Signal Transduction Pathway Targets for Anticancer Drug Discovery

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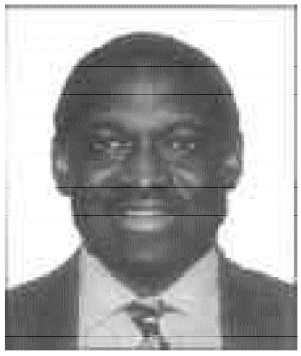

Abstract: There are currently over 80 agents officially approved for the treatment of cancer world-wide. However, the most common epithelial cancers, which cause greater than 75% of cancer deaths, remain incurable. Most drugs have been developed empirically by testing large numbers of chemicals on rapidly growing transplantable rodent tumors, and more recently, human tumor xenografts. This approach has identified prodeminantly DNA-active drugs that are considerably toxic and have limited efficacy. Novel molecular targets, which are selective for neoplastic cells, are needed for chemotherapeutic agents to improve cure rates of epithelial malignancies, with acceptable toxicity. In recent years, agents inhibiting signal transduction pathway molecules have entered clinical trials. These include antibodies and small molecules, which inhibit growth factor receptors and their receptor tyrosine kinases, inhibitors of cytoplasmic second messengers such as ras, raf and MEK, inhibitors of protein trafficking, and inhibitors of protein degradation.

INTRODUCTION

Cell proliferation and differentiation are regulated by a number of hormones, growth factors and cytokines. These molecules interact with cellular receptors and communicate with the nucleus of the cell through a network of intracellular signaling pathways (Fig **1**). In cancer cells, key components of these pathways may be altered by oncogenes through over-expression or mutation, leading to disregulated cell signaling and cell proliferation. The components of these abnormal signaling pathways, which are specific to neoplastic cells, represent potential selective targets for new anticancer therapies. These potential targets include ligands (typically growth factors), cellular receptors, intracellular second messengers and nuclear transcription factors. A detailed description of all these possible targets is beyond the scope of this review. The interested reader is referred to several recent, excellent reviews [1-5]. This discussion will focus exclusively on the targets for which promising anti-neoplastic agents are in clinical trials.

INHIBITION OF GROWTH FACTOR RECEPTOR BINDING

The first, obvious point of intervention in a signaling cascade is the neutralization of ligands before they can associate with their receptors. This approach has been investigated, initially through the use of antibodies, which interact with growth factors. Small cell lung cancer, and other malignancies secrete bombesin-like peptides, such as gastrin releasing peptide. A recently published phase I trial established that repeated doses of monoclonal antibody *2A11*, which binds to the bombesin-like peptide, GRP with high affinity, could be given safely to SCLC patients, and sustained plasma levels could be achieved on a 1-week schedule of antibody administration [6]. This approach is limited by the need to prospectively identify patients whose tumors express the receptor, and whose plasma contains significant amounts of circulating growth factor. Non-specific inhibition of several growth factors was explored through the use of the polysulfonated naphthylurea, suramin [7]. This agent neutralized a number of growth factors, but presented challenging dosing problems because of an extremely long half-life and significant toxicities. In spite of evidence of modest activity in prostate cancer, its development has been discontinued, because of toxicity problems.

The second approach to abrogating signaling pathways is the prevention of the binding of growth factors to their receptors. Several strategies have been under development in an attempt to block growth factor receptors. The most successful approach to date has been the development of monoclonal antibodies which bind to receptors and by so doing prevent the binding of the endogenous ligands.

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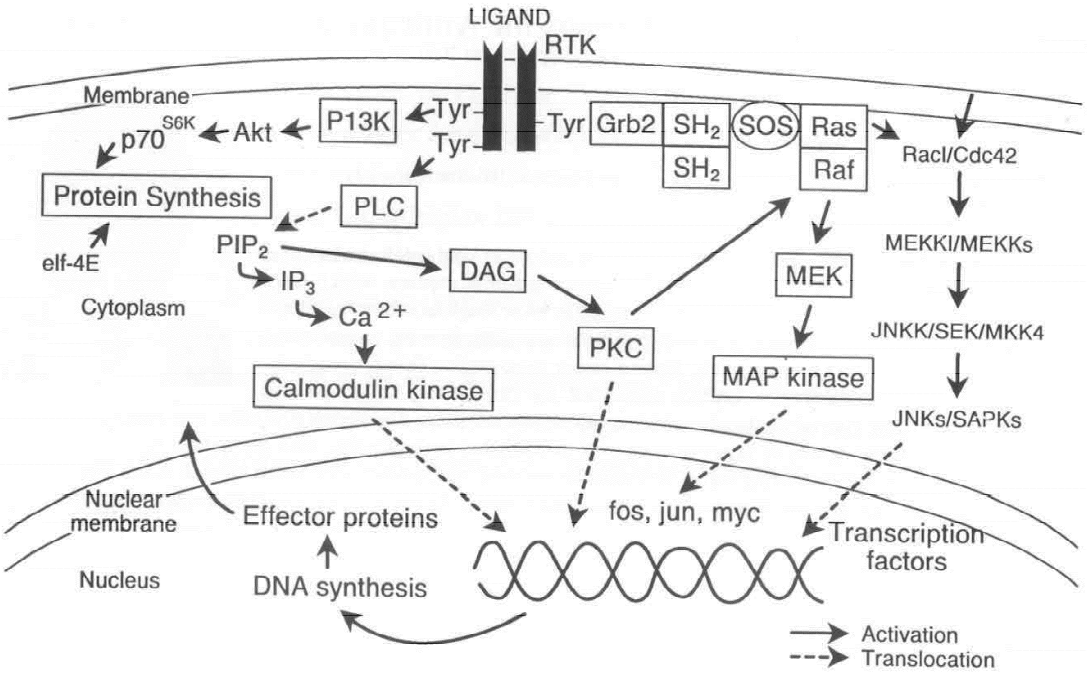

Fig. (1). Schematic representation of some important signal transduction pathways in cancer cell proliferation. DAG, diacylglcecrol; IP3, inositide triphosphate; PI3K, phosphoinositide-3-kinase; PLC, phospholipase C; PIP2, phosphoinositide diphosphate; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; MAP kinase, mitogen-activated protein kinase.

The Epidermal Growth Factor Receptor (EGFR) Family

The epidermal growth factor (EGF) receptor was cloned in 1984 by Ullrich *et al.* [8]. This receptor has two cysteine-rich regions in the extracellular domain and a single kinase domain. Three other members of this family, HER-2, HER-3 and HER-4 (referred to also as *erb*B2, *erb*B3 and *erb*B4) are known. The designation HER-2 stands for **H**uman **E**pidermal growth factor-like **R**eceptor type **2**. Members of the EGF receptor family and their ligands are overexpressed or expressed as an autocrine loop in a number of tumor types, including pancreatic, lung, ovarian, renal cell,gastric, hepatocellular and breast cancers [10-12].

Anti-EGFR Antibodies

Because over-expression of EGFR has been associated with a more aggressive disease and a poor prognosis, the blockade of EGFR activation has been proposed as a target for anticancer therapy. The most promising agent is the human-mouse chimeric monoclonal antibody 225 (C225), which inhibits activation of the EGFR receptor tyrosine kinase. This inhibition of EGFR activation causes cell cycle arrest in G1. The mechanism of growth inhibition has been shown to involve an elevation in the levels of $p27^{KIP1}$ and inhibition of cyclin-dependent kinase-2 activity [13]. Preclinically, C225 in combination with several chemotherapy agents, including cisplatin, doxorubicin and paclitaxel exhibited synergistic antitumor activity, with successful eradication of well-established tumor xenografts that were resistant to treatment with either C225 or drug alone [14]. Phase I clinical trials have established the safety of repeated administration of single-agent C225 at concentrations that maintain receptor-saturating blood levels for up to 3 months [15]. Phase I trials exploring C225 treatment in combination with the chemotherapy agents mentioned above, are ongoing [16], and single-agent phase II trials are in progress. Preliminary data indicate that antitumor activity is significantly augmented when the antibody is utilized in combination with cytotoxic chemotherapy.

Anti-HER-2 Antibodies

The proto-oncogene HER-2/neu is localized to chromosome 17q, and encodes a 185kDA

transmembrane glycoprotein receptor with intrinsic tyrosine kinase activity. No endogenous ligands for the HER-2/neu protein are known. When HER-2/neu protein is activated, it can interact with many different cellular proteins such as shc, PLC, GAP and the ras MAP kinase pathway [17]. The HER-2 receptor can also form heterodimers with other members of the EGFR family. Amplification of the HER-2/neu gene or over-expression of the HER-2/neu protein has been identified in 10-34% of breast cancers. Possible techniques for evaluating HER-2/neu status in breast cancer cells include gene-based assays such as polymerase chain reaction methods and in-situ hybridization utilizing both fluorescent (FISH) and non-fluorescent approaches. Qualitative protein measurements are the most common techniques used. Immunohistochemistry, typically on archival tissues is utilized. A significant discordance between HER-2/neu detection methods has been reported [18]. The discordance has been between immunohistochemistry methods and between immunohistochemistry and gene-based assays [19]. A number of clinical studies involving over 10,000 women have examined the relationship of HER-2/neu gene and/or protein abnormalities and breast cancer outcome. Results of these studies have not been uniform. Several studies including the original study published by Slamon *et al.* [20] found that HER-2/neu over-expression independently predicted poor overall survival and disease-free survival. Some immunohistochemical studies have found significant correlation between HER-2/neu protein immunoreactivity and disease outcome in univariate, but no independent predictive status in multivariate analysis [21]. A few studies have found no correlation whatsoever with disease outcome [22.23]. Compared to the prognostic information outlined above, there are fewer studies correlating the expression of HER-2/neu protein with response to therapy. Several studies have found HER-2/neu over-expressing tumors to be resistant to tamoxifen therapy [24, 25]. One large study in 200 patients, however, failed to show resistance to tamoxifen in HER-2/neu over-expressing tumors [26]. While HER-2/neu over-expression has been associated with enhanced response to chemotherapy regimens containing doxorubicin in clinical samples, a poor response to CMF (cyclophosphamide, methotrexate, 5-fluorouracil) therapy has been found in the same population of patients [27]. In cultured breast cancer cell lines, HER-2/neu expression is associated with resistance to paclitaxel [28]. However, another study indicated a 3-fold increased response to paclitaxel in the same population [29]. The preponderance of evidence would suggest a poorer prognosis and poor response to some therapeutic agents in patients with HER-2/neu expressing tumors. The conflict in the data may be explained in part, by the different methods used to document HER-2/neu expression.

Trastuzumab (rhuMAb HER-2, Herceptin®)

Trastuzumab is a humanized monoclonal antibody that targets the HER-2 receptor with demonstrated activity in metastatic breast cancer. This is the first monoclonal antibody to be approved for the treatment of a solid tumor. In cultured cells that express high levels of HER2, trastuzumab causes growth arrest in the G0/G1 phase of the cell cycle [30]. The growth inhibitory effects have been explained by a marked induction of the cyclin dependent kinase-2 kinase inhibitor, p27, as well as the retinoblastoma-related protein p130 [30]. These data suggest that treatment of HER-2 overexpressing cells is antiproliferative, and that cytostasis may result from an inhibition of cell cycle progression.

In early preclinical studies, Slamon *et al.* demonstrated interactions between trastuzumab and several anticancer agents [31], using *in vitro* clonogenic studies. Synergistic interactions at clinically relevant drug concentrations were observed for trastuzumab in combination with cisplatin, thiotepa and etoposide. Additive cytotoxic effects were observed with trastuzumab plus doxorubicin, paclitaxel, methotrexate and vinblastine. 5-fluorouracil, was found to be less than additive with trastuzumab. Studies were further conducted with drug/trastuzumab combinations in HER-2/neu-transfected, MCF-7 human breast cancer xenografts in athymic mice. Combinations of trastuzumab and cyclophosphamide, doxorubicin , paclitaxel, methotrexate, etoposide, and vinblastine *in vivo* resulted in a significant reduction in xenograft volume compared to chemotherapy alone. Combinations of trastuzumab and 5-fluorouracil yielded equivalent results to those achieved by 5-fluorouracil alone. The 5-FU results were consistent with the sub-additive effects observed with this combination *in vitro*. The synergistic interaction of trastuzumab with alkylating agents, platinum analogs and topoisomerase II inhibitors, as well as the additive interaction with taxanes, anthracyclines and some antimetabolites in HER-2/neu over-expressing breast cancer cells guided the choice of combination studies in clinical trials. Currently, data from six breast cancer trials have

been published. Three studies have evaluated trastuzumab alone for the treatment of metastatic breast cancer. Two studies evaluated trastuzumab in refractory breast cancer [32]. Response rates were 11.6 and 15%, respectively [33]. The antibody was well tolerated. The third trial evaluatedtrastuzumab as first-line treatment of metastatic breast cancer. A preliminary response rate of 24% has been reported [34]. The pivotal multinational phase III study reported by Slamon *et al.* evaluated trastuzumab ± paclitaxel, or doxorubicin/cyclophosphamide in 469 first-line metastatic breast cancer patients. Overall, the median time to progression was improved from 4.6 to 7.6 months by the addition of trastuzumab, with an improvement in overall response from 32% to 48%. Toxicity was generally mild, consisting of febrile episodes and mildly increased myelossuppression. An increased incidence of symptomatic cardiac toxicity was noted when trastuzumab was added to anthracycline-based chemotherapy (19% vs. 3%). The etiology of this increased cardiotoxicity is unclear, but continues to be investigated [35]. Trastuzumab plus cisplatin was evaluated in patients with refractory breast cancer, yielding a response rate of 24% [36].

INTRACELLULAR SIGNALING PATHWAYS

The intracellular signaling pathways that are activated after growth factors and other cell-proliferation-associated ligands bind to their receptors are complex and incompletely understood. Major components of these effector systems such as the protein tyrosine kinases, protein kinase C and the ras/MAP kinase pathway have, however, been identified.

PROTEIN TYROSINE KINASES

Protein tyrosine kinases (PTKs) catalyze the phosphorylation of tyrosine residues on target proteins [37]. Two major groups of PTKs have been described to date, receptor and non-receptor tyrosine kinases. Non-receptor tyrosine kinases are cytoplasmic proteins which transduce extracellular signals to downstream intermediates in pathways that regulate cell growth, activation and differentiation. Many non-receptor tyrosine kinases are linked to transmembrane receptors including those for peptide hormones and cytokines. Unlike receptor tyrosine kinases, they lack transmembrane domains and ligand binding. They are activated by ligand binding to their associated receptors or events such as cell adhesion, calcium influx or cell cycle progression [38]. More than 30 members are classified in 10 families including src, abl, JAK, MKK2, FES [38].

Receptor tyrosine kinases (RTKs) share several structural features. They are glycoproteins possessing an extracellular ligand-binding domain, which conveys ligand specificity, and a single hydrophobic transmembrane domain, which anchors the receptor to the membrane. Intracellular sequences typically contain regulatory regions in addition to the catalytic domain. Ligand binding induces activation of the intracellular tyrosine kinase domain leading to the initiation of signaling events specific for the receptor. The RTKs have been organized into families based on sequence homology, structural characteristics and distinct motifs in the extracellular domain. There are currently 19 known families in vertebrates. The various subfamilies include receptors for epidermal growth factor (EGFR), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF). Ligand binding to a RTK induces receptor dimerization with conformational changes that result in intermolecular phosphorylation at tyrosine residues at multiple sites. Receptor heterodimerization can also occur, as reported with transforming growth factor alpha interaction with receptor heterodimers comprising HER-2 and EGFR [39]. In malignant tumors, a number of these receptors are over-expressed or mutated, leading to abnormal cell proliferation.

PLATELET DERIVED GROWTH FACTOR (PDGF)

PDGF is a major mitogen for endothelial cells, fibroblasts, smooth muscle cells and glial cells. PDGF exists as disulfide-linked homodimers and heterodimers of A and B chains, resulting in 3 isoforms (PDGF-AA, PDGF-BB, PDGF-AB). PDGF and its receptors are expressed in a wide variety of cultured neoplastic cells including breast, prostate and colon cancers. Expression has also been documented in tumor biopsies of ovarian cancer and gliomas [40].

The tyrphostins are synthetic protein tyrosine kinase inhibitors derived from erbstatin, a natural product with broad spectrum activity against protein tyrosine kinases and protein kinase C. SU101 is a tyrphostin derivative, which predominantly inhibits the PDGF receptor tyrosine kinases. In a completed phase I study, the most common toxicities were mild to moderate nausea, vomiting, and fever. Neutropenia was uncommon, and occurred only at

the highest dose levels [41]. Phase III studies are ongoing in recurrent gliomas, and phase II studies have been completed in lung, prostate and ovarian cancers [42].

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF has 5 isoforms, which are splice variants and exist as disulfide-linked homodimers, with some structural similarities to PDGFs. These isoforms bind with high affinity to 2 receptors, *fms*-like tyrosine kinase (flt-1) and fetal liver kinase (flk-1). The biologic significance of these multiple VEGF receptor forms is not understood. VEGF stimulates the growth of endothelial cells during the process of angiogenesis, but has also been identified as a vascular permeability factor. A recombinant humanized monoclonal antibody, rhuMAb VEGF, has been developed to inhibit the effects of VEGF in the treatment of solid tumors. Phase II trials have been completed in lung and colon cancer. Results are awaited with interest A small molecule inhibitor of VEGF tyrosine kinase, SU5416 is undergoing phase I testing, and a phase I/II study in AIDS-related Kaposi's sarcoma is ongoing [43].

A second generation broad spectrum RTK inhibitor, SU6668 is currently undergoing phase I testing. This agent is a small organic molecule, that possesses anti-angiogenic and anti-proliferative properties. It inhibits the autophosphorylation of three distinct tyrosine kinases, Flk-1/KDR; PDGFR, and FGFR with IC_{50} values of 0.2 μM, 0.2 μM, and 4.1 μM, respectively. EGFR kinase activity remains uninhibited (IC_{50} > 100 μM). *In vitro* kinetic analyses demonstrate that SU6668 is a competitive inhibitor of ATP binding. This mechanism is similar to that of the bioflavonoids such as genistein and quercetin, and distinct from the tyrphostins and their derivatives, which compete for the substrate binding site of RTKs [44].

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

EGFR differs from the other receptor tyrosine kinases in that there is a single isoform, from a single 26 exon gene located across 110kb on chromosome 7p11-13. It serves as the predominant receptor for multiple distinct ligands, including EGF, TGF-α, amphiregulin and HB-EGF (Table 1). EGFR interacts with most members of the EGFR (c-*erb*B) family of RTKs. As previously mentioned, the ligand for c-erbB2 is unknown, while erbB3 and erbB4 serve as heregulin and neuregulin receptors. The major function of these other receptors appears to be as downstream effectors of each other. They cross-phosphorylate and modulate signaling from each other in specific pairs. EGFR interacts with HER-2 and HER-3 but not HER-4. HER-4 pairs with HER-2.

It is noteworthy that HER-3 lacks kinase activity, but serves as a docking protein to recruit a broader spectrum of downstream effectors after phosphorylation by EGFR or HER-2 [45]. Currently, 3 EGFR tyrosine kinase inhibitors are undergoing phase I clinical testing. One of these is ZD1839, which is an orally active, and selective inhibitor of the EGFR (HER-1) tyrosine kinase. In preclinical studies, administration of this agent daily for 4 months resulted in significant tumor growth delay in rodents bearing human xenografts. *Ex vivo* examination of xenograft tissues revealed a time and dose-dependent decrease in c-fos mRNA, a marker for EGFR signaling [46]. An ongoing phase I trial has enrolled 38 patients. A partial response has been noted in NSCLC, with minor responses in head and neck and renal cell cancers. The most common toxicity is a skin rash [47, 48]. The second agent in this class, CP-358774 is also a selective EGFR tyrosine kinase inhibitor. An acneform skin rash, diarrhea and mild hepatic transaminase elevations were the most common toxicities [48]. The third agent in this class, CI-1033 is a non-specific inhibitor of the EGFR family (HER-1, HER-2, HER-4) tyrosine kinases [49].

Table 1. Members of the Human Epidermal Growth Factor Gene Family

Gene	Ligand
HER-1 (c-erbB-1)	EGF, TGFα, Beta cellulin, Amphiregulin, Heparin binding growth factor
HER-2 (c-erbB-2)	? Heregulin
HER-3 (c-erbB-3)	Heregulin, neu differentiation factor 1 / 2
HER-4 (c-erbB-4)	Heregulin, neu differentiation factor 1+2

This agent demonstrates an *in vitro* IC_{50} in the low nanomolar range with all four RTKs. Results of phase I studies with this agent are awaited with interest.

PROTEIN KINASE C INHIBITORS

Protein kinase C (PKC) is a family of serine/threonine directed protein kinases consisting of a group of at least 10 related isoenzymes that are involved in the transduction of signals for cell proliferation and differentiation. PKC has therefore been considered a suitable target for novel antineoplastic drugs. The PKC family is divided into Ca^{2+}-responsive and Ca^{2+}-unresponsive subtypes [50, 51]. The enzyme consists of an N-terminal regulatory domain, which binds and inhibits the C-terminal catalytic domain.

PKC modulatory agents, which affect the catalytic domain, are the staurosporine congeners. Staurosporine is the parent compound derived from microbial sources two decades ago, and has poor selectivity. It is inhibitory towards tyrosine kinases and cAMP-dependent protein kinases as well as PKC at similar concentrations [52]. Its analogs 7-hydroxy-staurosporine (UCN-01) and CGP 41251 have greater selectivity, and effectively arrest the growth of several human-derived tumor cell lines *in vitro*. They also possess antineoplastic activity *in vivo* in human tumors grown as xenografts in nude mice. CGP 41251 reverses the multidrug-resistance phenotype of cancer cells [53]. Both agents are currently under clinical evaluation as potential antitumor drugs. These agents are also potent modulators of the cyclin dependent kinase system, which determines the progression of cells through the cell cycle. The nature of this interaction is complex. UCN-01 blocks cells in GI phase by promoting accumulation of dephosphorylated retinoblastoma protein as a consequence of inhibition of the activity of certain cyclin-dependent kinases, down-regulation of their partner cyclins and an increase in the expression of cyclin-dependent kinase inhibitor proteins [54]. Preliminary results of early clinical trials suggest that UCN-01 and CGP41251 are without remarkable toxicity but display high binding to human plasma protein [55].

Several studies indicate a role for PKC in the regulation of the multi-drug resistance (MDR) phenotype, since several PKC inhibitors are able to partially reverse MDR and inhibit P-glycoprotein (Pgp) phosphorylation. The MDR phenotype is also associated with variation in PKC iso-enzyme content, in particular with PKC-α over-expression. Based on these results, it has been hypothesized that PKC inhibitors may synergize with cytotoxic agents through modulation of MDR. However, other potential mechanisms of PKC interaction with anticancer drugs exist and have been documented, such as the enhancement of chemotherapy-induced apoptosis by safingol, a specific PKC inhibitor [56]. Safingol is undergoing a phase I clinical trial in combination with doxorubicin. While no final data are presently available, it appears that plasma levels of safingol approach those associated with potentiation of chemotherapy in animals, and no pharmacokinetic interaction between the two drugs exists. Drugs targeting PKC are therefore felt to possess potential as modulators of cytotoxic agents.

Inhibitors of the regulatory domain of PKC include the ether lipids and bryostatins. Bryostatin 1, a macrocyclic lactone isolated from the marine organism *Bugula nerutina*, is a partial PKC agonist, and has shown potent antineoplastic properties *in vitro and in vivo*. Bryostatin 1 has both antineoplastic and immune-stimulatory properties, including the induction of cytokine release and expansion of tumor-specific lymphocyte populations. Bryostatin I has demonstrated antitumor activity in phase I trials in patients with malignant melanoma, lymphoma and ovarian carcinoma and is undergoing broad phase II testing, both as a single agent, and in combination with standard cytotoxic agents [57, 58]. The dose-limiting toxicity is myalgia. In a completed phase II study, 16 previously treated patients with malignant melanoma were treated with bryostatin at 25 $\mu g/m^2$ weekly for three courses followed by a rest week. The principal toxicities were myalgia, phlebitis, fatigue and vomiting. Of 15 patients evaluable for tumor response, 14 developed progressive disease. One patient developed stable disease for 9 months after bryostatin treatment. The authors concluded that single-agent bryostatin was ineffective in the treatment of metastatic melanoma in patients previously treated with chemotherapy, but suggested that it should, however, be investigated further in previously untreated patients [59].

ISIS 3521 (ISI 641A), an antisense phosphorothioate oligonucleotide to protein kinase C-α, has been studied in a phase I trial. The antisense approach involves targeting specific ribonucleotide (RNA) sequences in order to block translation of the RNA message into protein. Drug-related toxicities included mild to moderate nausea, vomiting, fever, chills, and fatigue, hematologic toxicity was limited to mild to moderate

thrombocytopenia. Dose escalation on this study was discontinued on attaining peak plasma concentrations which approached that associated with complement activation in primates. No dose limiting toxicity was identified, and clinical activity was observed in two patients with non-Hodgkin's lymphoma who achieved complete responses [60].

THE ras/MITOGEN ACTIVATED PROTEIN KINASE PATHWAY

As previously mentioned, effector molecules recruited by receptor tyrosine kinases (RTKs) include phospholipase C (PLC), phosphoinositide-3-kinase (PI3-kinase) and ras. The ras pathway is fairly well understood. The effector molecules bind to phosphorylated tyrosine residues on the RTKs via src homology 2 (SH2) domains or an adapter protein, Grb2/SOS in the case of ras. Activated ras activates raf, which is a serine/threonine kinase. Raf activates mitogen activated protein kinase kinase (MEK, MAPKK) which in turn activates mitogen activated protein (MAP) kinase. MAP kinase activation results in phosphorylation and activation of ribosomal S6 kinase and transcription factors such as c-jun, c-myc and c-fos, resulting in the switching on of a number of genes associated with proliferation [61-63]. Signaling by this pathway is more complex than outlined above, since ras and raf-independent pathways for MAP kinase activation exist. Secondly, a subfamily of 54 kDa MAP kinases which function independently of MEK has been identified [64].

The ras protein plays an essential role in signal transduction, as already described. Three mammalian *ras* proto-oncogenes encode four related and highly conserved proteins, H-ras, N-ras, and the splice variants K-ras 4A, and K-ras 4B [65]. The $p21^{ras}$ proteins bind guanosine triphosphate (GTP) and guanosine diphosphate (GDP) with high avidity. When GTP is bound, the ras protein is in an "active" state. When GDP is bound in the resting state, $p21^{ras}$ is inactive. One means of negative regulation of $p21^{ras}$ activity is via the GTPase-activating protein (GAP). Single point mutations of the ras gene can lead to its constitutive activation. These mutated forms have impaired GTPase activity. Although they bind GAP, there is no "off" sign since GTPase is not activated. This results in continuous stimulation of cellular proliferation, and inhibition of apoptosis [66]. Oncogenic ras mutations have been identified in approximately 30% of human cancers [67]. K-ras mutations are frequent in non-small cell lung, colorectal and pancreatic carcinomas; H-ras mutations are found in bladder, kidney and thyroid carcinomas; and N-ras mutations are found in melanoma, hepatocellular carcinoma and hematologicmalignancies [67]. Association of ras with the plasma membrane, and consequent activation require prenylation, i.e. the attachment of a farnesyl group, at the carboxy-terminus.

ras INHIBITORS

Because of the high percentage of human tumors harboring oncogenic *ras* mutants, interrupting the ras signaling pathway has been a major focus of new drug development efforts. The current promising approaches taken are i) the inhibition of ras protein expression through antisense oligonucleotides, ii) the prevention of membrane localization of ras, and iii) the inhibition of ras function through inhibition of downstream ras effectors.

Antisense Oligonucleotides

Oligonucleotides that are complementary to messenger RNA (mRNA) transcripts of the activated *ras* oncogene, have been utilized to decrease ras protein expression. These oligonucleotides hybridize to complementary mRNA sequences and decrease protein expression through a variety of mechanisms, including RNase H-mediated cleavage of hybridized ras mRNA. Roth and co-workers developed a retroviral antisense K-*ras* expression vector, which has been shown to eliminate the expression of human ras protein in lung cancer cells [68]. This K-*ras* retroviral construct was successfully administered intratracheally to nude mice bearing implanted human lung cancers. Significant activity was observed with 87% of treated mice being tumor-free compared to 10% of control mice [69]. A persistent problem had been the inability to deliver intact antisense RNA agents into tumor cells. In recent years, the distribution of phosphorothioate oligodeoxynucleotides,presumably by endocytosis in rodent tissues after intravenous administration has been demonstrated [70]. An investigational phosphorothioate antisense oligodeoxynucleotide, ISIS 2503 designed to hybridize to the 5'-untranslated region of human H-ras mRNA, has completed testing in phase I clinical trials. Interim results indicate a tolerable toxicity profile, with moderate thrombocytopenia and fatigue as the only adverse events. An indication of biologic activity has been seen, with mild tumor shrinkage in a patient with sarcoma [71]. The schedule that has

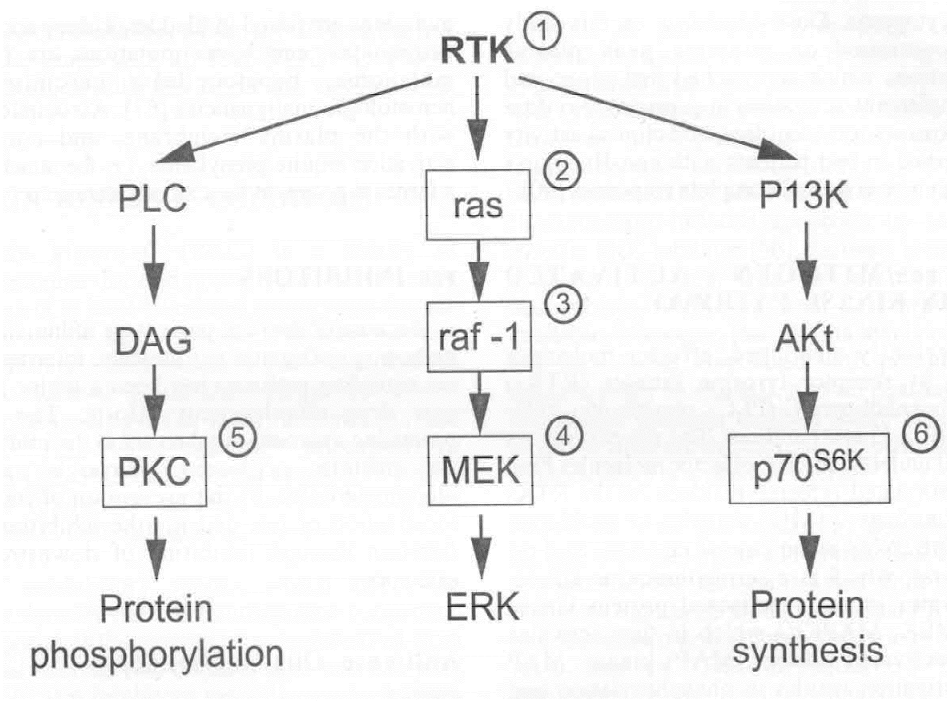

Fig. (2). Simplified scheme of signal transduction pathways, with an indication of the molecules to which clinical agents are targeted.

1) anti-receptor antibodies such as trastuzumab, receptor tyrosine kinase inhibitors such as monoclonal antibody C225, ZD1839, CP258774 and CI-1033

2) farnesyltransferase inhibitors (SCH66336, R115177, L748259, BMS214662); antisense oligonucleotides (ISIS 2503)

3) antisense oligonucleotides (ISIS 5132), HSP90 inhibitor (17AAG)

4) MEK inhibitor (PD 184322)

5) PKC inhibitors (bryostatin, UCN-01, CGP41251) antisense oligonucleotide (ISIS 3521)

6) CCI-779

been selected for further testing is a 14-day continuous infusion every 21 days. This agent is currently undergoing phase I combination studies with cytotoxic chemotherapy agents, and phase II testing in pancreatic carcinoma.

As described above, C-raf kinase acts downstream of ras in the mitogen-activated-protein kinase pathway. Recent evidence also suggests that C-raf kinase may also be activated by other mechanisms such as bcl-2 [72]. ISIS 5132 is a 20-mer phosphothiorate antisense oligonucleotide, which inhibits c-raf kinase. Phase I testing of different infusion schedules have been completed. Toxicities have been mild fever, and evidence of complement activation, which is clinically insignificant [73]. Phase II studies are underway in prostate, colorectal and ovarian cancer.

Farnesyltransferase Inhibitors

Mammalian farnesyltransferase (FT) is a zinc metalloenzyme, which exists as a heterodimer [74]. The 48 kDa α-subunit is identical to that of geranylgeranyltransferase-1 (GGT-1). The 45 kDa β-subunit appears to contain the binding sites for both substrates (peptide and isoprenoid). The CAAX motif, which is the tetrapeptide sequence of the carboxyterminus, is crucial in recognizing peptide substrates. The A_1 position has a relaxed amino acid specificity with basic and aromatic side chains tolerated whereas the A_2 and X positions have strict specificity criteria [75, 76]. For example, the substitution of an aromatic side chain at A_2 creates a molecule, which is a competitive inhibitor of FT [77]. Either substrate is able to bind independently to FT, but the preferred sequence is isoprenoid binding followed by peptide binding.

Product release after these processes appears to be the rate-limiting step [78]. In addition to zinc, FT requires Mg2+ for activity. However, the exact role of zinc and magnesium ions in catalysis of the transferase reaction, remains unknown at present.

Known substrates for FT in mammalian cells include the ras proteins, the nuclear lamin proteins lamin B [79], and prelamin A [80], cyclic GMP phosphodiesterase α [81], rhodopsin kinase [82], a peroxisomal protein PxF of unknown function and the γ-subunit of the retinal protein transducin [83]. The historical development and structural diversity of FT inhibitors has been reviewed recently [84-86]. The first true inhibitor of FT was the tetrapeptide CVFM, which competed with the CAAX motif. While this agent was not pharmacologically viable because of inability to enter cells, it served as a prototype for the development of other therapeutic candidates. Subsequent developments focused on peptide CAAX inhibitors, which were modified to enter cells.

The FT inhibitors fall into two main classes, the CAAX competitive inhibitors such as L731735, L744832, SCH 66336, R 115777 and the FPP competitive inhibitors such as PD 169451, and RPR 130401 [87-92]. BMS241261 belongs to a third class of bispecific inhibitors. Currently, four FT inhibitors (SCH66336, R115777, L778123, BMS241261) are in clinical trials in the US and Europe, with several more at different levels of preclinical development. One of these agents, SCH 66336 belongs to a novel non-peptide tricyclic group of FT inhibitors, which compete with the protein substrate for binding to FT [93]. SCH 66336 inhibits the farnesylation of H-ras and K-ras-4B *in vitro* by purified human farnesyl protein transferase with an IC_{50} of 1.9 and 5.2 nM, respectively. There is no inhibition of a related protein prenyl transferase, geranylgeranyl protein transferase (GGPT-1) at concentrations of up to 50 uM, confirming the selectivity of this agent for FT. SCH 66336 inhibits H-Ras processing in whole cells. In addition, SCH 66336 blocks the transformed growth properties of human neoplastic cell lines and fibroblasts expressing activated Ki-ras proteins. SCH 66336 also demonstrated potent antineoplastic activity in nude mice bearing human xenograft models of lung, prostate, pancreas, colon and urinary bladder carcinomas [94]. In a completed phase I trial at Mayo Clinic, this agent was found to be well tolerated. Gastrointestinal toxicities of diarrhea, nausea and vomiting were dose limiting. A greater than 50% tumor shrinkage has been observed in a previously treated patient with non-small cell lung cancer, who has been on treatment for one year [95]. Phase I studies have also been completed with R115777 [91]. Phase II studies are currently ongoing with these two agents. While this class of agents holds some promise as anticancer agents, their mechanism of action need to be further elucidated.

Prenylation of ras proteins is complex. Recent *in vitro* studies suggest that both N-ras and Ki-ras are low-affinity substrates for GGPT-1. Geranylgeranylation (addition of a 20-carbon isoprene lipid) of Ki-ras and N-ras proteins has been observed in intact cells treated with FT inhibitors [96]. Furthermore, geranylgeranylated forms of ras have oncogenic transformation potential when overexpressed. Despite this alternative prenylation pathway, FT inhibitors demonstrate *in vitro* and *in vivo* antitumor activity in cells with activated Ki-ras mutations. In addition, several cell types with no ras mutations are sensitive to FT inhibitors, both *in vitro* and *in vivo*. These findings argue that inhibition of the farnesylation of other proteins in addition to ras may contribute to the observed antitumor properties of these agents. Because of the large number of proteins that are farnesylated, the use of FT inhibitors has been expected to lead to unanticipated side-effects. Of particular theoretical importance are the sequelae of inhibiting the farnesylation of transducin and rhodopsin kinase, which are retinal proteins involved in vision. However, in addition to SCH 66336, a recently completed phase I study of R115777 also revealed mild to moderate side effects, consisting of gastrointestinal effects (nausea, diarrhea, constipation, heartburn), arthralgia, fatigue and headache. Interestingly, no ocular toxicities have been observed in these two studies [91, 95].

MEK Inhibitors

The MAP kinase pathway is essential in cellular growth and differentiation. Sequential activation of MAPK kinase 1 (MAPKK1 or MEK1) and MAP kinase (ERK1/2) occurs downstream of ras. MAP kinase in turn phosphorylates downstream substrates involved in cellular responses such as cytoskeletal changes and gene transcription. Conditional MAP kinase activation is important in gene regulation, promoting G1 cell cycle progression before DNA replication, and spindle assembly during both meiotic and mitotic cell division, among other processes. Inappropriate activation of the MAP kinase pathway, through mutations introduced via oncogenes, is a feature of

many neoplasms. Molecules, such as MEK, are therefore potential targets for cancer therapy. Leopold-Sebolt *et al.* have reported the discovery of PD184322, a highly potent and selective inhibitor of the upstream kinase MEK, that is orally active. Tumor growth was inhibited as much as 80% in mice implanted with colon carcinomas of both mouse and human origin after treatment with this inhibitor. Efficacy was achieved with a wide range of doses with no signs of toxicity, and correlated with a reduction in the levels of activated mitogen-activated protein kinase in excised tumors. These data indicate that MEK inhibitors represent a promising, non-cytotoxic approach to the interruption of the ras-MAP kinase pathway for cancer therapy [97].

It has been argued that blocking a downstream target such as MEK may abrogate a number of different signaling pathways, which impinge on this intermediary molecule. This may allow for targeting of a broad range of tumors, but increases the potential of toxic effects. On the other hand, blocking an upstream target such as EGFR (HER-1) may limit the range of tumors that can be treated to only those that aberrantly express this receptor. This selectivity may however reduce the potential for toxicity. It is unclear at this time if either of the two approaches will be superior. Ongoing clinical trials will resolve this issue.

Inhibition of p70S6 Kinase

Phosphoinositide 3'- kinase (PI3K) is a member of a family of lipid kinases that phosphorylate phosphoinositides [98]. Activation of PI3K results in the production of a number of phosphoinositides, which function as second messengers. Two downstream targets for PI3K activation are known. Protein kinase B (PKB; also known as Akt) [99], and $p70^{S6k}$ [100]. It is known that $p70^{S6k}$ mediates phosphorylation and activation of the 40S ribosomal protein S6, which is necessary for cell cycle progression from G_1 into S phase [101-103]. Thus inhibition of $p70^{S6k}$ could abrogate the uncontrolled proliferation of malignant cells. A class of novel antibiotics, rapamycin and its analogs inhibit $p70^{S6k}$.

Rapamycin Analogues

The progression of growth factor-stimulated mammalian cells from G1 to M phase occurs through precisely timed activation and inactivation of cyclin-associated protein kinase activities. The timing and sequence of activation of each cyclin-cyclin dependent kinase (cdk) complex is controlled in part by cell-cycle checkpoints, which ensure that the appropriate cyclin-cdk complex becomes active only after obligatory earlier events have occurred [70]. The potent immunosuppressive drug rapamycin (sirolimus), a macrocyclic lactone, and its analogues, are products of *Streptomyces hygroscopicus* which inhibit signaling pathways required for T cell activation and proliferation in response to cytokine stimulation. Rapamycin blocks progression of the cell cycle at middle to late G1, in the cytokine -induced proliferation of T cells, B cells, osteosarcoma, and rhabdomyosarcoma cell lines, among others [104-106]. In these systems, rapamycin blocks cell-cycle progression before S phase as measured by quantitation of the cellular DNA. Rapamycin exposure decreases the kinase activity of cdk 2/cyclin E and cdk 4/cyclin D complexes. These kinase activities normally peak in middle to late G1, and their activation involves the dissociation of the cyclin-dependent kinase inhibitors, $p21^{wafl}$ and $p27^{kip1}$, from the complex in activated T cells. $P27^{kip1}$ is a member of the family of endogenous protein cdk inhibitors, which are negative regulators of the cell cycle. In quiescent cells, this protein is present in high concentration and leads to a block of G1 progression. In the presence of cytokine or growth factor stimulation, the $p27^{kip1}$ induced block is overcome through degradation of $p27^{kip1}$ via the ubiquitin-proteasome pathway [107]. In rapamycin treated cells, the down-regulation of $p27^{kip1}$ is blocked, and the cdk/cyclin complexes are not activated. This also prevents downstream events after activation of cdk4/cyclin D and cdk 2/cyclin E complexes, including phosphorylation of the tumor suppressor retinoblastoma protein (pRb), dissociation of the pRb:E2F complex, and increased activity of E2F transcription factors, which are all essential for progression through the G1 phase of the cell cycle [108].

The biochemical effects of rapamycin described above were shown to be reversible by a molar excess of FK506, indicating that these effects are dependent on the formation of a rapamycin-FKBP complex in cells. However, it was shown that the FKBP-rapamycin complex affected neither the p70 S6 kinases, nor the G1 cdks in cell-free systems, indicating that the complex targeted upstream regulators of the enzymes in cells. The target of the FKBP-Rapamycin complex was first identified in yeast and termed the target of rapamycin (TOR). Two proteins, TOR1 and TOR2 were identified. These two proteins have 67% amino acid identity and 80% similarity overall [109]. Currently, the

exact biochemical function of the mammalian target of rapamycin (mTOR) remains undefined. Recent evidence suggests that mTOR has cell cycle regulatory functions and may represent the initial molecular switch in a new pathway of signal transduction. This polypeptide is structurallyrelated to the phosphoinositide kinase PI-3-kinase [110] and has protein kinase activity. These drugs also inhibit the proliferative responses of fibroblasts and several neoplastic cell types to a variety of growth factors including FGF, PDGF and IGF [110].

Out of several derivatives of rapamycin, CCI-779 was selected for further development, based on its favorable anti-proliferative profile *in vitro* and *in vivo*. In studies with human tumor cell lines, prostate and breast cell lines were most sensitive (IC_{50} <10^{-9}M). PDGF-induced proliferation of the human glioblastoma line T98G was also inhibited by CCI-779 (IC_{50} = 0.5-2.0 x 10^{-12}M). *In vivo* activity has been documented against prostate, glioblastoma, melanoma, and colon, breast, and pancreatic carcinoma models in nude mice. Different intermittent dosing regimens of CCI-779 (e.g. daily times five, every 2 to 3 weeks, and single dose weekly) were effective in these animal models. Phase I clinical studies are currently underway with different schedules.

Inhibitors of Protein Trafficking

The heat shock proteins are cellular chaperone proteins, which assist in relocation of proteins from sites of synthesis to sites of action. HSP90 belongs to this family, and participates in multiple signal transduction pathways and is required for the proper function of MAP kinase, the activity of several tyrosine kinases, activity of several transcription factors including p53, retinoid receptors, steroid receptors, and the activity of CDK4 [111,112]. HSP90, one of the most highly conserved members of the cellular chaperone machinery, is found in all mammalian cell lines. It is a predominantly cytosolic cellular protein and makes up 1-2% of total protein in unstressed cells, but increases to 4-6% of the cellular protein under stress. It is the core of a multi-molecular chaperone complex [111], which includes co-chaperone proteins such as p23 [112]. HSP90 functions as a scaffold for this complex.

17-Allylaminogeldanamycin (17-AAG)

Geldanamycin (GA) is a member of the benzoquinoid ansamycin class of agents. The original prototype structure of this class is herbimycin, which contains a unique ring system. Japanese investigators demonstrated in the 1980s that herbimycin could reverse the transformed phenotype of the src-transformed rat kidney cell line. Additional studies have revealed that herbimycin can reduce or inhibit transformation by a large number of tyrosine kinase oncogene products including *erb*B2, among others [113,114]. The development of GA was terminated because of unacceptable hepatotoxicity [115]. 17-Allylaminogeldanamycin (17-AAG), is a derivative of geldanamycin, which is less toxic than GA and was found to be active in breast, melanoma and ovarian mouse xenograft models. 17-AAG treatment also produced a dose-dependent induction of heat shock protein expression, that correlated with inhibition of tumor cell growth. 17-AAG is cytotoxic and/or cytostatic to tumor cells *in vitro,* at concentrations that also reduce cellular levels of Raf-1, $p185^{erbB2}$, mutant p53, and other cell regulatory proteins [116]. Down-regulation of these onco-proteins is thought to be related to the ability of 17-AAG to specifically bind HSP90 and its homologue GRP94, thereby disrupting specific hetero-protein complexes in the cell. As a consequence, decreased stability and impaired cellular trafficking of these critical onco-proteins is observed [117]. As an example of this general phenomenon, 17-AAG decreases *erb*B2 under conditions where overall transcription and translation are barely affected; both the *erb*B2 phosphotyrosine and *erb*B2 protein levels are decreased 4 hours following drug treatment [117]. 17-AAG is undergoing broad phase I trials in North America and Europe, with evaluation of various schedules of administration.

INHIBITORS OF PROTEIN DEGRADATION

The Ubiquitin-Proteasome System

The ubiquitin-proteasome pathway plays an important role in regulating signal transduction pathways through regulation of cell cycle proteins. In this way, this protein degradation system regulates neoplastic growth and metastasis [118]. A number of key regulatory proteins are temporally degraded during the cell cycle by the ubiquitin-proteasome pathway; and the ordered degradation of these proteins is required for the cell to progress through the cell cycle to mitosis. One of the targets of ubiquitin-proteasome mediated degradation is the tumor suppressor p53. Cyclins and the cyclin-dependent kinase inhibitors p21 and p27 are another set of growth regulatory proteins that are

regulated by proteasome-dependent proteolysis [118]. Both p21 and p27 can induce G_1 cell cycle arrest by inhibiting the cyclin D-, E- and A-dependent kinases [119]. In addition, the ubiquitin-proteasome pathway is required for transcriptional regulation. Nuclear factor-κB (NF-κB) is a key transcription factor, whose activation is regulated by proteasome-mediated degradation of the inhibitor protein I-κB [120, 121]. Cell adhesion molecules (CAM) such as E-selectin, ICAM-1, and VCAM-1 are regulated by NF-κB and are involved in tumor metastasis and angiogenesis *in vivo* [122]. During metastasis these molecules direct the adhesion and extravasation of tumor cells from the vasculature to distant tissue sites within the body. As such, tumor cell metastasis will also be limited by the down-regulation of NF-κB dependent cell adhesion molecule expression. Moreover, NF-κB is also required in a number of cell types to maintain cell viability as an anti-apoptotic controlling factor [123]. Inhibiting NF-κB activation by stabilizing the I-κB protein makes cells more sensitive to environmental stress and cytotoxic agents, ultimately leading to apoptosis.

Altered degradation of cell cycle control proteins can result in accelerated and uncontrolled cell division, and promote neoplastic growth and metastasis. Interfering with the temporal degradation of these regulatory molecules by inhibiting the ubiquitin-proteasome system can lead to the inhibition of cell growth and thereby arrest or blunt disease progression in cancer. Taken together, it is clear that inhibitors of the proteasome can act through multiple mechanisms to arrest tumor growth, tumor spread and angiogenesis. The combination of these mechanisms offers a novel approach to treating cancer. Tumor cells that bear multiple genetic defects, including impaired repair mechanisms and faulty cell cycle checkpoint controls, are sensitive to the growth inhibitory actions of proteasome inhibitors both *in vitro* and *in vivo* [124].

PS-341

PS-341 is a small, cell permeable molecule that specifically and selectively inhibits the proteasome reversibly by binding tightly the enzyme's active sites (Ki = 0.6 nM). PS-341 is a modified dipeptidyl boronic acid derived from leucine and phenylalanine. PS-341 inhibits the growth of cultured tumor cells by blocking cell division in the G_2-M phase of the cell cycle, leading to cytotoxicity via apoptosis. PS-341 inhibits the degradation of the wild-type tumor suppressor protein p53, but not mutated forms of this protein. PS-341 stabilizes the cdk inhibitor p21, and inhibits the activation of NF-κB by the stabilization of the inhibitor protein I-κB.

PS-341 demonstrates a unique pattern of activity when screened against the National Cancer Institute panel of 60 human cancer cell lines [125]. In these screens, PS-341 demonstrated growth inhibitory and cytotoxic activity for many human tumor cell types. The average IC_{50} of PS-341 across the 60 cell lines was 3.8 nM. The pattern of growth inhibition and cytotoxicity was unique, suggesting that PS-341 represents a novel class of cytotoxic compounds. Biological activity of PS-341 has been observed in the hollow fiber assay system, as well as the murine Lewis Lung carcinoma model and in two human xenograft models, HT-29 colon tumors and PC-3 prostate tumors. PS-341 is currently undergoing multi-institution Phase I trials. This agent has proven to be tolerable to date. Inhibition of the 20S proteasome has been demonstrated *ex vivo* in patient samples. Based on the importance of the ubiquitin-proteasome system in degrading a host of cellular proteins, there are theoretical reasons to be concerned about significant toxicity if this system is inhibited. To date, however, such concerns have been proven to be unwarranted.

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