Protein Phosphorylation and Signal Transduction Modulation: Chemistry Perspectives for Small-Molecule Drug Discovery

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Abstract: Protein phosphorylation has been exploited by Nature in profound ways to control various aspects of cell proliferation, differentiation, metabolism, survival, motility and gene transcription. Cellular signal transduction pathways involve protein kinases, protein phosphatases, and phosphoprotein-interacting domain (e.g., SH2, PTB, WW, FHA, 14-3-3) containing cellular proteins to provide multidimensional, dynamic and reversible regulation of many biological activities. Knowledge of cellular signal transduction pathways has led to the identification of promising therapeutic targets amongst these superfamilies of enzymes and adapter proteins which have been linked to various cancers as well as inflammatory, immune, metabolic and bone diseases. This review focuses on protein kinase, protein phosphatase and phosphoprotein-interacting cellular protein therapeutic targets with an emphasis on small-molecule drug discovery from a chemistry perspective. Noteworthy studies related to molecular genetics, signal transduction pathways, structural biology, and drug design for several of these therapeutic targets are highlighted. Some exemplary proof-of-concept lead compounds, clinical candidates and/or breakthrough medicines are further detailed to illustrate achievements as well as challenges in the generation, optimization and development of small-molecule inhibitors of protein kinases, protein phosphatases or phosphoprotein-interacting domain containing cellular proteins.

Key Words: Protein kinase, protein phosphatase, phosphoprotein-binding domain, Src, Src family kinase, EGFR, VEGR, KIT, Flt-3, PDGFR, Bcr-Abl, Src, Src family kinases, Raf, MAPK, MEK, PTP1B, SH2 domain, PTB domain, WW domain, ATP, peptide substrate, phosphotyrosine, phosphoserine, phosphothreonine, phosphopeptide, peptidomimetic, nonpeptide, small-molecule, pTyr mimetic, ATP mimetic, purine, pyrrolopyrimidine, pyrazolopyrimidine, indolinone, pyridopyrimidinone, quinazoline, quinoline, quinolinone, phenylaminopyrimidine, STI-571, ZD1839, OSI-774, GW-572016, PKI-166, CI-1033, EKB-569, SU-5416, SU-6668, SU-11248, PTK-787, ZD-6474, CEP-7055, CP-547632, PKC-412, CEP-701, MLN-518, PD180970, AP23848, AMN-107, BMS-354825, ON012380, SKI-606, CGP-76030, AP23464, AZM-475271, SU-6656, AP23451, SB-203580, LY-364947, HTS-466284, flavopiridol, UCN-01, CYC202, BMS-387032, BAY43-9006, PD184352, rapamycin, AP23573, FK506, cyclosporin, cantharidin, X-ray crystallography, virtual screening, chemoinformatics, structure-based design, mechanism-based design, SMART drug design.

1. INTRODUCTION

From nearly three decades of research and, more recently, the sequencing of the human genome, the study of protein phosphorylation and its intimate relationship with various cellular processes (e.g., proliferation, differentiation, metabolism, survival, motility and gene transcription) has led to the identification and validation of promising therapeutic targets for drug discovery. A hallmark of such studies includes the mapping of signal transduction pathways involved in disease, especially including the molecular genesis and/or sustainment of various cancers, inflammatory, immune, metabolic and bone diseases [1-20]. Protein phosphorylation is a complex phenomena with molecular triggering, compensatory mechanisms, and both spatial and temporal factors each contributing to biological specificity and functional endpoints within the cellular milieu. The global phosphorylation state of proteins in cells is at present essentially impossible to decipher at high resolution, albeit sophisticated methods (e.g., mass spectrometry, functional interaction traps, affinity chromatography) are emerging to carefully analyze the so-called phosphoproteome [21-24]. The dramatis personae in protein phosphorylation include protein kinases, protein phosphatases and phosphoprotein-interacting domains (e.g., SH2, PTB and WW, FHA, 14-3-3) of various catalytic/noncatalytic intracellular proteins to provide a multidimensional, dynamic and reversible orchestration of the phosphoprotein formation, hydrolysis and molecular recognition at specific sites involving Tyr, Ser and/or Thr residues (Fig. **1**) [1-20]. A significant portion (estimated at 30%) of proteins encoded by the human genome may be phosphorylated by ATP as the phosphoryl donor. With respect to Tyr phosphorylation, it is interesting to point out that pTyr represents only approximately 0.05% of total phosphorylated protein in normal cells (the remaining 99.95% being pSer and pThr), whereas oncogenic constitutively activated tyrosine kinases (*vide infra*) may increase this percentage upwards to 0.5% within transformed

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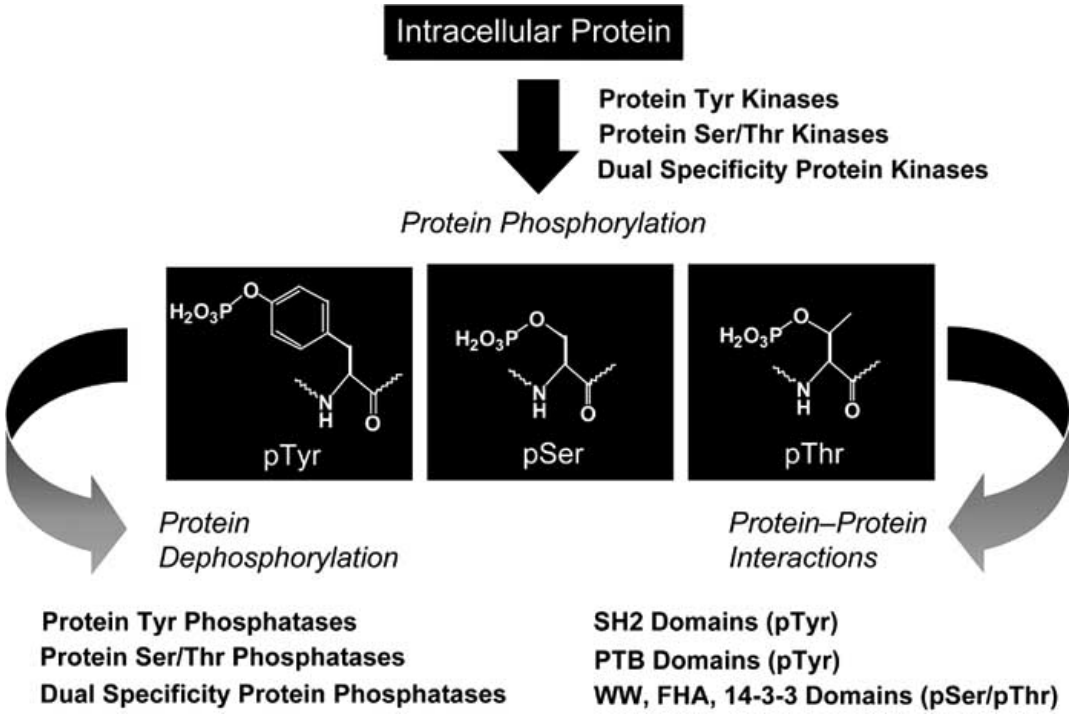

Fig. (1). Protein phosphorylation (at specific Tyr, Ser and Thr sites) and the relationship of protein kinases, protein phosphatases, and phosphoprotein-interacting domain containing cellular proteins.

cells [25]. It is remarkable how much knowledge has been gained since the first observations of protein kinase activity in 1954 and throughout numerous milestone achievements over the past five decades [26], including the discoveries of the Src [27, 28] and EGFR [28], as the first determined non-receptor and growth factor receptors tyrosine kinases, respectively, the discovery of PTP1B [29] as the first determined protein tyrosine phosphatase, and the discovery of the Src SH2 domain [30] as the first determined phospho-protein interaction motif conferring molecular recognition to pTyr-containing cellular proteins.

Molecular genetics and cell biology studies (e.g., signal transduction pathway) have correlated possible relationships between gene deletion, fusion, mutation and/or overexpress-ion of several known protein kinases, protein phosphatases and phosphoprotein-interacting domain containing cellular proteins with aberrant cellular activities and *in vivo* phenotypes correlating to known cancers or other diseases (Table **1**) [1, 7, 10, 12-17, 31-33]. In the case of cancer, it is now understood that the causative factors relate to imbalances in cell-cycle progression, cell growth, and programmed cell death (apoptosis). Of the nearly 300 cancer genes that have been reported to date, protein kinases represent the largest group (nearly 10%) having structural homology. Many cancers have been correlated to somatic mutations of protein kinases of which both receptor and non-receptor tyrosine kinases have emerged as particularly significant therapeutic targets for cancer drug discovery. Oncogenic transformation of protein kinases in humans may arise from fusion products of genomic re-arrangements (e.g., chromosomal translocations), mutations (e.g., gain-of-function), deletions, and overexpression resulting from gene amplification. Such transformations typically result in enhanced or constitutive kinase activity which then effects subsequent altered downstream signal transduction. Gene knockout and related functional genomic and cellular biology studies have further characterized a number of protein kinases in terms of signal transduction pathways and *in vivo* phenotypes as related to cancer or other diseases (e.g., Src gene KO and osteopetrosis). Some examples of oncogenic protein tyrosine kinases (*vide infra* for further discussion and literature references) include EGFR, HER-2, HER-3, VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/ KDR), Flt-3, Flt-4, PDGFR-α, PDGFR-β, KIT, RET, MET, IGF-1R, Abl, Src and Src family, FAK, Pyk2, and JAK family. Some examples of protein serine/threonine kinases that have been identified as key therapeutic targets with respect to oncogenic signaling (*vide infra* for further discussion and literature references) include TGFβR, CDK family, Raf, MEK, PKC, PI-3K, Akt, mTOR, and aurora kinases.

Beyond protein kinases, molecular genetics and signal transduction pathway studies have also correlated possible relationships between key protein phosphatases and phosphoprotein-interacting domain containing cellular proteins and certain diseases (Table **1**) [12-17]. Some examples of protein phosphatase therapeutic targets (*vide infra* for further discussion and literature references) include PTP1B, CD45 and CDC25A for diabetes (and obesity), immune and inflammatory disease, and cancer, respectively. Some examples of phosphoprotein-interacting domain con-taining cellular protein therapeutic targets (*vide infra* for further discussion and literature references) include SH2 (e.g., Grb2, Shc, Src, ZAP-70) and PTB (e.g., Shc and IRS-1) as related to cancer, autoimmune disease, osteoporosis, autoimmune disease, type-2 diabetes mellitus, and autosomal recessive hypercholesterolemia.

Table 1. Some Molecular Genetic Relationships of Protein Kinases, Protein Phosphatases, and Phosphoprotein-interacting Domain Containing Cellular Proteins with Disease Phenotypes. Refer to Text for Discussion and References Therein

Therapeutic Target	Gene modification	Cancer (or Other Disease)
Protein Kinase		
EGFR/ErbB-1	Overexpression, point mutations	Breast, NSCL, ovarian, glioblastoma
ErbB-2/HER-2/Neu	Overexpression. point mutations	Breast, ovarian, gastric, NSCL, colon
ErbB-3/HER-3	Overexpression	Breast
IGF-1R	Overexpression	Cervical, sarcomas
PDGFR-α	Overexpression	Glioma, glioblastoma, ovarian, GIST
PDGFR-β	Fusions (Tel-PDGFR-β)	Leukemias
	Overexpression	Glioma
Flt-3, Flt-4	Point mutation	Leukemias, angiosarcoma
KIT	Point mutations, overexpression	GIST, AML, myelodysplastic syndromes
MET	Point mutations, overexpression	Renal, hepatocellular
RET	Point mutations, fusions	Thyroid, parathyroid, adrenal
VEGFR-1/ Flt-1	Expression	Tumor angiogenesis
VEGFR-2/ Flk-1	Expression	Tumor angiogenesis
Src	C-terminal truncation, point mutations, overexpression	Colon, breast, pancreatic; metastasis
	Deletion (KO)	Osteopetrosis
Yes	Overexpression	Colon, melanoma
FAK	Overexpression	Metastases, adhesion, invasion
Pyk2	Overexpression	Metastases, adhesion, invasion)
Abl	Fusions (Bcr-Abl), pont mutations	CML, ALL
JAK1, JAK3	Overexpression	Leukemias
JAK2	Translocation	Leukemias
Protein Phosphatase		
PTP1B	Deletion (KO)	Increased sensitivity to insulin, resistance to weight gain
Phosphoprotein-Interacting Domain (Cellular Protein)		
SH2 (SAP)	Point mutations	X-linked lymphoproliferative disease
SH2 (Btk)	Point mutation	X-linke agammaglobulinemia
SH2 (SHP-2)	Point mutations	Noonan's syndrome
PTB (IRS-1/2)	Point mutations	Type 2 diabetes mellitus
PTB (ARH)	Point mutations	Autosomal recessive hypercholesterolemia

Structural biology has also provided insight into the molecular architecture and mechanistic properties of protein kinases, protein phosphatases, and phosphoprotein-interacting domain containing cellular proteins intimately involved in signal transduction pathways. Several hundred X-ray crystallographic structures have been determined for this collective group of signal transduction therapeutic targets [4-6, 13-17, 19, 34-39]. In the case of protein kinases, both structural biology and drug design studies of small-molecule inhibitors has led to the determination of varying

modes of enzyme inhibition, (i.e., both active and inactive conformations of the catalytic domain), and opportunities to exploit binding interactions beyond those existing for ATP and peptide substrate [5, 6, 9, 34-50]. Such findings have extraordinary impact to both understanding and overcoming the challenges of resistance to small-molecule inhibitors resulting from mutations of the protein kinase (e.g., Imatinib-resistant Bcr-Abl mutants) [51].

Without question, small-molecule drug discovery to advance novel signal transduction modulators has emerged to be a major focus of many pharmaceutical/biotechnology companies. Significant progress has been realized within the scope of protein phosphorylation as exemplified by a number of protein kinase inhibitors which have advanced into clinical trials as well as noteworthy progress with respect to key proof-of-concept lead compounds for key protein kinase, protein phosphatase and phosphoprotein-interacting domain containing cellular protein therapeutic targets (*vide infra*). From a chemistry perspective, such work is fascinating relative to the integration of structural biology, drug design, chemoinformatics and chemical diversity technologies to generate and optimize novel small-molecule lead compounds having promising biological specificity profiles, bioavailability, pharmacokinetics, and efficacy with minimal toxicity *in vivo*.

2. PROTEIN KINASE THERAPEUTIC TARGETS AND INHIBITORS

More than 500 protein kinase genes (excluding additional protein kinase pseudogenes) have been identified from the human genome and constitute nearly 2% of all human genes. Furthermore, chromosomal mapping studies have revealed that 244 protein kinases map to disease loci or cancer amplicons [2]. A case study using high-throughput sequencing and analysis of the tyrosine kinome from a panel of colorectal cancer cell lines revealed several genes (e.g., *NTRK3, FES, KDR, EPHA3, NTRK2*) having somatic mutations of otherwise conserved residues in functionally important regions of the catalytic domain, including the autoinhibitory activation loop and, in several cases, the mutations correlated with known point mutations of genes of other protein kinases that have been determined to be pathogenic.

In retrospect, since the identification of the non-receptor tyrosine kinase, Src, about twenty-five years ago [26], a plethora of biological studies have steadily unraveled the complex orchestration of signal transduction pathways involving protein kinases of varying structural architecture and functional properties [1, 7-10]. The superfamily of protein kinases (Table **2**) is exemplified by: (i) growth factor receptor tyrosine kinases (*e.g.*, EGFR family, VEGFR family, PDGFR, KIT, TEL, Flt-3, FGFR, and IGFR); (ii) non-receptor tyrosine kinases (e.g., Abl, Src family, and FAKl); (iii) growth factor receptor serine/threonine kinases (e.g., TGFβR); and (iv) non-receptor serine/threonine as well as dual specificity kinases (e.g., MAPK family, CDK family, Raf kinase, PI-3K, PKC, PKB/Akt, mTOR, and aurora kinase). A number of such protein kinases have proven to be particularly significant as therapeutic targets for cancer drug discovery (*vide infra* for examples).

2.1 Receptor and Nonreceptor Tyrosine Kinases

In retrospect, the seminal identifications of Src and EGFR as oncogenic protein tyrosine kinases launched a campaign in both basic research and drug discovery to enhance the understanding of protein kinases in signal transduction pathways (and related cellular activities), provide correlation with disease (e.g., cancer), and to develop first-generation therapies, including small-molecules inhibitors and monoclonal antibodies. Approximately 60 receptor tyrosine kinases are known, and each has cell membrane-spanning sequences with extracellular ligand binding domains and intracellular catalytic (kinase) domains (Fig. **2**). Ligand (e.g., growth factor) binding induces dimerization or an increased association of already dimerized subunits and subsequent autophosphorylation or cross-phosphorylation of proximate receptors at specific tyrosine residues on their intracellular C-terminal sequences. The resultant phosphotyrosine (pTyr) sequences of such activated receptor tyrosine kinases may then interact with various nonreceptor signal transduction proteins by virtue of pTyr molecular recognition by SH2 domains (e.g., Src, phospholipase-Cγ, and phosphatidyl-inositol-3-kinase, Grb2) or PTB domains (e.g., IRS-1) to modulate varying downstream biological pathways. As representative of nonreceptor tyrosine kinases, Src and Abl each illustrate multiple domain architectures which include noncatalytic SH2 and SH3 domains (Fig. **2**).

Some noteworthy examples of both basic research and drug discovery achievements made relative to a few receptor and nonreceptor protein tyrosine kinases are described hereinafter.

EGFR Family Receptor Tyrosine Kinases and Small-molecule Inhibitors

Extracellular-mediated triggering of EGFR by EGF (or other cognate ligands) results in the activation of a network of signal transduction pathways which modulate cell division, apoptosis, motility and adhesion [52-55]. EGFR (HER-1/erbB-1), HER-2 (Neu/erbB-2), HER-3 (erbB-3) and HER-4 (erbB-4) constitute four members of the EGFR family. Dysregulation of EGFR family signaling can result from gene amplification and/or mutations, effecting overexpression and, is some cases, constitutive activity correlating to certain cancers (e.g., gliomas, breast, ovary, and lung).

First-generation EGFR tyrosine kinase inhibitors ZD1839 (**1**, Iressa™, gefitinib) and OSI-774 (**2**, Tarceva™, erlotinib, CP-358774) are particularly noteworthy ATP-competitive, small-molecules based on a quinazoline template (Fig. **3**). ZD1839 [56-61] is a potent EGFR tyrosine kinase inhibitor (IC_{50} = 23 nM) with high selectivity relative to other protein tyrosine kinases (e.g., HER-2 and VEGFR-1 and VEGFR-2) and serine/threonine kinases. ZD1839 inhibits the proli.

Table 2. Some Examples of Protein Kinases, Protein Phosphatases, and Phosphoprotein-interaction Domain Containing Cellular Proteins (Further Categorized with Respect to Receptor/Nonreceptor and/or Phosphotyrosine-, Phosphoserine/ Phosphothreonine- or Dual Specificity Subgroups as Relevant)

Receptor Tyrosine Kinases	***Receptor Serine/Threonine Kinase***
Epidermal growth factor receptor (EGFR) Fibroblast growth factor receptor (FGFR) Vascular endothelial growth factor receptor (VEGFR) Platelet-derived growth factor receptor (PDGFR) Stem cell reeptor (KIT) Hematopoietic classs III receptor (Flt) Insulin receptor (IRK) Insulin-like growth factor receptor (IGFR) Colony-stimulating factor receptor (CSFR) Nerve growth factor receptor (NGFR) Hepatocyte growth factor receptor (Met) Glial-derived neurotrophic factor receptor (RET)	Transforming growth factor receptor-β (TGFR-β) ***Nonreceptor Serine/Threonine Kinases and Dual Specificity Kinases*** cAMP-dependent protein kinase (PKA) Phosphoinositol-3-kinase (PI-3K) Cyclin-dependent kinase (CDK) Mitogen-activated protein kinase (MAPK) MAPKK (ERK) MAPKKK (MEK) Raf kinase Aurora kinases Protein Kinase-C (PKC) Protein Kinase-B (PKB/Akt) mTor (FRAP) Polo-like kinases (Plk) Integrin-linked kinase (ILK) Glycogen synthase kinase-4 (GSK-3)
Nonreceptor Tyrosine Kinases Src and Src-family kinase (SFK) Abl FAK, Pyk2 Janus kinase (JAK) family	
Protein Tyrosine Phosphatases and Dual Specificity Phosphatases	***Protein Serine/Threonine Phosphatases***
Protein tyrosine phosphatase-1B (PTP1B) SH2-containing tyrosine phosphatase (SHP2) Protein tyrosine phosphatase-α (PTPα) Cyclin-dependent kinase/cyclin-25 (CDC25) Protein tyrosine phosphatase receptor-C (PTPRC/CD45) Phosphatase and tensin homolog on chromosome ten (PTEN)	Protein phosphatase-1 (PP1) Protein phosphatase-2B (PP2A) Protein phosphatase-2B (PP2B/calcineurin) Protein phosphatase-2C (PP2C) Protein phosphatase-3 (PP3)
SH2 and PTB Domain Containing Cellular Proteins	***WW, FHA and 14-3-3 Domain Cellular Proteins***
Src and Src family (SH2 domain) Abl (SH2-domain) Grb2 (SH2 domain) Shc (PTB and SH2 domains) IRS-1 (PTB domain)	Pin1 (WW domain Rad53 (FHA domain) 14-3-3 family (14-3-3 domain)

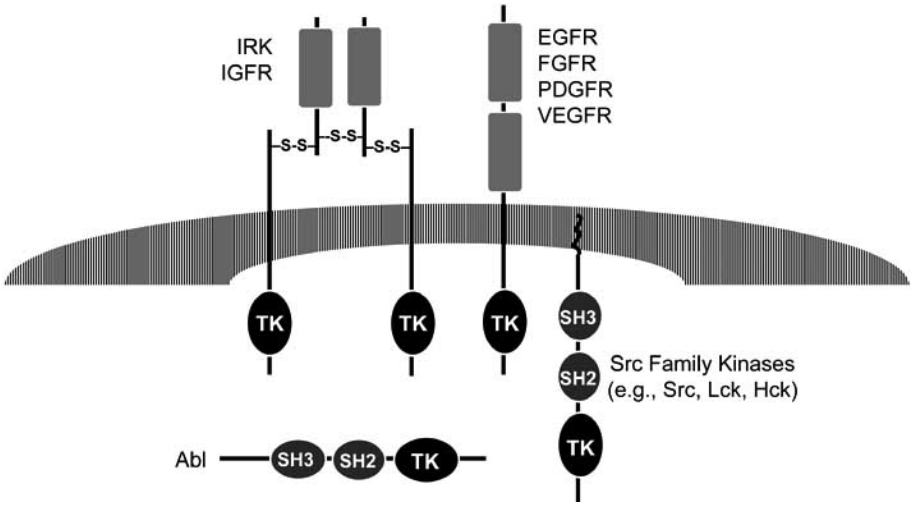

Fig. (2). Schematic representation of molecular architectures of receptor tyrosine kinases (IRK, IGFR, EGFR, FGFR, PDGFR, and VEGFR families) and nonreceptor tyrosine kinases (Src family and Abl) showing the catalytic tyrosine kinase (TK) as well as noncatalytic (SH2 and SH3) domains.

feration of several cancer cell lines (e.g., NSCLC, breast, ovarian, and colon), shows synergistic enhancement of the inhibitory action of single-agent cytotoxic drugs, and effects dose-dependent antitumor activity *in vivo* in nude mice bearing several human cancer cell xenografts. Very recently, a proteomics study identified cellular targets (beyond EGFR) for ZD1839 in cells and showed that several other protein tyrosine kinases (e.g., Brk, Met, Yes, Hck, and EphB4) as well as protein serine/threonine kinases (e.g., RICK, GAK, Aurora-B, and p38 MAPK) were inhibited [62]. Iressa™ has been clinically tested and first approved (in Japan) for the treatment of NSCLC as related to inoperable or recurrent disease, and it has gained further approvals for previously treated NSCLC in many countries including the U.S.) OSI-774 [63-66] is also a quinazoline template-based inhibitor of EGFR tyrosine kinase (IC_{50} = 20 nM) with high selectivity to other protein tyrosine kinases and efficacy in vivo in nude mice bearing human cancer cell xenografts. Recently determined X-ray crystal structures of EGFR tyrosine kinase domain, including both apoprotein and a complex with OSI-774, have provided the first experimental analysis of the EGFR family. OSI-774 effects long-lasting inhibition of EGFR autophosphorylation in tumor xenografts (*ex vivo* analysis) following high dose single administration. In combination with cisplatin, OSI-774 showed additive antitumor activity and enhanced apoptosis via suppression of the PKB/Akt survival pathway. Tarceva™ has been clinical tested and it has just been approved in the U.S. for the treatment of patients with locally advanced or metastatic NSCLC after failure of at least one prior chemotherapy. Very recent studies that have captured significant clinical attention and which impact protein kinase inhibitor drug discovery are those related to the identification of activating mutations in EGFR tyrosine kinase that underly the responsiveness of NCLC (primary tumors from patients) to Iressa™ [67]. In brief, this study indicated a correlation with NCLC patients having marked response to Iressa™ with a preponderance of somatic mutations in the ATP-binding domain of the EGFR gene, and it is conceptualized that such mutations may stabilize the binding of some ATP-competitive inhibitors such as ZD-1839 to afford greater sensitivity relative to wild-type EGFR.

Some noteworthy second-generation EGFR tyrosine kinase inhibitors that have also advanced to clinical testing include GW-572016 (3), PKI-166 (**4**), PD183805 (**5**, CI-1033, canertinib) and EKB-569 (**6**) (Fig. **3**). Such small-molecules reflect different biological profiles and

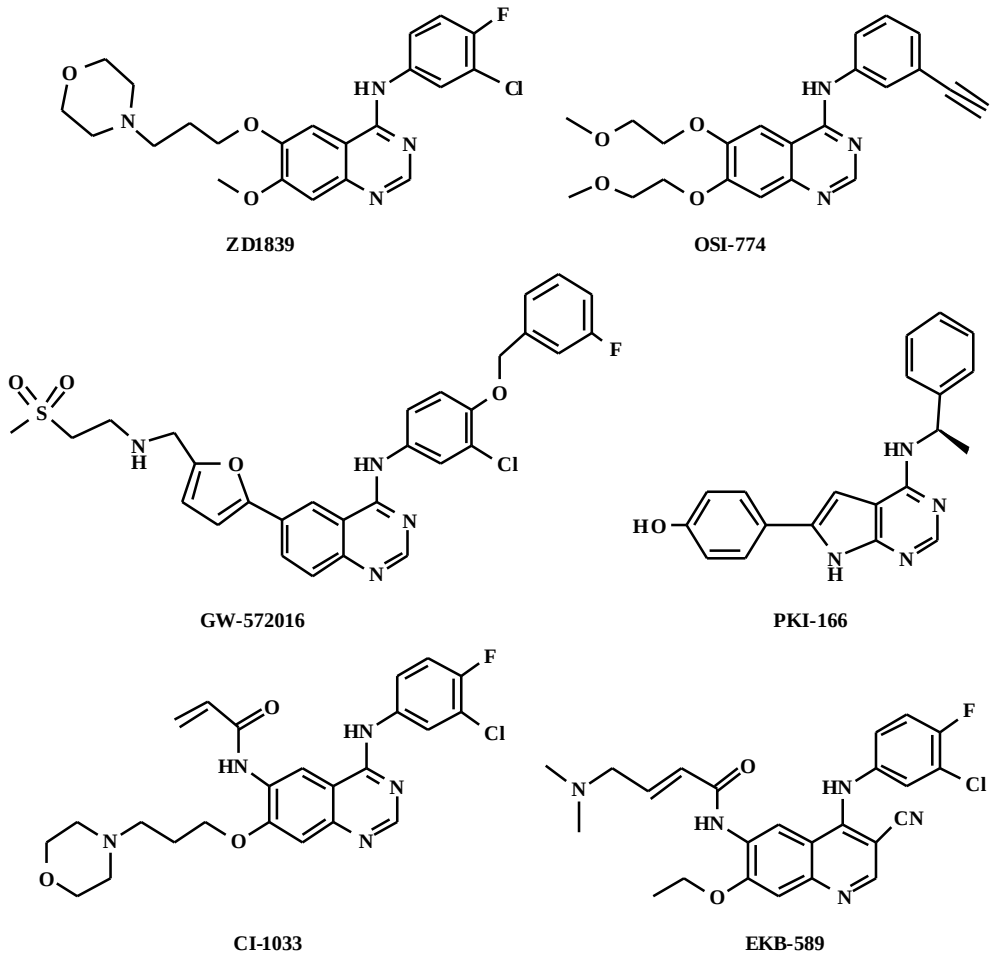

Fig. (3). Some EGFR family tyrosine kinase inhibitors that have advanced into clinical testing.

mechanisms of action relative to ZD-1839 and OSI-774. GW-572016 [68-71] is a potent quinazoline-based inhibitor of both EGFR (IC_{50} = 11 nM) and HER-2 (IC_{50} = 9.2 nM) which confers an ability to block signaling from either receptor which is known to be overexpressed in cancers (e.g., breast). GW-572016 inhibits proliferation in EGFR/HER-2 overexpressing cancer cells and was effective *in vivo* at high dose to suppress tumor growth in breast as well as head and neck cancer xenograft mice. PKI-166 [72-75] illustrates a potent pyrrolopyrimidine-based dual inhibition of EGFR (IC_{50} = 1 nM) and HER-2 (IC_{50} = 11 nM) with additional inhibiton against Abl, Src and VEGFR-2 tyrosine kinases (IC_{50} = 26, 103, and 327 nM, respectively). CI-1033 [76-78] illustrates a potent quinazoline-based inhibitor of EGFR (IC_{50} = 1.5 nM) and other EGFR family members. CI-1033 is chemically novel relative to its incorporation of an acrylamide electrophilic moiety which was designed to covalently interact (as a Michael acceptor) with a proximate cysteine residue to the ATP binding pocket. This hypothesis was confirmed experimentally, and the uniqueness of this cysteine relative to other receptor or nonreceptor tyrosine kinases may account for the extraordinary selectivity properties of CI-1033 to inhibit EGFR. CI-1033 was a potent inhibitor *in vitro* and *in vivo*, including several EGFR/HER-2-dependent cancer xenograft models, and it has been found to synergize with various cytotoxic agents and radiation. EKB-569 [79-82] is also an acrylamide-containing molecule, but based on a novel cyanoquinoline template, and it potently inhibits both EGFR (IC_{50} = 1.3 nM) and HER-2 (IC_{50} = 15 nM). EKB-569 effects potent inhibition of EGFR and HER-2 expressing cell proliferation, and it is also found to be effective in human squamous cancer xenografts in mice. EKB-569, alone or in combination with a non-steroidal, anti-inflammatory agent was determined to be effective *in vivo* in a mice model of human colon cancer.

VEGFR, Flt3 and KIT Family Receptor Tyrosine Kinases and Small-molecule Inhibitors

Beyond EGFR family growth factor receptor tyrosine kinases, there has emerged an increasing effort to understand and exploit the biology of tumors with respect to angiogenesis and lymphangiogenesis to identify therapeutic targets critical for the recruitment and formation of blood vessels and lymphatics [83-85]. One of the major pathways involved in both processes is the VEGF family of ligands and receptors (VEGFR-1/Flt-1 and VEGFR-2/Flk-1/KDR for angiogenesis and VEGFR-3/Flt-4 for lymphangiogenesis). Overexpression of VEGF ligands has been associated with various cancers (e.g., colorectal, gastric, pancreatic, breast, prostate, lung and melanoma). Upregulation of VEGFR expression occurs in activated endothelium and is high in blood vessels surrounding tumor tissue that is growing and invading.

First-generation VEGFR tyrosine kinase inhibitors SU-5416 (**7**, semaxanib), SU-6668 (**8**), SU-11248 (**9**), PTK-787 (**10**, CGP79787, vatalanib), ZD-6474 (**11**), CEP-7055 (**12**), and CP-547632 (**13**) are particularly noteworthy ATP-competitive, small-molecules based on a variety of different templates (Fig. **4**). SU-5416 [86-89] was the forerunner of a series of indolinone template-based small-molecule inhibitors of VEGFR, and it also was the first to advance into clinical testing with a focus on metastatic colorectal cancer in combination with chemotherapy. Unfortunately, the Phase-II/III clinical studies showed no survival advantage for SU-5416, hence further drug development was discontinued. The second indolinone analog, SU-6668 [89-91], was determined to be a very potent inhibitor of PDGFR (IC_{50} = 8 nM) relative to VEGFR-2 (IC_{50} = 2.1 μM) and it was quite effective to inhibit VEGF-driven mitogenesis in cells. SU-6668 effected significant inhibition of a panel of human tumor xenografts by oral administration in nude mice, and it was further shown to exhibit *in vivo* antiangiogenic and antimetastatic activities in a colon cancer cell-injected mice. The SU-6668 is in Phase-II clinical trials for solid tumors. The third indolinone analog, SU-11248 [92-95], was determined to be a highly potent inhibitor of VEGFR-2 (IC_{50} = 9 nM) and PDGFR-β (IC_{50} = 8 nM) as well as relatively potent inhibition of other receptor tyrosine kinases (FGFR-1, Flt-3 and KIT). SU-11248 effects potent inhibition of cell proliferation as driven by VEGF, PDGF-β and FGF. SU-11248 was determined to be orally effective and showed potent *in vivo* antitumor activity in mice relative to several human xenografts, and recent studies have shown its *in vivo* antiangiogenic activities in a lung cancer metastasis model. SU-11248 is in Phase-III clinical trials for various cancers. PTK-787 [96-99], a novel small-molecule inhibitor designed from a phthalazine-based template, is a potent inhibitor of VEGFR-1 (IC_{50} = 77 nM) and VEGFR-2 (IC_{50} = 37 nM). PTK-787 inhibits VEGF-driven cell proliferation, survival and cell migration in cells, and it has also been shown to effect antiangiogenic activity *in vitro*. PTK-787 demonstrates effective *in vivo* antiangiogenic and antitumor activity in several animal models by oral administration, and it has further shown *in vivo* antimetastatic activity both orthotopic renal cell carninoma and human pancreatic xenograft models. ZD-6474 [100-104] is a relatively potent inhibitor of VEGFR-2 (IC_{50} = 40 nM), VEGFR-3 (IC_{50} = 110 nM), EGFR (IC_{50} = 500 nM), and RET (IC_{50} = 100 nM). ZD-6474 effectively inhibits endothelial cell proliferation driven by VEGF, EGF and FGF. ZD-6474 has been shown to inhibit the growth of various human tumor xenografts in a dose-dependent manner, and it also was found to inhibit primary tumor growth in a spontaneous metastasis model of breast cancer at high dose. ZD-6474 is in Phase-II clinical trials for solid tumors. CEP-7055 [105, 106], a water-soluble prodrug of a indenopyrrolocarbazole template-based parent molecule (CEP-5214), is a potent inhibitor of VEGFR-1, VEGFR-2 and VEGFR-3 (IC_{50} ranging 12-18 nM). CEP-7055 is a potent inhibitor of tumor growth in several subcutaneous tumor xenografts, including decreasing the number of metastasis. CEP-7055 is in Phase-I clinical trials with indications for solid tumors. CP-547632 [107], a isothiazole template-based molecule, is a potent inhibitor of VEGFR-2 (IC_{50} = 11 nM) and FGFR-2 (IC_{50} = 9 nM). CP-547632 was an orally active inhibitor of VEGF-induced corneal angiogenesis in mice, and it was also effective to significantly inhibit human xenograft in nude mice. CP-547632 is in Phase-I clinical trials with indications for solid tumors.

The relationship between Flt-3 and acute myeloid leukemia (AML) has been implied by the fact that about 30% of AML patients have constitutively activating internal

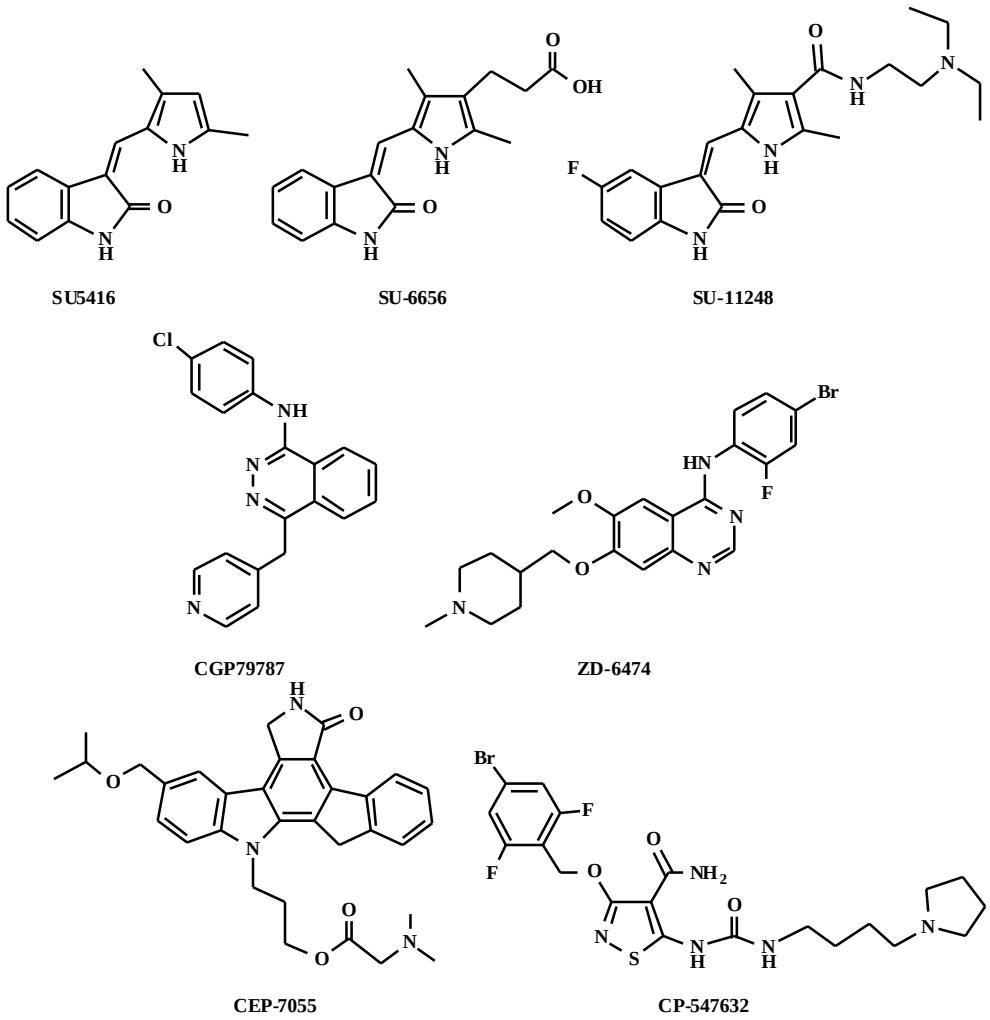

Fig. (4). Some VEGFR family tyrosine kinase inhibitors that have advanced into clinical testing.

tandem duplications of the juxtamembrane domain or mutations in the activation loop of the Flt-3 receptor tyrosine kinase [108-110]. First-generation Flt-3 tyrosine kinase inhibitors PKC-412 (**14**, CGP41251, midostaurin), CEP-701 (**15**, KT-5555), SU-11248 (**9**, *vide supra*), and MLN-518 (**16**, CT-53518) are particularly noteworthy small-molecules based on a variety of different templates (Fig. **5**). Also, each of these compounds have advanced into clinical testing in patients with AML harboring Flt-3 activating mutations. PKC-412 [111, 112], a staurosporine derivative, is an inhibitor of Flt-3 and several other protein kinases, including VEGFR-2, PDFGR, KIT, and Syk. CEP-701 [113], an indolocarbazole derivative, is a potent inhibitor of Flt-3 and Trk tyrosine kinases. SU-11248 (*vide supra*) is also a potent inhibitor of Flt-3 tyrosine kinase. MLN-518 [114], a quinazoline template-based molecule, is a potent inhibitor of Flt-3, PDGFR and KIT tyrosine kinases. A number of studies have been described [111-117] to show the effectiveness of these compounds *in vitro* and *in vivo*, including induction of apoptosis in cell lines with Flt-3 activating mutations as well as prolonging survival of mice expressing mutant Flt-3 in their bone marrow cells. Prediction of resistance to some of the above small-molecule Flt-3 tyrosine kinase inhibitors using an *in vitro* screen designed to identify mutations of the ATP-binding pocket has shown several key amino acid mutations, including G697R, that confer resistance and potential clinical problems with a single agent Flt-3 drug therapy [118]. In this regard, mutations and translocations involving both receptor (e.g., Flt-3 and KIT) and nonreceptor tyrosine kinases (e.g., Abl, *vide infra*) are now well-recognized within the scope of transformed hematopoietic cells and underlying myeloid and acute leukemias [119]. Such mutations enhance the complexity of small-molecule drug discovery from the standpoint of lead optimization as well as both *in vitro* and *in vivo* testing in disease models which must reflect the inclusion of known mutant tyrosine kinase therapeutic targets.

KIT is a member of a family of growth factor receptor tyrosine kinases which include RET and PDGFR, and it is known to contribute to the growth and survival of cancers, especially those such as gastrointestinal stromal tumors (GISTs) in which it is frequently mutated and activated [120]. Co-expression of KIT and its ligand, stem cell factor

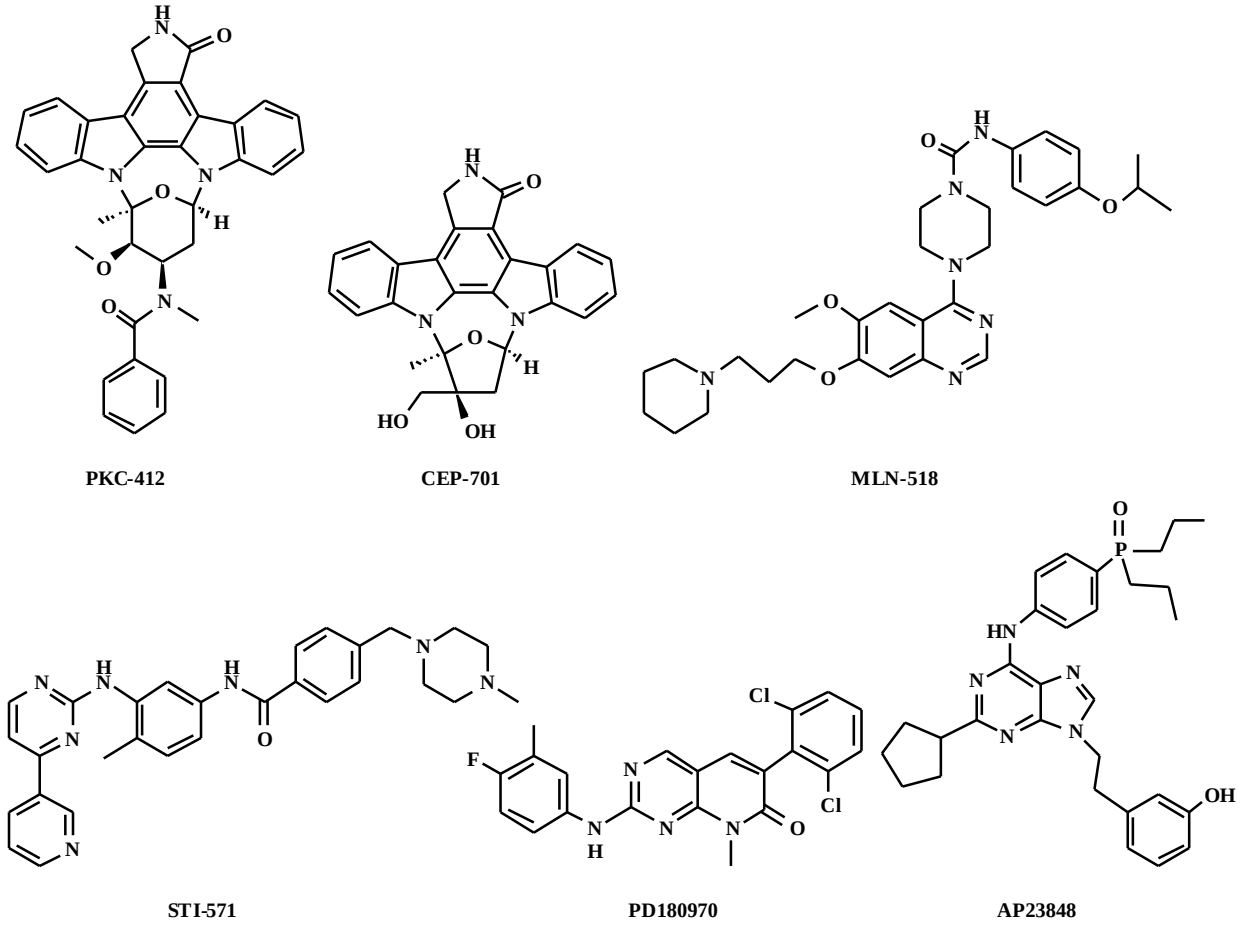

Fig. (5). Some Flt3 and KIT tyrosine kinase inhibitors that have advanced into clinical testing.

(SCF), are known to exist in breast cancer, small-cell lung carcinoma, gynecological tumors and malignant glioma cells. More than 30 gain-of-function mutations of KIT have been identified [10]. Juxtamembrane domain mutations of KIT are common in GISTs, while kinase activation loop mutations (e.g., D816V) are common in both systemic mastocytosis and acute myelogenous leukemia (AML). First-generation KIT tyrosine kinase inhibitors STI-571 (**17**, CGP57148, Gleevec/Glivec™, imatinib), SU-6668 (**8**, *vide supra*), SU-11248 (**9**, *vide supra*), PTK-787 (**10**, *vide supra*), ZD-6474 (**11**, *vide supra*), PKC-412 (**14**, *vide supra*), MLN-518 (**16**, *vide supra*), PD180970 (**18**) and AP23848 (**19**) are particularly noteworthy small-molecules based on a variety of different templates (Fig. **5**). Several compounds, including MLN518 and PKC-412 (each also being Flt-3 tyrosine kinase inhibitors, *vide supra*) are in clinical testing for AML. Initial proof-of-concept studies to investigate a KIT tyrosine kinase inhibitor with respect to KIT-driven cell lines, including both wild-type and mutant KIT, were achieved with STI-571 [121-123]. Consistent with preclinical data showing that KIT wild-type and juxtadomain mutants were highly sensitive to STI-571, clinical testing of imatinib revealed that GIST patients having these mutations had a 30-70% response rate to drug therapy [124]. In contrast, cells expressing KIT activation loop mutant D816V are resistant to imatinib, and patients with systemic mastocytosis involving this mutant are completely resistant to imatinib therapy [121, 125-127]. In regard to identifying small-molecule inhibitors of such KIT tyrosine kinase mutants (i.e., imatinib-resistant), noteworthy progress has been achieved with respect to several compounds, including SU-11652, MLN-518, PD180979 and AP23848 [128-130]. AP23848 has been very recently shown to be a highly potent inhibitor of KIT D816V mutant-expressing cell proliferation as well as to induce cell cycle arrest and apoptosis *in vitro*, and it was further effective *in vivo* to inhibit KIT D816V mutant expressing tumor growth at high dose.

Bcr-Abl and Src Family Non Receptor Tyrosine Kinases and Small-molecule Inhibitors

Bcr-Abl is encoded by the Bcr-Abl oncogene, a translocation gene product found on the Philadephia (Ph) chromosome in hematopoietic stem cells and is causative of chronic myelogenous leukemia (CML) and Ph+ acute lymphoblastic leukemia (ALL) [131]. The chimeric Bcr-Abl protein is expressed with a constitutively active tyrosine kinase, and it has been found to be essential for Bcr-Abl driven transformation and excessive proliferation of Ph+ cells of myeloid and lymphoid lineage. The discovery of STI-571 (**17**) [132, 133] provides one of the most intriguing

and high impact case examples of small-molecule protein kinase inhibitors that have been advanced into clinical testing and being the first-in-class compound approved in the U.S. for the treatment of CML (and an increasing number of other cancer indications, including GIST, *vide supra*). STI-571 is a potent inhibitor of Bcr-Abl (IC_{50} = 250 nM) and several other protein kinases, including KIT (IC_{50} = 100 nM) and PDGFR (IC_{50} = 100 nM). The novel chemical structure of STI-571 (relative to ATP-mimetics of the 'classic' templates such as purines, pyrrolopyrimidines, pyrazolopyrimidines, pyridopyrimidines, and quinazolines) and novel mechanism of inhibition (binding to an inactive conformation of the enzyme) was revealed by recent X-ray structures of STI-571 and analogs complexed with the Abl tyrosine kinase) [134. 135]. STI-571 was determined to bind to an inactive conformation of Abl kinase in which the active loop mimics bound peptide substrate. The potency of STI-571 against activated forms of Bcr-Abl likely results from the dynamic nature of protein kinases relative to switching between active and inactive conformations, thus allowing STI-571 to gain entry and effect inhibition. STI-571 was first determined to be effective *in vivo* with respect to the inhibition of tumor growth in a model developed using syngeneic mice injected with Bcr-Abl transformed cells [136], and a plethora of both *in vitro* and *in vivo* studies have further examined in cellular mechanisms of action and pharmacological properties relative to Bcr-Abl as well as other therapeutic targets [132]. However, despite the incredible successful drug development of imatinib, the issue of Bcr-Abl mutations effecting resistance in CML and ALL patients has provided an opportunity for second-generation, small-molecule inhibitors having superior potency to Bcr-Abl wild-type and many of the known clinically-relevant mutants of Bcr-Abl tyrosine kinase.

Second-generation Bcr-Abl tyrosine kinase inhibitors AMN-107 (**20**), BMS-354825 (**21**), ON012380 (**22**), SKI-606 (**23**), PD180970 (**18**, *vide supra*), PP1 (**24**), CGP-76030 (**25**), and AP23464 (**26**) are particularly noteworthy small-molecules based on a variety of different templates (Fig. **6**). Several compounds, including SKI-606, AZM-475271, PD-180970, PP1, CGP-76030, and AP23464 were first described as Src or Src family tyrosine kinase inhibitors (*vide infra*). Both AMN-107 and BMS-354825 are in clinical trials for Bcr-Abl-dependent leukemias. AMN-107 [137], an analog of STI-571, is a highly potent inhibitor of Bcr-Abl kinase (IC_{50} < 30 nM) as well as several STI-571-resistant mutants (e.g., E255K, E255V, F317L, and M351T) except T315I. AMN-107 is a potent inhibitor of the proliferation of STI-571-resistant Bcr-Abl-expressing cells (except for T315I mutant). An X-ray structure of AMN-107 complexed with Abl kinase mutant M351T showed that it binds in a similar mode as STI-571, however the protein α-helix containing M351T shifts slightly to accommodate binding of AMN-107 relative to its unique disubstituted aniline moiety reflecting the chemical modification relative to STI-571. In contrast, the Bcr-Abl T315I mutant presumably blocks interaction of AMN-107 by sterically preventing the ability of the inhibitor to penetrate into the so-called 'hydrophobic specificity' pocket that exists proximate to the ATP binding site. AMN-107 is in Phase-I clinical trials with indications for CML and ALL. BMS-354825 [138-140], a novel pyrimidinylamino-thiazole template-based molecule, is a highly potent inhibitor of Bcr-Abl (IC_{50} < 1 nM), Src (IC_{50} = 0.5 nM), and KIT (IC_{50} = 5 nM), and it was determined to be further effective to inhibit both PDGFR-β (IC_{50} = 28 nM) and EGFR (IC_{50} = 180 nM). An X-ray structure of BMS-354825 revealed its binding to the ATP pocket relative to a number of H-bonding interactions between the inhibitor and protein, and the disubstituted benzamide moiety fitting well into the hydrophobic specificity pocket (and further implicating the lack of potency against the T315I mutant). BMS-354825 was effective to inhibit several Bcr-Abl mutants (except for T315I) *in vitro*, and *in vivo* studies showed that it prolonged survival of mice infused with Bcr-Abl wildtype or M351T mutants. Further comparative analysis of BMS-354825 and STI-571 saturation mutgenesis experiments showed that each molecule gave rise to an overlapping but different population of mutants, and that combination of the two inhibitors significantly reduced recovery of drug-resistant clones. BMS-354825 is in Phase-II clinical trials for CML. ON012380 [141], a novel small-molecule that is not ATP competitive, is a highly potent inhibitor of Bcr-Abl (IC_{50} = 1.5 nM). In fact, initial biochemical analysis indicate that ON012380 is substrate-competitive, and if further supported by structural studies, ON012380 would constitute a break-through in the identification of small-molecule inhibitors of protein kinases that are mechanistically related to the substrate rather than ATP. ON012380 was further determined to induce apoptosis of cells expressing Bcr-Abl as well as a number of mutants (including T315I) at low nanomolar concentrations. ON012380, at high dose, was effective *in vivo* to cause regression of leukemias induced by Bcr-Abl T315I mutant expressing cells injected into mice. SKI-606 [142, 143], a novel quinoline-carbonitrile template-based molecule, is a highly potent inhibitor of Abl kinase (IC_{50} = 1 nM) as well as Src (IC_{50} = 1.1 nM). SKI-606 effects potent antiproliferative activity against CML cells *in vitro*, and *in vivo* studies further showed SKI-606, at high dose, to cause complete regression of CML xenografts in nude mice. PD180970 [144-146] is a highly potent inhibitor of Abl (IC_{50} = 2.2 nM). PD180970 induces apoptosis in CML cells *in vitro*, and it was further shown to block Stat5 signaling and induce apoptosis in a Bcr-Abl high-expressing cell line that is resistant to STI-571. PD180970 was also determined to inhibit several STI-571-resistant Bcr-Abl mutants *in vitro* with the exception of T315I. A related pyridopyrimidine, PD166326 [147, 148], has been shown to be highly potent againt Bcr-Abl tyrosine kinase and several Bcr-Abl mutants *in vitro*. PP1 [149, 150] and a related pyrazolopyrimidine template-based analog, PP2 [151], were found to be effective inhibitors of Bcr-Abl tyrosine kinase *in vitro*. CGP76030 [150], a pyrrolopyrimidine template-based molecule, has been shown to be an effective inhibitor of Bcr-Abl tyrosine kinase and STI-571-resistant Bcr-Abl mutants (except for T315I) *in vitro*. AP23464 [152], a purine template-based molecule, has been determined to be a highly potent inhibitor of Abl tyrosine kinase (IC_{50} = 1 nM) and effective to inhibit Bcr-Abl and STI-571-resistant Bcr-Abl cell lines (except for T315I) *in vitro*. AP23464 effectively ablated Bcr-Abl tyrosine phosphorylation, blocked cell cycle progression, and promoted apoptosis in Bcr-Abl-expressing cells. Other than the development of Bcr-Abl tyrosine kinase inhibitors capable

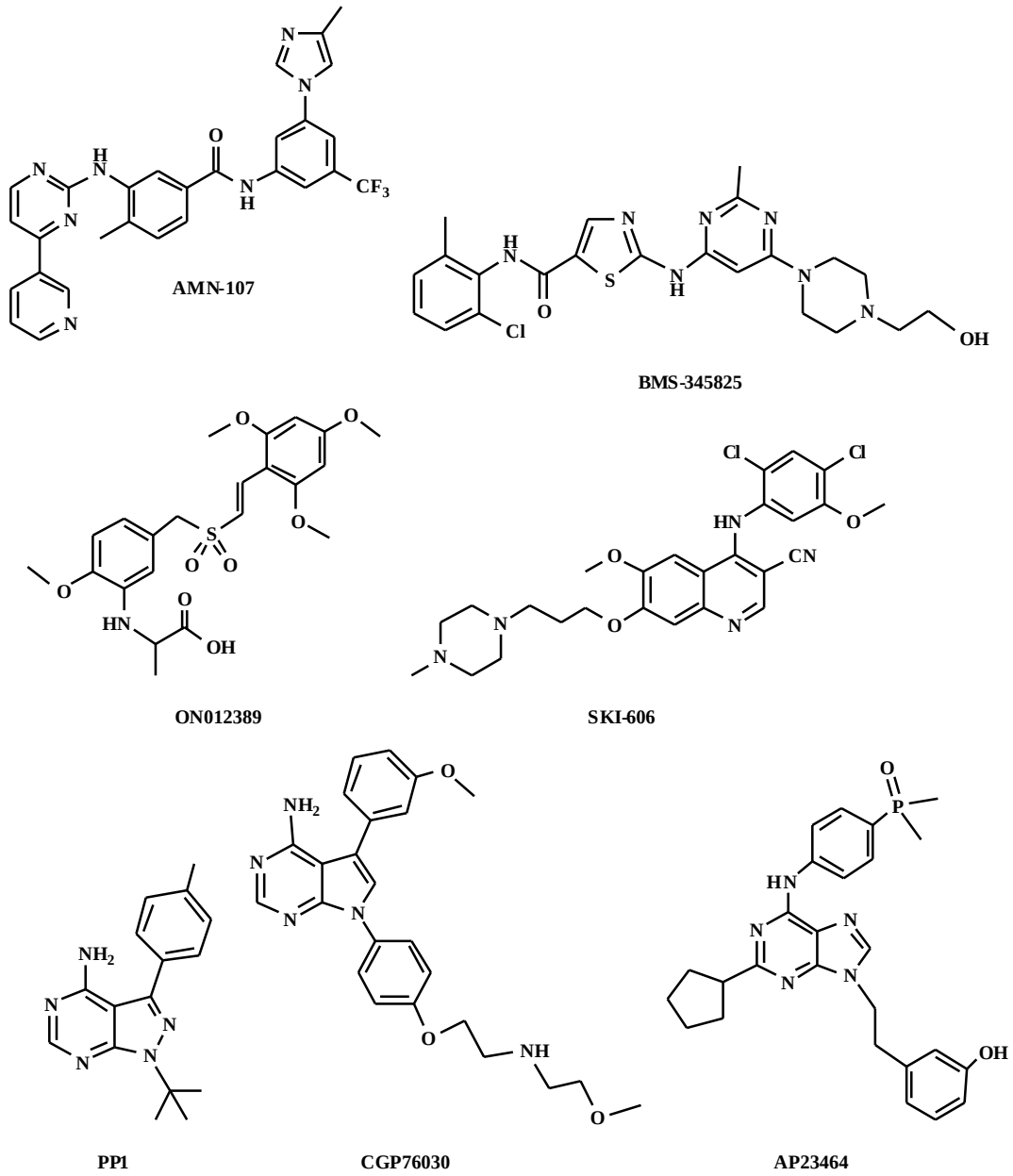

Fig. (6). Some Bcr-Abl and dual Src-Abl tyrosine kinase inhibitor lead compounds (including several that have advanced into clinical testing).

of inhibiting essentially all clinically-relevant Bcr-Abl mutants (e.g., ON012380), a combination of Bcr-Abl tyrosine kinase inhibitors may provide a strategy to provide a potentially fail-safe therapy for CML or Ph+ ALL patients. Furthermore, combination strategies may include imatinib (or other Bcr-Abl tyrosine kinase inhibitor) with other drugs which act on different, but mechanistically-related signal transduction therapeutic targets that might overcome STI-571 resistance [153]. Recently, the farnesyl protein transferase inhibitor SCH66336 [154, 155] and PDK-1 inhibitor OSU-03012 [156] have been found to be effective to enhance the antiproliferative properties of imatinib in both Bcr-Abl- and STI-571-resistant Bcr-Abl-expressing cells (including T315I) *in vitro*.

As previously noted, Src is the prototype of the superfamily of protein tyrosine kinases and is amongst the first protein kinases to be characterized by functional genomics, structural biology and a plethora of biochemical and cellular biology studies designed to understand its role in signal transduction pathways and its role in disease processes, including cancer, osteoporosis, and both tumor- and inflammation-mediated bone loss [10, 36, 156-165]. Pioneering studies on Src provided some of the first evidence correlating protein kinase activity and substrate protein phosphorylation in the regulation of various signal transduction pathway critical to cell growth, cell cycle, cell migration, cell survival and malignant transformation of mammalian cells. Src family kinases include Fyn, Yes, Yrk,

Blk, Fgr, Hck, Lyn, and Frk subfamily members Frk/Rak and Iyk/Bsk. A broad spectrum of functional properties exists for these Src family kinases, including cell growth, differentiation, survival, cytoskeletal alterations, adhesion and migration. The molecular mechanisms of Src-dependent cancer cell biology (e.g., cell-cell and cell-matrix adhesion, cell motility, invasion), including cancer metastasis and angiogenesis have been revealed (Fig. **7**) as a result of many landmark studies [166-184]. Elevated Src expression and/or activity has been correlated with tumor growth in specific cancers having HER2 or c-Met receptors by studies using Src-specific antisense DNA. Elevated Src expression and/or activity has been found in breast cancer cell lines and malignant breast tumors. Src has been implicated in metastatic colon cancer, head and neck cancers, and pancreatic cancer. Activating Src mutations in advanced human colon cancer have also been identified. Src has been implicated in malignant transformations for certain cancers, such as breast cancer and multiple myeloma, *via* EGFR or interleukin-6 receptor (IL6R) signaling pathways, respectively, that commonly activate the transcription factor known as signal transducer and activator of transcription-3 (STAT3). Aberrant activation of STAT signaling pathways have been linked to oncogenesis with respect to the prevention of apoptosis. Relative to integrin receptors and the dynamic relationship between cell-cell and cell-matrix interactions, Src-induced de-regulation of E-cadherin in colon cancer cells has been determined to require integrin signaling, and Src tyrosine kinase activity is required for adhesion turnover associated with cancer cell migration. Collectively, such findings are consistent with the correlation of Src kinase activity (*via* overexpression of CSK and/or dominant-negative mutants of Src) with cellular and *in vivo* metastasis. With respect to VEGFR, Src in intimately involved in VEGF-mediated angiogenesis and vascular permeability. In particular, the ability of VEGF to disrupt endothelial barrier function, which has been correlated to tumor cell extravasation and metastasis, is mediated through Src tyrosine kinase.

Src possesses noncatalytic regulatory motifs, namely the SH3 and SH2 domains *(vide infra)*, which also are functionally important in signal tranduction processes. The molecular basis of Src activation has been further revealed by structural biology studies, including X-ray structures of near full-length Src (i.e., SH3-SH2-tyrosine kinase) [186-188]. These studies have shown that Src exists in an assembled, inactive conformation by virtue of its SH3 and SH2 domains (Fig. **8**). Specifically, the inactive conformation involves intramolecular binding of the SH2 domain with the C-terminal tail (phosphorylated at Tyr-527) as well as intramolecular binding of the SH3 domain with a linker sequence between the SH2 domain and the N-terminal lobe of the tyrosine kinase. The process of Src activation is believed to involve displacement of the imperfect intramolecular SH3 and SH2 interactions within the inactive conformation by intermolecular binding with SH3 and/or SH2 cognate proteins, and subsequent phosphorylation at Tyr-416 (kinase domain) and dephosphorylation at Tyr-527.

An X-ray structure of the Src family member Lck has been determined [189] as complexes of the tyrosine kinase domain with AMP-PNP, staurosporine and PP2 (Fig. **9**). The Lck tyrosine kinase–PP2 complex revealed the detailed molecular interactions of the ATP-mimetic pyrazolopyrimidine template with respect to conserved H-bonding (as experimentally comparable to the AMP-PNP complex). Noteworthy was the binding of PP2 into the large hydrophobic specificity pocket which is not accessed by AMP-PNP. With respect to the entire superfamily of protein kinases, this hydrophobic specificity pocket varies in size,

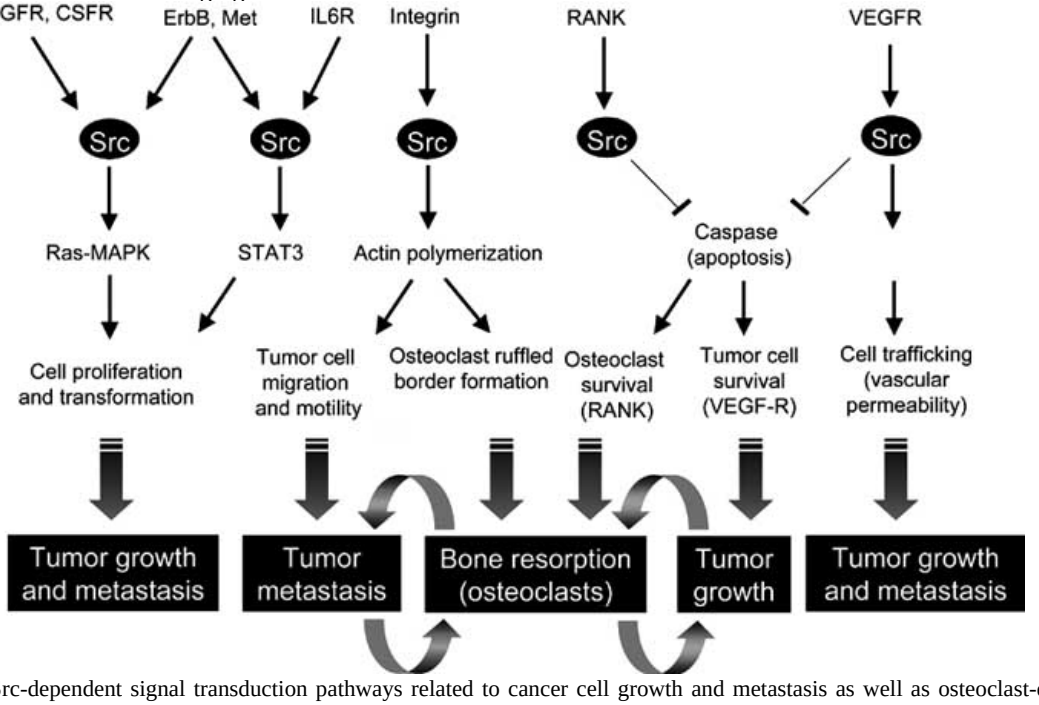

Fig. (7). Some Src-dependent signal transduction pathways related to cancer cell growth and metastasis as well as osteoclast-driven bone resorption (adapted from [160]).

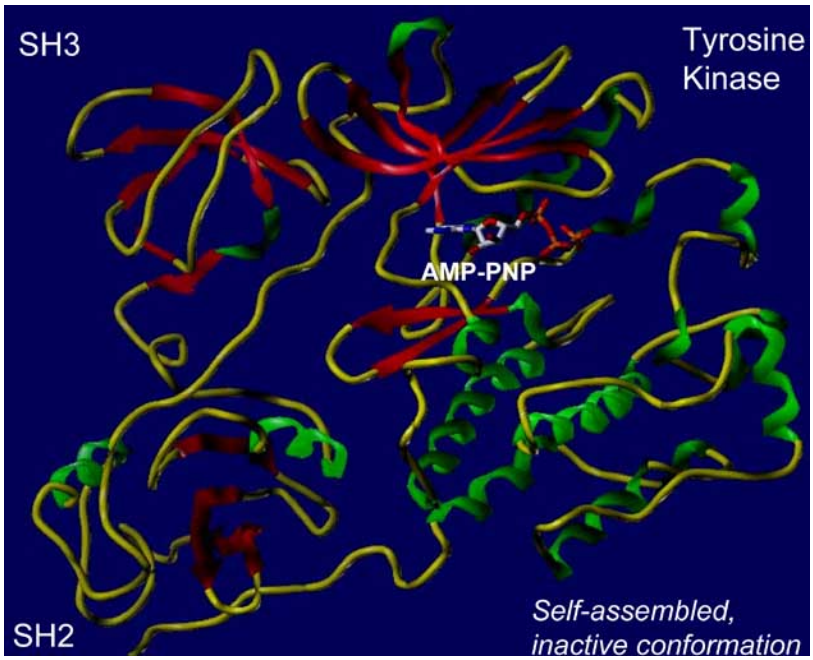

Fig. (8). X-ray structure of Src SH3-SH2-tyrosine kinase complexed with AMP-PNP in a down-regulated, inactive conformation of the protein (adapted from [186]).

shape and amino acid composition with further relationship to the conformation (active/inactive) of the enzyme.

A strategy to exploit protein engineering to mutate the ATP-binding pockets of protein kinase with the objective of enhancing selectivity for synthetic ATP analogs or inhibitors has been developed [190-192] using Src tyrosine kinase as a prototype model. In brief, mutation of a conserved amino acid in the ATP binding pocket was made to create a unique new site that would accommodate a synthetic ATP substrate analogue, namely, [γ-^{32}P]-N^6-(benzyl)-ATP which then provides a matched set of enzyme-substrate to explore signal transduction pathways with respect to the identification of cellular substrates under varying experimental conditions.

The design of Src kinase inhibitors has focused on a number of strategies [5, 35, 36, 79, 160, 161, 193, 194] including ATP template-related mimetics, novel heterocyclic leads (generally from screening corporate chemical collections, but also including combinatorial libraries), natural products, and peptide substrate-based compounds. A schematic model of the Src tyrosine kinase active site

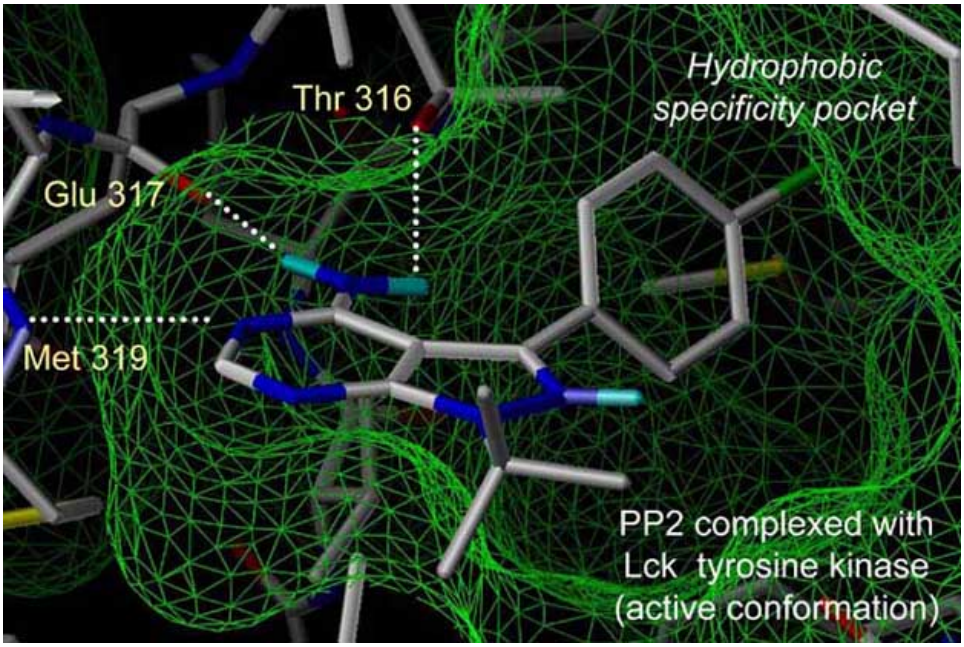

Fig. (9). X-ray structure of Lck tyrosine kinase complexed with ATP-mimetic inhibitor PP2 in an active conformation of the protein (adapted from [189]).

(Fig. **10**) illustrates ATP and peptide substrate binding relative to predicted conserved H-bonding interactions and the hydrophobic specificity pocket (proximate to ATP).

First- and second-generation Src (and Src family) tyrosine kinase inhibitors BMS-354825 (**21**, *vide supra*), SKI-606 (**23**, *vide supra*), PD180970 (**18**, *vide supra*), PP1 (**24**) *vide supra*), CGP-76030 (**25**, *vide supra*), AP23464 (**26**, *vide supra*), AZM-475271 (**27**), SU-6656 (**28**), and AP23451 (**29**) are particularly noteworthy small-molecules based on a variety of different templates (Fig. **11**). BMS-354825 and AZD-0530 (an analog of AZM-475271 with undisclosed chemical structure) are in clinical trials for Src-dependent cancers. BMS-354825 [138] is a highly potent inhibitor of Src tyrosine kinase as well as Bcr-Abl tyrosine kinase (*vide supra*), however there is nothing yet reported to describe its biological properties further with respect to Src-dependent disease models *in vitro* or *in vivo*. SKI-606 [142, 143, 195-197], is a highly potent inhibitor of Src kinase (IC_{50} = 1.1 nM). SKI-606 exhibits antiproliferative efficacy against Src-transformed fibroblasts *in vitro* and *in vivo* relative to a xenograft model. PD180970 and related pyridopyrmidine analogs [144-146, 198-200] have been advanced as potent inhibitors of Src tyrosine kinase with varying selectivities to PDGFR, FGFR and EGFR tyrosine kinases. PP1 and its pyrazolopyrimidine analog PP2 [201] were first described as potent inhibitors of Src-family kinases with marked selectivity versus ZAP-70, JAK2, EGF-R and PKA kinases. PP1 has provide a key Src tyrosine kinase inhibitor to determine the role of Src in VEGF-mediated angiogenesis and vascular permeability [184, 185]. PP1 is an effective inhibitor of Src-driven human breast cancer cell lines with respect to both heregulin-dependent or independent growth [202]. PP1 has also been reported [203] to inhibit collagen type-I-induced E-cadherin down-regulation and consequent effects on cell proliferation and metastatic properties. CGP-76030 and related pyrrolopyrimidine analogs have been described as potent and selective inhibitors of Src tyrosine kinase *in vitro* and *in vivo* relative to animal models of osteoporosis [204, 205]. CGP-76030 has been determined to reduce growth, adhesion, motility and invasion of prostrate cancer cells [206]. AP23464 [35] is a highly potent inhibitor of Src tyrosine kinase (IC_{50} < 1 nM), and it has been recently utilized [207] to examine the functional relationship of Src and FAK in adhesion turnover associated with migration of colon cancer cells and provide mechanistic proof-of-concept correlating Src tyrosine kinase as a key therapeutic target. AZM-475271 [208, 209], a quinazoline template-based molecule, is a potent inhibitor of Src tyrosine kinase and has been determined to be effective to inhibit tumor growth in a Src-transfomed cell xenograft mice as well as in an orthotopically-implanted pancreatic cancer cells in nude mice, thereby providing *in vivo* proof-of-concept for the potential of a Src kinase inhibitor for cancer invasion and metastasis. The combination of AZM-475271 and gemcitabin was also shown to be particularly effective in the pancreatic cancer model. SU-6656 [208] has been described as a potent inhibitor of Src tyrosine kinase (as well as Lck, Fyn, and Yes tyrosine kinases) and to inhibit PDGF-stimulated DNA synthesis and Myc induction in a fibroblast cell line. AP23451 [36, 183, 209] is a potent inhibitor of Src kinase (Ki = 8 nM) and incorporates a bone-targeting moiety in its trisubstituted purine template to confer it a unique property, relative to any other known protein kinase inhibitor, of seeking bone surface (hydroxyapatite) and inhibit Src-dependent osteoclast activity. A related analog of AP23451 has been described [210] which details the structure-activity properties of varying bone-targeting substituents, and other templates (e.g., pyridopyrimidine, pyrrolopyrimidine, pyrazolopyrimidine) have also been elaborated with bone-targeting groups to advance novel Src inhibitors for use in osteolytic bone metastasis and bone diseases [211, 212]. Other Src kinase inhibitors have also been advanced [213-215], including natural products, combinatorial library-based lead compounds, and substrate-based analogs. These molecules further illustrate the extraordinary chemical

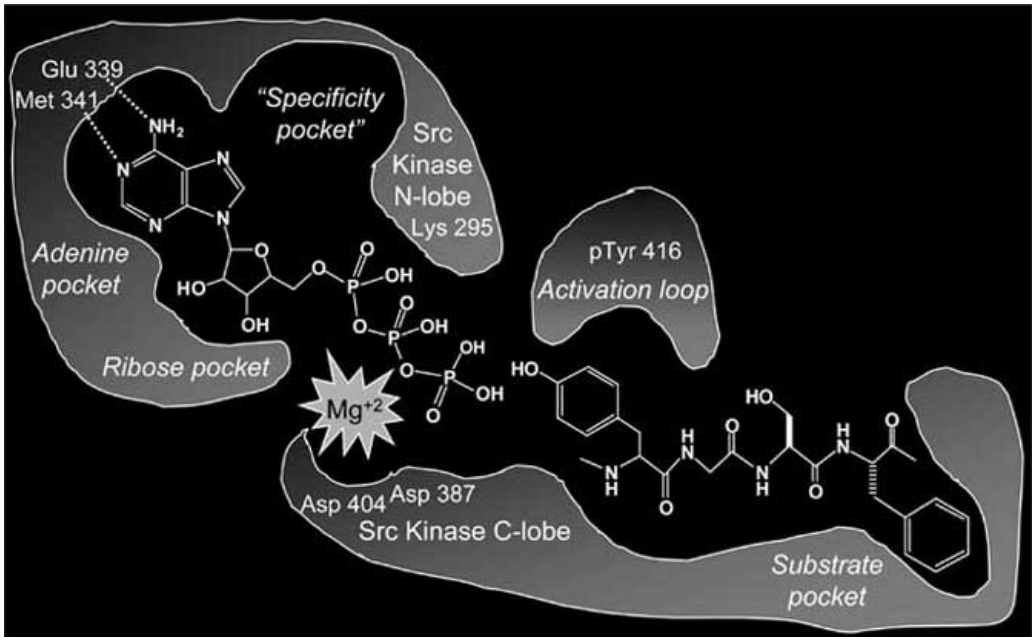

Fig. (10). Schematic model of Src tyrosine kinase complexed with ATP and peptide substrate.

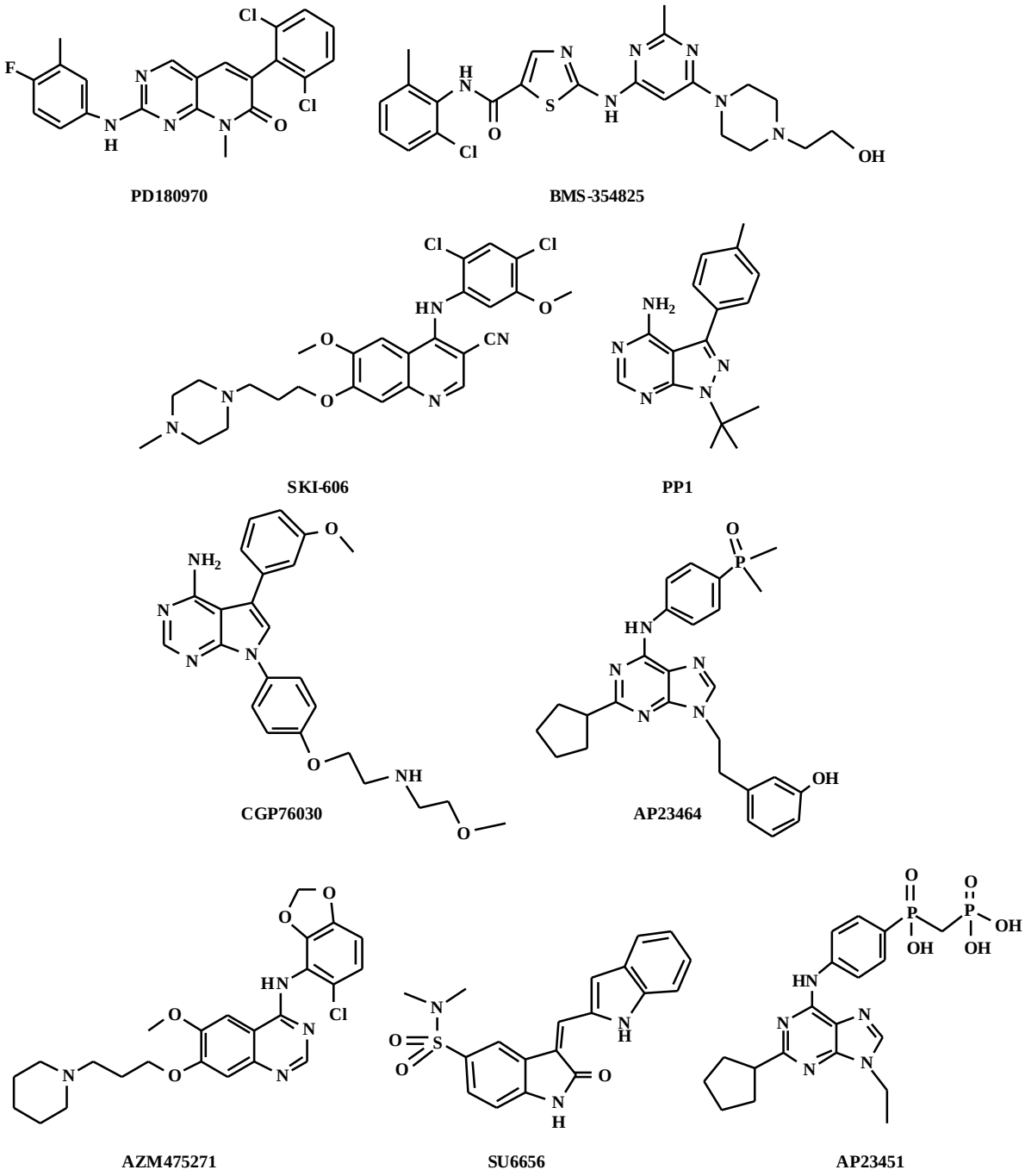

Fig. (11). Some Src and Src family tyrosine kinase inhibitor lead compounds highlighting the structural groups that are predicted to bind to the hydrophobic "specificity pocket" (see Fig. 10 and refer to text for discussion).

diversity and drug design approaches that have emerged relative to this intriguing oncogenic protein kinase therapeutic target. A recent determination [216] of X-ray structures of Src tyrosine kinase complexed with ATP-mimetic inhibitors AP23464 and AP23451 provides further opportunity to understand the varying modes of binding and comparative similarities as well as differences in molecular recognition amongst many of compounds described above.

2.2. Receptor and Nonreceptor Serine/Threonine and Dual Specificity Kinases

Protein serine/threonine and dual specificity protein kinases are represented by both receptor types (e.g., TGFβR) and a large number of nonreceptor types (e.g., MAPK family, CDK family, Raf kinase, PI-3K, PKC, PKB/Akt, mTOR, and aurora kinase). Several have been identified as promising cancer therapeutic targets, and more are emerging

for future drug discovery. In this sense, some noteworthy examples include TGFβR, CDKs (and related cell cycle protein serine/threonine kinases), and a few key therapeutic targets within two oncogenic signaling pathways, namely the Ras-Raf-MEK and PI-3K-Akt/PKB-mTOR pathways (e.g., Raf and MEK, and Akt/PKB and mTOR, respectively). Without question, there has been an extraordinary effort to develop small-molecule inhibitors of various protein serine/threonine and dual specificity kinases, and the few examples illustrated below represent only a fraction of progress made in both basic research and drug discovery for such therapeutic targets.

TGFβR Serine Kinase and Small-molecule Inhibitors

TGF-β is critically involved in the progression of fibrosis as well as tumor invasiveness and metastasis [217, 218] through binding to the receptor serine kinase TGFβR, of which two subtypes (TGFβR1 and TGFβR2) exist. TGFβR1 activation subsequently leads to phosphorylation of transcription factors (e.g., SMADs). Disregulated cell growth due to decreased growth inhibitory activity is a major feature of a defect in TGF-β function, and evidence is emerging that common variants of the TGF-β pathway (ligand and receptors) that alter TGF-β signaling modify cancer risk, including known genetic correlation with a variant of the TGFβR1 gene known as *TGFβR1*6A* which correlates with decrease TGF-β-mediated growth inhibition and increased cancer risk. Interestingly, while decreased TGF-β signaling increases cancer risk, TGF-β secretion and activated TGF-β signaling enhances the aggressiveness of several types of tumors, therefore pointing to drug discovery efforts which have converged on the TGFβR1. First-generation TGFβR1 serine kinase inhibitors SB-203580 (**30**), SKF-104365 (**31**) and LY-364947/HTS-466284 (**32**) are particularly noteworthy small-molecules based on substituted imidazole or pyrazole templates (Fig. **12**). SB-203580 [219], a trisubstituted imidazole moderately inhibits TGFβR1 serine kinase autophosphorylation (IC_{50} = 20 μM) and was discovered by cross-screening small-molecule inhibitors of p38 MAPK. SKF-104365 [220] was found to be a relatively potent inhibitor of TGFβR1 phos-phorylation of SMAD3 (IC_{50} = 1.6 μM) and selective relative to p38 MAPK. LY-364947/

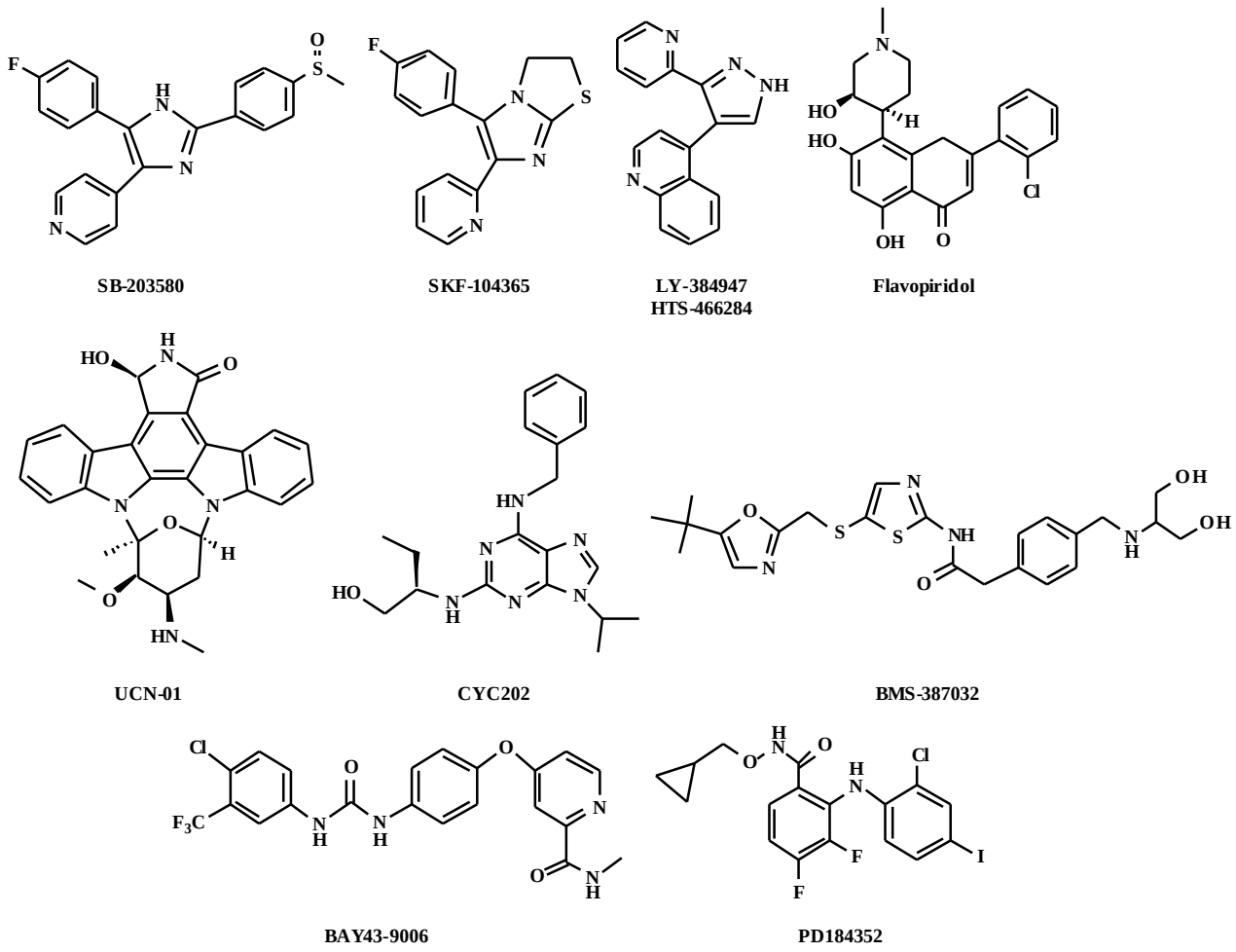

Fig. (12). Some TGFβR1, CDK, PKC, Raf, and MEK kinase inhibitor lead compounds, (including CDK, PKC, Raf, and MEK inhibitors that have advanced into clinical testing).

HTS-466284 [221, 222] was independently discovered by screening- and structure-based strategies and is a highly potent inhibitor of TGFβR1 autophosphorylation (IC_{50} = 51 nM). The X-ray structure of LY-36494/HTS-466284 complexed with TGFβR1 serine kinase was also independently determined in these studies.

CDK Serine Kinases and Small-molecule Inhibitors

Aberrations in cell cycle progression are commonly found in a majority of cancers, and it has been determined that the tumor suppressor retinoblastoma (Rb) family of proteins act as a master switch to regulated cell cycle. Cell proliferation is driven by Rb proteins *via* their phosphorylation by cyclin-dependent kinases (CDKs) [224,-226].The CDK family of serine kinases include those activated by D-type cyclins (D1, D2, and D3) and cyclin-E, and are inhibited by two families of CDK inhibitors (CKIs) which include Ink and Cip/Kip subfamilies. Rb is an important regulator of G1/S transition and its function is abnormal in most cancers. Loss in Rb function occurs by CDK hyperactivation, hence implicating CDK inhibition as a promising strategy for drug discovery. CKIs have further been shown to have additional functions such as the regulation of Rho signaling and the control of cytoskeletal organization and cell migration, and upregulated cytoplasmic CKIs appear to be involved in tumor invasion and metastasis [227]. With respect to the CDK family, the prominent therapeutic targets for small-molecule inhibitor drug discovery have been CDK1, CDK2, CDK4 and CDK5. First- and second-generation CDK serine kinase inhibitors flavopiridol (**33**, L86-8275/HMR1275), UCN-01 (**34**), CYC202 (**35**, roscovitine), and BMS-387032 (**36**) are particularly noteworthy small-molecules which have advanced into clinical testing (Fig. **12**, *vide supra*). Flavopiridol [224, 228-231], is a semisynthetic flavonoid natural product derivative is a potent inhibitor of CDKs (IC_{50} values ~ 100 nM). Flavopiridol effects G1/S and G2/M arrest by inhibition of CDK1 and CDK2. It further has been determined to induce apoptosis and effect antiangiogenesis properties. An X-ray structure of a flavopiridol analog complexed with CDK2 has revealed its binding to the ATP pocket. UCN-01 [232, 233], a staurosporine analogue initially developed as a protein kinase C (PKC) inhibitor, is a potent inhibitor of CDK1 (IC_{50} = 31 nM) and CDK2 (IC_{50} = 30 nM) as well as PKC (IC_{50} = 6.8 nM). UCN-01 inhibits the production of phosphorylated Rb, and induces apoptosis in cancer cells. Recently, UCN-01 has been determined to be potent inhibitor of Pdk1 serine/threonine kinase which then results in the inactivation of Akt/PKB, thus modulating the PI-3K survival pathway. UCN-01 effects antitumor activities in xenograft mice models relative to breast cancer, renal cancer and leukemias. CYC202 [235, 236], a purine template-based molecule, is a potent inhibitor of CDK2/ cyclin E kinase (IC_{50} = 100 nM) and is effects cytotoxicity against many human cancer cell lines (IC_{50} ~ 10 μM range). CYC202 induces cell death from all compartments of the cell cycle as determined in colorectal cancer cells *in vitro*, and *in vivo* studies in nude mice bearing this cancer showed CYC202 effected significant antitumor activity at high dose. BMS-387032 [237], a substituted aminothiazole-template-based molecule, is a potent and selective inhibitor of CDK2/cyclin-E (IC_{50} = 48 nM). BMS-387032 effects potent antiproliferative activity in cancer cells *in vitro*, and *in vivo* it produced significant antitumor activity in a human ovarian carcinoma xenograft model. Collectively, such CDK serine kinase inhibitors have been shown to be quite effective, and combination studies with either conventional cytotoxic drugs or other novel signal transduction modulatory drugs are expected to be useful in the clinical treatment of certain cancers.

Ras-Raf-MEK and PI-3K-Akt/PKB-mTOR Pathway Therapeutic Targets and Small-molecule Inhibitors

The relationship of Ras signal transduction pathway and cancer [238, 239] is hallmarked by the first identification of oncogenic and mutationally activated forms of *Ras* genes more than two decades ago. Mutated Ras alleles are known to exist in about 30% of human cancers, especially those with poor survival indications (e.g., lung and pancreatic carcinomas). Ras can also be abnormally activated by other mechanisms such as overexpressed or mutationally activated EGFR. Amongst therapeutic targets intimately involved in the the Ras pathway (Ras-Raf-MEK-MAPK) are Ras farnesyl protein transferase, Raf [240] and MEK kinase inhibitors [241], EGFR inhibitors (both small-molecule tyrosine kinase inhibitors and monoclonal antibodies to the extracellular receptor), inhibitors of gene expression (i.e., H-*Ras* and *Raf*). First-generation Raf serine kinase and MEK dual specificity kinase inhibitors BAY43-9006 (**37**) and PD184352 (**38**, CI-1040) are particularly noteworthy small-molecules which have advanced into clinical testing (Fig. **12**, *vide supra*). BAY43-9006 [242, 243], a novel biaryl-urea template-based molecule, is a potent inhibitor of Raf-1 serine kinase as well as several receptor tyrosine kinases, including VEGFR-2, PDGFR-β, Flt-3 and KIT. BAY 43-9006 effectively inhibits the MAPK pathway in cancer cell lines (e.g., colon, pancreatic, and breast) expressing mutant K-RAS or wild-type or mutant BRAF. BAY 43-9006 was effective *in vivo* against a broad-spectrum antitumor activity in colon, breast, and non-small-cell lung cancer xenograft models, and additional evidence showing its inhibition of neovascularization in these xenograft models implicates BAY43-9006 as an *in vivo* dual inhibitor of both Raf and VEGFR. PD184352 [244, 245], a novel substituted anthranilic acid template-based molecule, is a potent and highly specific inhibitor of MEK (IC_{50} = 1 nM) to block the phosphorylation of ERK and downstream signal transduction thereof. PD184352 is not ATP-competitive, but is an allosteric inhibitor of MEK. PD184352 effects significant antitumor activity *in vitro* and *in vivo* (particularly pancreas, colon, and breast cancers), of which there has been determined a correlation with the inhibition of ERK phosphorylation.

The relationship of PI3K-Akt/PKB-mTOR signal transduction pathway and cancer has been intensively studies [246-253], and significant evidence exists that dysregulation of the PI3K-Akt/PKB-mTOR pathway may include loss of the PI3K suppressor protein PTEN, PI3K mutations (constitutive activating), and/or other downstream complications involving AKT and mTOR of which the latter integrates signal transduction from both growth factors and

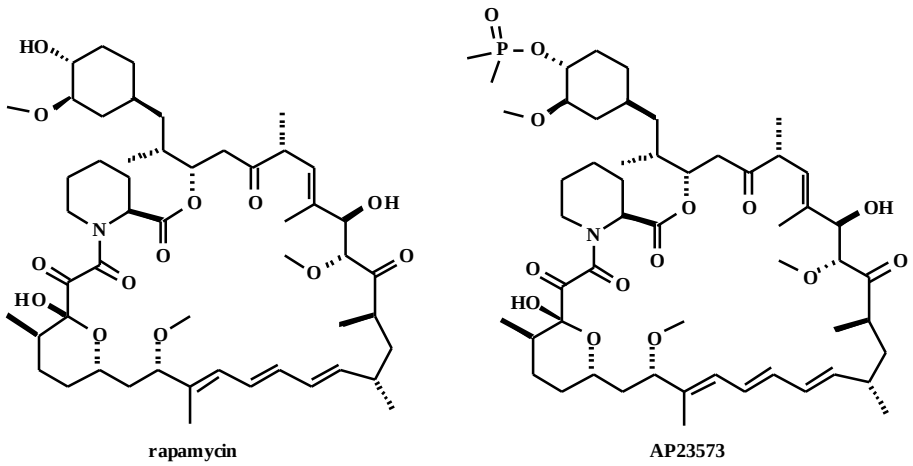

Fig. (13). Some mTOR inhibitor lead compounds.

nutrients. Activation of the PI3K-Akt/PKB-mTOR pathway potentiates cell survival and proliferation as well as cytoskeletal function and motility to impact tumor invasion. Furthermore, the PI3K-Akt/PKB-mTOR pathway is implicated in angiogenesis, including upregulation of angiogenic cytokines. First-generation mTOR inhibtor rapamycin (**39**, sirolimus) and second-generation rapamcyin analog AP23573 (**40**) are noteworthy natural product-based molecules which have advanced into clinical testing (Fig. **13**). The antiproliferative properties of rapamycin were identified more than two decades ago, and it has been developed as an immunosuppressive and antifungal agent. Rapamycin has provided a powerful proof-of-concept lead compound relative to exploiting its potent and specific inhibitory properties against mTOR [246-248, 251, 252] in both *in vitro* and *in vivo* cancer models. AP23573 [246-248] exemplifes a highly potent analog of rapamycin which incorporates a novel modification of the macrolide template with a dimethylphosphoryl moiety. AP23573 and other rapamycin analogs (e.g., CCI-779 and RAD-001) [246-248] have advanced into clinical testing for the treatment of many cancers.

3. PROTEIN PHOSPHATASE THERAPEUTIC TARGETS AND INHIBITORS

More than 100 protein phosphatases have been identified from the human genome [11-14] which constitute a superfamily (Table **2**) which includes; (i) protein tyrosine phosphatases (e.g., PTP1B, SHP2, PEST, PTPH1, PTPα, and CD45); (ii) dual specificity protein phophatases (e.g., VHR, CDC25, PTEN, and MKP-4), and (iii) protein serine/threonine phosphatases (e.g., PP1, PP2A, PP2B/calcineurin). Receptor and nonreceptor tyrosine phosphatases have a vital role in intracellular signal-transduction pathways and regulate such physiological processes as cell growth and proliferation, cell-cycle progression, cytoskeletal integrity, differentiation and metabolism. In particular, there is intense interest in the tyrosine phosphatase PTP1B, which dephosphorylates activated insulin receptor kinase, as result of PTP1B gene knockout and other studies correlating its functional role as a negative regulator of insulin receptor signaling [254-256]. Such work suggests that novel small-molecule inhibitors that might improve insulin sensitivity in type II diabetes and/or be effective for the treatment of obesity [257-258]. Dual-specificity phosphatases are generally categorized as a subclass of protein tyrosine phosphatases, and they are uniquely able to hydrolyse a phosphate ester bond on either a tyrosine, serine or threonine residue of a substrate phosphoprotein. Dual-specificity phosphatases have crucial roles in intracellular signal-transduction pathways and are most prominently known for regulating the mitogen-activated protein kinase (MAPK) signal transduction pathways and cell-cycle progression. Key dual-specificity phosphatases include VHR, a MAPK phophatase, and cell-division cycle 25 (CDC25) which dephosphorylates CDKs. Protein tyrosine kinases differ both in terms of substrate structural recognition and catalytic mechanism of substrate hydrolysis from protein serine/threonine phosphatases. Protein serine/threonine phosphatases are metalloenzymes, whereas protein tyrosine phosphatases hydrolyze phosho-tyrosine substrates in a two-step mechanism involving a conserved cysteine residue to form a thiol-phosphate intermediate followed by water-mediated hydrolysis assisted by key active site residues in the enzyme.

First-generation PTP1B tyrosine phosphatase inhibitors **41**, **42**, and **43** are particularly noteworthy small-molecules which have advanced drug discovery efforts towards the goal of creating a highly potent, orally bioavailable and *in vivo* effective lead compound (Fig. **14**). Compound **41** [260], which was generated using a combinatorial library strategy, is a highly potent inhibitor of PTP1B (Ki = 2.4 nM) and very selective relative to other protein phosphatases (e.g.,Yersinia PTPase, SHP1, SHP2, LAR, HePTP, PTPα, CD45, VHR, MKP3, Cdc25A, Stp1, and PP2C). Compound **42** [261, 262], designed from a lead tripeptide (Ac-Asp-Tyr[SO_3H]-Nle-NH_2), is a potent inhibitor of PTP1B tyrosine phophatase (Ki = 220 nM) and highly selective relative to other protein phosphatases (e.g., SHP-2, LAR, CDC25b, and calcineurin). Prodrug modifications provided cellular activity as measured in terms enhanced 2-deoxyglucose uptake in cells (relative to the parent molecule) with concomitant augmentation of the tyrosine phosphorylation levels of insulin-signaling

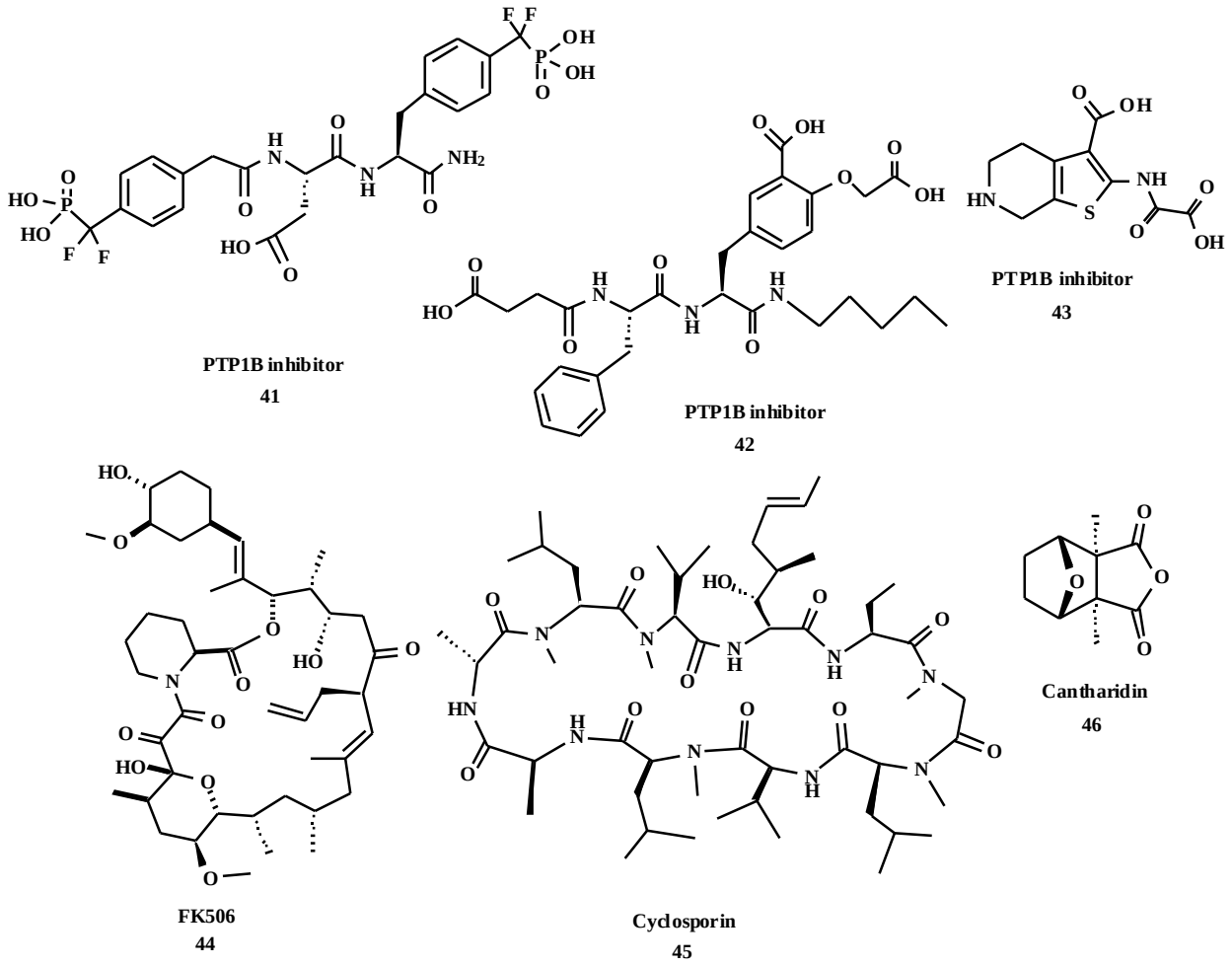

Fig. (14). Some PTP1B, PP1, PP2A and PP2B phophatase inhibitor lead compounds.

molecules. Compound **43** [263, 264], a tetrahydrothieno-pyridine template-based molecule, is a moderately potent inhibitor of PTP1B (Ki = 290 nM) and provided a key lead compound for further optimization to achieve oral bioavailability. It is noted that there has been significant drug discovery efforts to develop PTP1B tyrosine phosphatase (as well as other protein phosphatase) inhibitors and the aforementioned small-molecule lead compounds are only representative of such progress.

First-generation PP1, PP2A and PP2B phosphatase inhibitors **44** (FK506), **45** (cyclosporin), and **46** (cantharidin) are particularly noteworthy natural product and small-molecules (Fig. **14**, *vide supra*) which have advanced drug discovery efforts towards the goal of creating a highly potent, orally bioavailable and *in vivo* effective lead compounds for several diseases, including neurological and metabolic disorders, respiratory disease, immunosuppression, and cancer (as particularly related to cell cycle modulation) [265] FK506, a macrolide isolated from bacteria, is a highly potent inhibitor of PP2B (IC_{50} = 0.5 nM). Cyclosporin-A, a cyclic peptide isolated from fungus, is also a highly potent inhibitor of PP2B (IC_{50} = 5 nM). Cantharidin, a natural product isolated from insects, is a highly potent inhibitor of PP1 and PP2A (IC_{50} = 500 and 200 nM, respectively).

4. PHOSPHOPROTEIN-INTERACTION DOMAIN CONTAINING CELLULAR PROTEIN THERAPEUTIC TARGETS AND INHIBITORS

The complex orchestration of signal transduction and related cellular activities that have been unraveled with respect to both protein kinases and protein phosphatases also include the intimate participation of a relatively large number of cellular proteins which contain phosphoprotein-interacting domains [3, 4, 15-20], including those recognizing phosphotyrosine such as SH2 (e.g., Grb2, Shc, Src, ZAP-70) and PTB (e.g., IRS-1 and ARH) as well as others that recognize phosphoserine/phosphothreonine such as WW and 14-3-3 (Table **2**). Collectively, such phosphoprotein-interacting domains underly cellular mechanisms that have been related to cancer, autoimmune disease, osteoporosis, autoimmune disease, type-2 diabetes mellitus, and autosomal recessive hypercholesterolemia. With respect to small-molecule drug discovery, the overwhelming focus on such phosphoprotein-interacting motifs has been on the SH2 domain [5, 15, 36, 266-270]. SH2 domains are non-catalytic

motifs of approximately 100 amino acids which have been determined to be one of the top twenty-five most frequently occurring protein structural types that have been identified from the human genome. Numerous X-ray and/or NMR structures have been determined for SH2 domains (e.g., Src, Grb2, and Zap70) and complexes thereof with phosphopeptide, peptidomimetic or nonpeptide inhibitors. As the prototype SH2 domain, the Src SH2 domain and phosphopeptide (e.g., pTyr-Glu-Glu-Ile sequences) complexes were first determined by X-ray structural studies to provide detailed molecular maps, especially concerning the pTyr moiety.

A major challenge of SH2 inhibitor drug design was the pTyr moiety in terms of developing metabolically-stable pTyr mimics that exhibit high affinity to a SH2 domain. Another major challenge was the transformation of the peptide ligand scaffold to peptidomimetic or nonpeptide templates that would afford more drug-like properties with respect to cellular and *in vivo* activity. Two successful drug design campaigns that have advanced key proof-of-concept lead compounds against Src SH2 and Grb2 SH2 domains illustrate endeavors to overcome such pTyr and peptide scaffold challenges. First-generation nonpeptide Src SH2 inhibitors **47**, **48** (AP22161) and **49** (AP22408) are particularly noteworthy small-molecules which have advanced the first proof-of-concept *in vivo* effective lead compound (Fig. **15**). Compound **47** [271], a benzamide template-based molecule, was created using de novo drug design strategies and was determined to be a moderately potent inhibitor of the Src SH2 domain (IC_{50} = 6.6 μM). Compound **48** [272], a bicyclic-benzamide template-based molecule incorporating a novel pTyr mimic having an electrophilic aldehyde designed to interact with a cysteine residue in the pTyr binding pocket of Src SH2, was determined to be a potent inhibitor of Src SH2 (IC_{50} = 240 nM) and to be effective in cell-based assays to block Src-dependent activities *in vitro*. Compound **49** [273], a bicyclic-benzamide template-based molecule incorporating a novel pTyr mimetic designed to increase

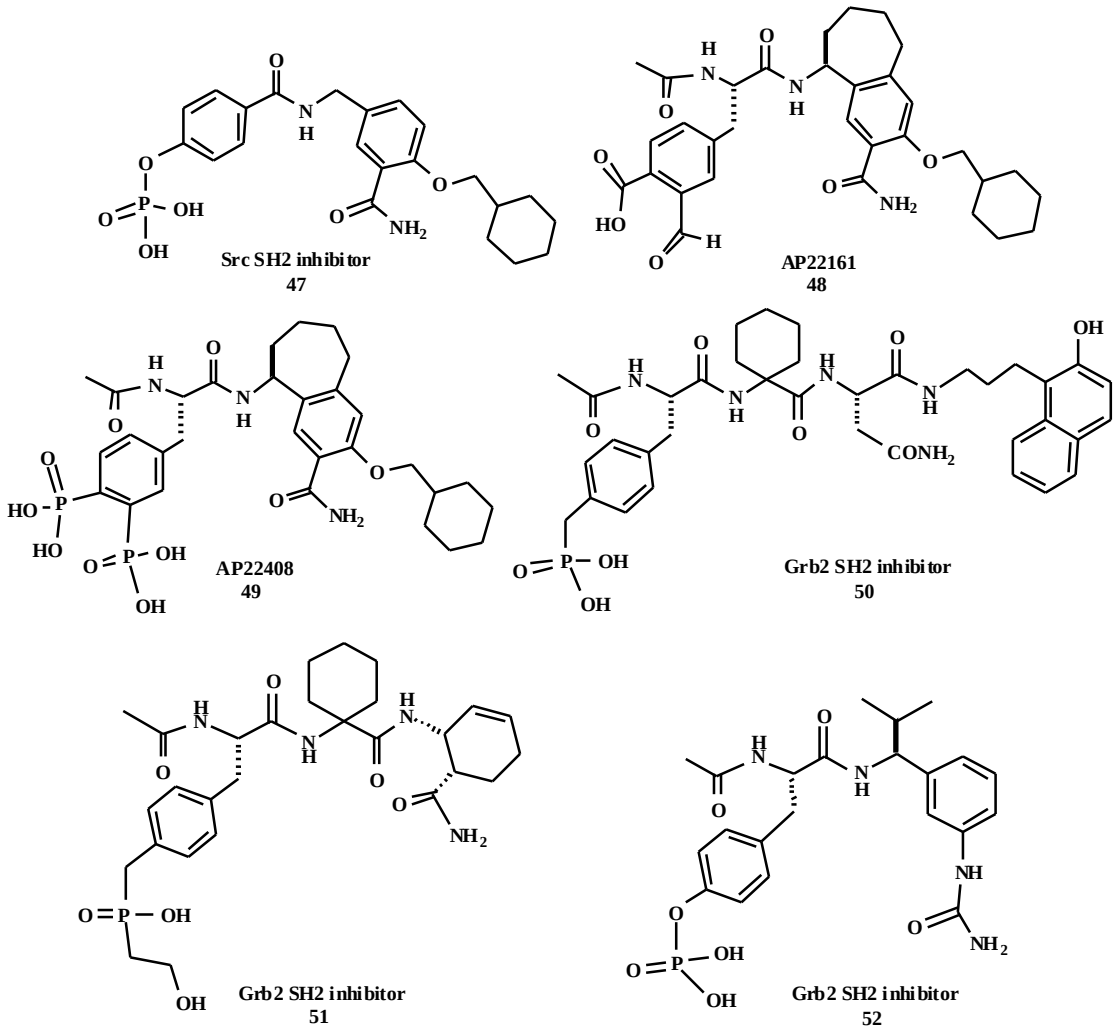

Fig. (15). Some Src SH2 and Grb2 SH2 inhibitor lead compounds.

Table 3. Some Small-molecule Oncogenic Protein Kinase Inhibitors that have Advanced to Clinical Investigation

Compound	Protein Kinase(s)	Clinical Status (cancer indications)	Company
STI-571 (Imatinib)	Bcr-Abl, PDGFR, Kit	Approved (chronic myelogenous leukemia,/CML) Approved (gastrointestinal stromal cell tumors/GIST)	Novartis
AMN-107	Bcr-Abl, PDGFR, Kit	Phase-I (CML, acute lymphocytic leukemia/ALL)	Novartis
BMS-354825	Bcr-Abl, SFK	Phase-II (CML)	Bristol-Myers Squibb
SKI-166	Bcr-Abl, SFK	Phase-II (CML, solid tumors)	Wyeth
AZD-0530	SFK, Bcr-Abl	Phase-I (solid tumors)	AstraZeneca
ZD-1839 (Iressa)	EGFR	Approved (non-small cell lung carcinoma/NSCLC)	AstraZeneca
OSI-774 (Tarceva)	EGFR	Approved (NSCLC)	OSI Pharmaceuticals
CI-1033	EGFR, HER2	Phase-II (various solid tumors)	Pfizer
PKI-166	EGFR, HER2	Phase-III (solid tumors)	Novartis
EKB-569	EGFR, HER2	Phase-II (NSCLC, colorectal)	Wyeth
GW-572016	EGFR, HER2	Phase-III (NSCLC, solid tumors)	GlaxoSmithKline
PTK787/ZK222584	VEGFR, PDGFR, Kit	Phase-III (colorectal, solid tumors)	Novartis/Schering AG
AMG-706	VEGFR, PDGFR, Kit, RET	Phase-II (NSCLC, colorectal, GIST)	AMGEN
SU-6668	VEGFR, PDGFR, Kit	Phase-II (solid tumors)	Pfizer (SUGEN)
SU-11248	VEGFR, PDGFR, Kit, Flt-3	Phase-III (GIST, solid tumors, acute myeloid leukemia/AML)	Pfizer (SUGEN)
CGP53716	PDGFR	Phase-III (brain tumors)	Novartis
ZD-6474	VEFGR, Kit	Phase-II (solid tumors)	AstraZeneca
CEP-7055	VEGFR	Phase-I (solid tumors)	Cephalon/Sanofi
CP-547632	VEGFR, FGFR	Phase-I/II (ovarian, NSCLC)	OSI Pharmaceuticals/Pfizer
MLN-518	Flt3, PDGFR, Kit	Phase-II (AML)	Millennium Pharmaceuticals
PKC-412	Flt3, VEGFR, PDGFR, Kit	Phase-II (AML, myelodysplastic syndrome)	Novartis
CEP-701	VEGFR, Flt3, Trk	Phase-II (AML)	Cephalon/Kyowa Hakko Kogyo
BAY-43-9006	Raf	Phase-II/III (kidney, breast, lung)	Onyx Pharmaceuticals/Bayer
PD-184352	MEK	Phase-II (pancreatic, breast)	Pfizer
Flavopiridol	CDK	Phase-II (head and neck cancer, solid tumors)	Aventis
CYC202	CDK	Phase-II (NSCLC, lymphoma)	Cyclacel
BMS-387032	CDK	Phase-I (metastatic refractory solid tumors)	Bristol-Myers Squibb
UCN-01	PKC, CDK	Phase-I/II (refractory solid tumors and lymphomas)	Kyowa Hakko Kogyo
AP23573	mTOR	Phase-II (hematologic malignancies, various solid tumors)	ARIAD Pharmaceuticals
CCI-779	mTOR	Phase-III (renal cell carcinoma, breast, prostate)	Wyeth
RAD001	mTOR	Phase-II (GIST, relapsed/refractory CML or AML)	Novartis

Adapted from Dancey and Sausville [4], Laird and Cherrington [8], Becker [24], and Garcia-Echevaria and Fabbro [54].

binding affinity and confer bone-targeting properties, was a potent inhibitor of the Src SH2 domain (IC_{50} = 300 nM) and effective to inhibit osteoclast-dependent bone resorption *in vitro* and *in vivo*. Noteworthy is the overall structure-based design of AP22408 with respect to both the pTyr and peptide scaffold replacement strategies, and further studies [274] have extended the scope of other pTyr mimetics by a strategy of multiple functional group replacement. Other drug discovery efforts have advanced Src SH2 inhibitors having nonpeptide templates [275-277]. First-generation nonpeptide Grb2 SH2 inhibitors **50**, **51** and **52** are particularly noteworthy small-molecules which have advanced the first proof-of-concept cellularly effective lead compound (Fig. **15**). Compound **50** and **51** [278-280] are peptidomimetics which exploit a β-turn conformation as determined from X-ray structural studies of cognate ligand phosphopeptides complexed with Grb2 SH2, and each are potent inhibitors of Grb2 SH2(IC_{50} = 43 nM and 1.6 μM, respectively). Compound **51** was effective in cells and provided the first proof-of-concept lead compound relative to disrupting the Ras-Grb2 pathway by the inhibition of Grb2 SH2. Compound **52** [281] is a novel nonpeptide molecule which is a moderately potent Grb SH2 inhibitor (IC_{50} = 6.2 μM) and provides a template for further lead compound optimization.

5. CHEMISTRY PERPECTIVES FOR SMALL-MOLECULE DRUG DISCOVERY

The plethora of scientific endeavor that has been focused on understanding the fundamental principles of protein phosphorylation from a structural, functional and disease standpoint is nearly beyond comprehension. We now know the complexity of Nature's design in a rather simplistic way, albeit well-enough to establish sophisticated experimentation to further explore signal transduction pathways that involve protein phosphorylation. As this perspective has highlighted, the generation, optimization and development of small-molecule inhibitors of protein kinases, protein phosphatases and phosphoprotein-interactions domain containing cellular protein therapeutic targets has provided an emerging armamentarium of powerful tools, proof-of-concept lead compounds and clinical candidates (Table **3**). Although the integration of structure-based drug design, chemoinformatics, library and high-throughput screening as well as other wondrous biological technologies that are now available have unquestionably enabled the iterative process of drug discovery, there are yet challenging issues for chemistry to face in the field of signal transduction. Biological selectivity is emerging to be a key factor in the probability of success to advance the next-generation breakthrough medicines. Multiple inhibition of several key therapeutic targets (e.g., protein kinases that may synergize with respect to signal transduction pathways converging in a particular disease scenario) may conceptually have tremendous advantages, and there exist examples of a protein kinase inhibitors that may be superior drugs as the result of modulating more than a single target. However, biological selectivity has transcended to include an ever-increasing number of mutations of signal transduction therapeutic targets, especially protein kinases (e.g., EGFR, Bcr-Abl, KIT, and Flt3). Combination drug therapy is an option to overcome resistance due to mutations that select against a particular small-molecule inhibitor. Lastly, Nature's creative exploitation of phosphate (not only for proteins but within the greater scope of nucleic acids, lipids, carbohydrates, and the multifunctional ATP molecule itself) have been recognized and further manipulated by chemistry efforts at ARIAD Pharmaceuticals to advance novel small-molecule lead compounds, ranging from peptidomimetics to natural products and various heterocyclic templates to advance drug discovery efforts tackling several of the signal transduction therapeutic targets described in this perspective.

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