors are short peptides designed to mimic the primary sequence around the tyrosine moiety, and substitute nonphosphorylatable tyrosine analogues such as phenylalanine, tyramine or iodotyrosine for the tyrosine moiety.[28,107] Although the catalytic domain of a receptor tyrosine kinase bound to a peptide substrate has been crystallised,[108] progress in developing peptides as anticancer agents has been slow, especially for the EGFR family of receptors. These peptide pseudosubstrates have poor solubility, are susceptible to proteolysis, and do not easily penetrate the cell membrane. In addition, the peptides are recognised in a region distant from the catalytic site, perhaps explaining their poor kinase inhibiting activity. None of these compounds are currently in active clinical investigation.

Another pseudosubstrate approach is to target the downstream signalling proteins that normally dock to phosphotyrosine residues in the cytoplasmic portion of the receptor via src homology 2 (SH2) domains. This means of interference requires the phosphorylated tyrosine residues, and thus SH2

pseudosubstrates are not true TKIs, but inhibit the immediate downstream TK signalling.

4. Advancing Compounds to Clinical Trials

The enormous number of candidate inhibitors generated against TKs in general, and the EGFR family of receptor tyrosine kinases in particular, attest to the clinical potential of selectively interrupting growth factor receptor signal transduction. Equally exciting, although not discussed in this review, are compounds active against other receptor and nonreceptor TK subfamilies including the platelet derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), stem cell factor receptor (c-kit) and the Bcr-Abl tyrosine kinase.[27-30] Targeting these TKIs could lead to inhibition of tumour growth as well as tumour neovascularisation and angiogenesis. Clinical trials are underway with small molecule inhibitors such as SU-5416 and SU-6668 developed by SUGEN (South San Francisco, California, USA), to inhibit tumour angiogenesis, growth and metastasis. Both of these compounds cause tumouristasis and even tumour regression in a variety of human solid tumour models.[29,109]

Advances in the treatment of lymphomas and leukemias have also been made with the development of immunogenistein conjugates active against CD19-positive B cell malignancies,[110,111] and other compounds that target the oncogenic Bcr-Abl fusion protein implicated in the majority of chronic myeloid leukemias and a significant percentage of acute lymphoblastic leukemias.[112-115] Renewed interest in src inhibitors has led to structure-based design of a number of potent small molecule inhibitors active against this and other cytoplasmic tyrosine kinases, including the STI-571 which is producing clinical remissions in patients with Bcr-Abl expressing leukaemia.[27-30,89,116,117]

Translating *in vitro* success into a therapeutic benefit is a difficult challenge, as illustrated by the relatively small fraction of compounds that actually advance to clinical trials. Monoclonal antibody therapies can lead to the development of host anti-mAbs that neutralise and immunoprecipitate the active compound. Immunotoxins can lead to targeted cell death of normal cells bearing the overexpressed receptor. As single agent therapeutics, small molecule TKIs are tumouristatic rather than tumouricidal and regrowth occurs upon termination of therapy. Irreversible inhibitors delay this regrowth, but only long enough for new receptors to be synthesised. Thus, preclinical and clinical trials must include cytocidal agents in combination with TKIs to develop optimal therapeutic regimens. Peptide and pseudopeptide inhibitors are unlikely to reach clinical trials in the near future, and will also need to be used in combination with current cytocidal chemotherapy regimens. In this regard, the additive and synergistic anticancer effects being seen when many of the new receptor TKIs are given in combination with standard chemotherapeutic agents are encouraging. Given that signal transduction pathways can be redundant, targeting critical tyrosine kinase pathways at multiple sites may lead to more effective anticancer therapeutic regimens.

5. Conclusion

The past 5 years have resulted in dramatic advances in the discovery of potent, specific, and well tolerated inhibitors of tyrosine kinase activity for anticancer purposes. Immunotherapeutics and small molecule inhibitors generated against the EGFR family of receptor kinases have been the major but not sole focus of these efforts. It is likely that the pace of research will only increase in the next 5 years. Improved molecular understanding of the signal transduction pathway in normal and malignant cells, coupled with advances in drug screening and molecular modelling will undoubtedly result in a greater number of compounds under clinical investigation. The next 5 years will also yield results from a number of promising phase II and III clinical trials currently in progress. It is hoped that these results will identify the strategies most able to produce anticancer effects by tyrosine kinase inhibition and also allow us to identify the subset of

cancers most responsive to this inhibition. This clinical data and perspective on the potential utility of tyrosine kinase inhibitors will undoubtedly pave the way for a second generation of anticancer signal transduction inhibitors.

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