Targeting signal transduction in the discovery of antiproliferative drugs

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Derangements in the signal transduction pathways controlling cell growth can lead to cancer. Two areas of growth factor signaling offer novel opportunities for therapeutic intervention: protein–protein interactions and phosphorylation cascades.

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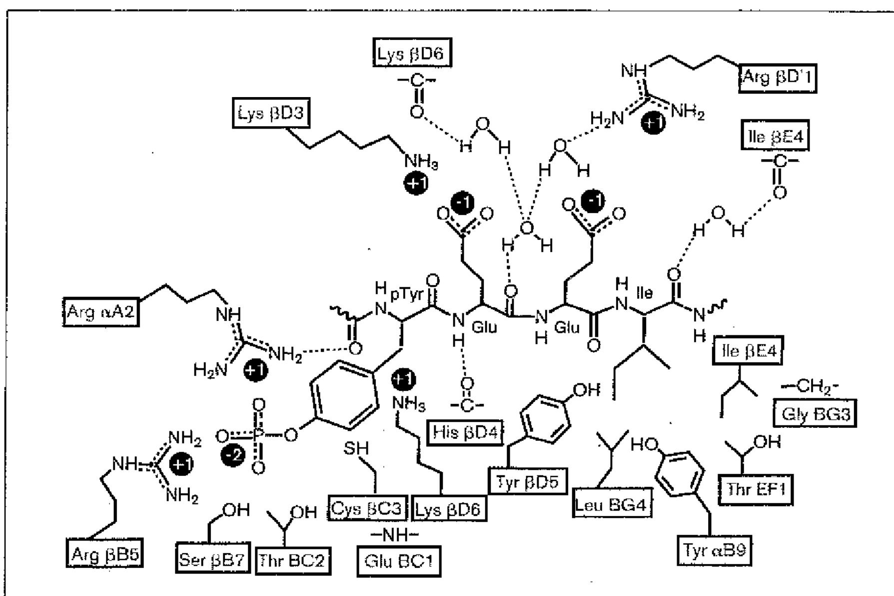
In the past few years, enormous progress has been made in elucidating the cellular events mediating the actions of hormones and growth factors. New intracellular pathways of signal transduction are being discovered at an extraordinary rate, revealing the complicated networks that underlie the hormonal regulation of metabolism, proliferation and differentiation of cells. Occasionally, these regulatory networks cease to function properly, and the subsequent disregulation of signaling pathways can result in disease. Many endocrine and immunological disorders arise from aberrant signal transduction. The most profound effect of the advances in our understanding of signal transduction will undoubtedly be in the area of understanding and treating proliferative diseases, such as cancer.

The intracellular pathways regulating cell growth in reponse to external signals all involve protein phosphorylation. This protein modification is widely used in cell regulation, and usually produces a conformational change that may modify the activity of a protein or its interaction with other molecules. The universal role of protein phosphorylation is seen most prominently in the case of the protein products of proto-oncogenes, the normal cellular counterparts of the oncogenes that are responsible for much or all of human cancer. A growth signal may be delivered to the nucleus either via a pathway triggered by a variety of extracellular stimuli or by the expression of intracellular oncogenes. The pathways used share many elements but each has some distinct features. This has led to the concept that combinatorial diversity of signaling proteins is used to ensure specificity in signal initiation. Upon activation, many growth factor receptors can specifically interact with different intracellular signaling proteins, often through distinct protein-interaction motifs in these proteins, typified by Src homology 2 (SH2) domains. These protein–protein interactions represent the first level of specificity in signal transduction. The next step is the activation of different signaling cascades, including those involving phospholipid metabolism, calcium mobilization, ion channels and protein kinases and phosphatases. Notable among these is the Ras–mitogen-activated protein (MAP) kinase pathway, a major route of signal transduction to the nucleus.

Many of the proteins involved in growth factor signaling were first identified as the products of oncogenes in human or animal tumors. Signal transduction pathways are currently some of the most attractive targets for therapeutic intervention in proliferative diseases. But, because of our lack of experience with pharmacological agents that target intracellular signaling, it is difficult to predict results of the selective blockade of a single pathway. Experiments in tissue culture cells suggest that many signaling mediators are universally utilized by growth factors, cytokines, hormones, neurotransmitters, biogenic amines and other extracellular stimuli. On the other hand, it may be misleading to extrapolate from studies *in vitro* to the physiological setting, since some of these agents do not cause growth *in vivo*, and the pathways may have taken on exaggerated importance in immortalized cell lines. Moreover, signaling pathways involved in growth control tend to be redundant in tissue culture cells, so that the failure of a single pathway may be compensated for by the activity of another. Thus, the selection of targets for drug discovery in proliferative diseases is less than straightforward. And even when the target is selected, difficulties remain. Is it possible to attain the specificity necessary for antitumor agents to be useful without inhibiting normal cell proliferation? Will signal transduction inhibitors merely stop the tumor from growing further, or will they change the behaviour of the tumor cell causing it to die or differentiate?

There is a raging debate about the ultimate usefulness of targeting signal transduction in human disease, which will probably be resolved only after the discovery of potent inhibitors of these pathways. An ideal therapeutic agent for proliferative diseases will have good oral bioavailability and cell permeability, a long half-life and specificity for its target tissue. These requirements indicate that non-peptide molecules that inhibit the function or the expression of an aberrant protein are likely to be most effective. The diversity of signaling pathways suggests many new targets for intervention. A great deal of attention has focused on growth factor receptors, particularly their tyrosine kinase activity [1]. Others have chosen as targets the ultimate effectors in signal transduction, the transcription factors responsible for regulating cell-cycle entry [2], or the cyclin-dependent kinases that regulate progression

Figure 1



Model of pTyr–Glu–Glu–Ile binding to the Src SH2 domain. The intermolecular binding interactions of pTyr–Glu–Glu–Ile with the Src SH2 domain are depicted as suggested from an X-ray crystallographic structure [7]. The binding pockets for the pTyr and P+3 Ile sidechains, as well as possible hydrogen-bonding interactions between the phosphopeptide, SH2 protein and several structural waters, are indicated.

through the cell cycle [3]. In this review we focus on two areas in growth factor signaling that represent opportunities for therapeutic intervention: SH2 domains, as a paradigm for protein–protein interactions, and the MAP kinase pathway, as an example of phosphorylation cascades.

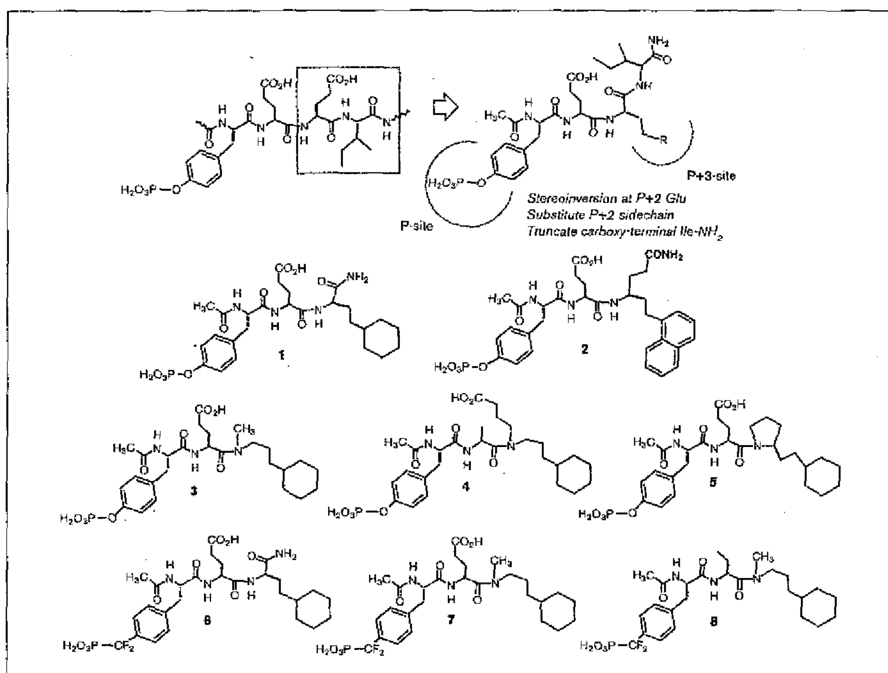
Src homology domains

The discovery of the noncatalytic regulatory regions referred to as Src homology (SH) domains has been critical in 'deconvoluting' interactions between enzymes and their substrates and regulatory proteins and their targets, for a number of signal transduction pathways [4]. SH2 domains bind specific phosphotyrosine (pTyr)-containing proteins, depending on the sequence of the amino acids on the carboxy-terminal side of the pTyr residue. For example, the Src SH2 domain prefers the sequence pTyr–Glu–Glu–Ile, and the adaptor molecule Grb2 prefers pTyr–Tyr–Asn–Tyr [5]. The SH3 domains, on the other hand, specifically bind proline-rich sequences of cognate proteins. Because of the pseudosymmetrical nature of the SH3 domains, target sequences may be bound either in the N→C direction or the C→N direction [6].

X-ray structures of the ligand-bound Src SH2 domain [7] have been used to design the first peptidomimetic antagonists of Src signaling [8]. A molecular map of the tetrapeptide sequence pTyr–Glu–Glu–Ile complexed with Src SH2 domain [7] shows the pTyr-binding pocket and a second binding site for the 'P+3' Ile residue (Fig. 1). Peptide scaffold-based approaches attempting to replace the internal dipeptide, Glu–Glu, with either flexible or rigid linkers [9], have thus far failed to yield potent analogs. However, peptidomimetics have been successfully designed in which stereoinversion at the second residue (P+2) to the D-configuration, and sidechain substitution to hydrophobic functionalities (e.g. cyclohexyl or naphthyl), allows the P+3 Ile sidechain to access its hydrophobic binding pocket [10,11]. Indeed, these compounds showed binding affinities essentially identical to those of the longer phosphopeptides containing the pTyr–Glu–Glu–Ile sequence ([1–3], Fig. 2). Recently, a series of potent peptidomimetics with novel carboxy-terminal functionalization (e.g. with a 'transposed' sidechain of the P+1 Glu or with a conformational constraint introduced by using a pyrrolidine ring; see Fig. 2, compounds 6–8) have been designed based on the structural information [7] and synthesized. Studies focused on the pTyr residue of peptide antagonists of the Src SH2 domain [9] have shown that the phosphate ester is particularly critical for molecular recognition, and that significant loss in binding occurs when it is replaced with sulfate, carboxylate, nitro, hydroxy or amino groups. Substitution of the pTyr residue by difluoromethylphosphonate-(F_2Pmp)-modified analogs is now known to give more stable derivatives ([8,10], Fig. 2), providing another step towards cellularly active 'second generation' compounds.

Recently, high resolution three-dimensional structures for the noncatalytic adaptor protein Grb2 (the apoprotein and its individual SH2 and SH3 domains) have been described [12,13]. An X-ray structure of a phosphopeptide complex provided insight into why Grb2 SH2 prefers to bind pTyr–Xxx–Asn–Yyy sequences. The binding interactions of Lys–Pro–Phe–pTyr–Val–Asn–Val showed that the phosphopeptide adopts a β-turn conformation about the P–P+3 residues (Fig. 3), and that the P+2 Asn sidechain carbox-

Figure 2



Discovery of peptidomimetic antagonists of the Src SH2 domain. The structure-based design of peptidomimetic antagonists of the Src SH2 domain is shown relative to the pTyr–Glu–Glu–Ile parent sequence. Key prototype leads include compounds **1** [10] and **2** [11]. Several 'second generation' compounds are also indicated, including F_2Pmp-modified analogs [8].

amide moiety is extensively hydrogen-bonded to the protein. In contrast to the well-defined binding pocket for the P+3 Ile in the Src SH2 ligand, the P+3 Val engages in limited surface hydrophobic interactions with Grb2 SH2, because the Trp121 residue of Grb2 SH2 sterically blocks the binding pocket.

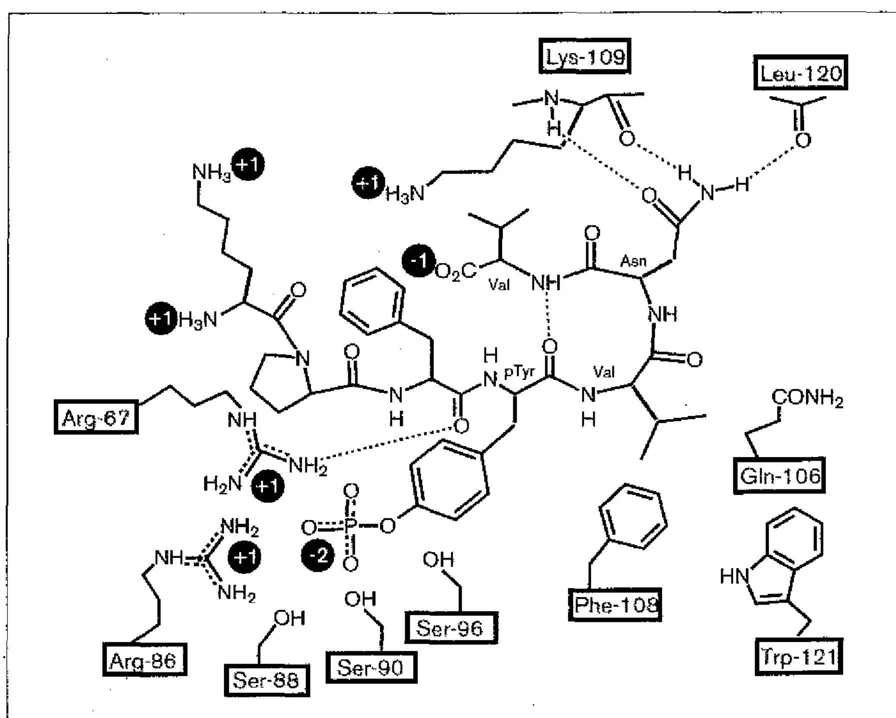
The Ras–MAP kinase pathway in growth factor signaling

Although there was general agreement among investigators in the early 1980s that protein tyrosine kinases are important in growth control, tyrosine phosphorylations were relatively scarce compared to those found on serine and threonine residues in cells treated with growth factors or transfected with tyrosine kinase oncogenes. Many investigators therefore suspected that serine kinases were activated directly or indirectly after tyrosine phosphorylation by growth factor receptors, dramatically amplifying the initial signal in the cell. Although no direct connection between tyrosine and serine protein kinases was found, genetic studies in the yeast *Saccharomyces cerevisiae*, the fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* revealed that the GTP-binding proteins that are members of the Ras family are important in regulation [14]. Ras proteins in these organisms are regulated by functional homologs of mammalian tyrosine kinase systems, suggesting that Ras might mediate the effects of tyrosine kinases on serine kinase activation.

We now know that Ras does indeed provide a link between growth factor receptors and serine kinases. In a sense, Ras functions as an amplifying 'switch' that converts the signals from tyrosine kinases into a language decipherable by serine kinases. In general, tyrosine kinases signal Ras to the 'on' state by promoting its binding to GTP, via the activity of a protein named for its *Drosophila* homolog, Son of sevenless (SOS). The subsequent hydrolysis of GTP to GDP is stimulated by a GTPase-activating protein, or GAP, turning Ras off. Ras proteins are mutated in human cancers. In fact, > 50 % of human tumors contain activating ras gene mutations. Our current understanding of Ras regulation suggests a number of therapeutic targets, both upstream and downstream of the protein. The domains that mediate the interactions of Ras with its inhibitor, GAP, and its activator, SOS, have been localized. Peptides modeled from these regions block these interactions and inhibit Ras function when microinjected into cells. Potent, nonpeptidic inhibitors of these interactions have not yet been developed.

Since many of the oncogenic mutations found in *ras* maintain the protein in the GTP-bound configuration, efforts have focused primarily on inhibiting the already activated forms of the protein. One approach is to block the post-translational attachment of isoprenoid (or prenyl) lipids to the protein, required for Ras to localize at the plasma membrane [15]. This prenylation reaction involves thioether linkages of either farnesyl or the longer geranylgeranyl groups to sequences near the carboxyl terminus of the protein. The enzymes that catalyze these lipid attachments, called prenyltransferases, recognize a specific

Figure 3



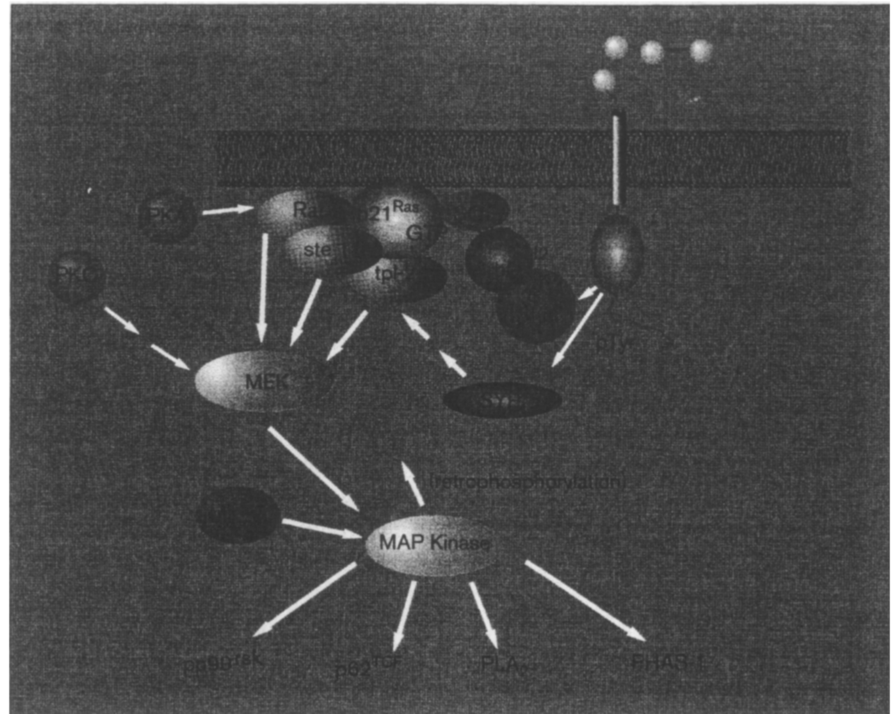
Model of pTyr–Val–Asn–Val binding to the Grb2 SH2 domain. The intermolecular binding interactions of pTyr–Val–Asn–Val with the Grb2 SH2 domain are depicted as suggested from an X-ray crystallographic structure [13]. The binding pockets for the pTyr and the P+1 Val as well as possible hydrogen-bonding between phosphopeptide and SH2 protein as well as intramolecular hydrogen-bonding of the phosphopeptide are indicated.

tetrapeptide motif in the Ras protein, called a 'CAAX' box, in which C represents cysteine, the two A's are aliphatic amino acids, and X is a methionine, serine or leucine residue. Both substrate-based and non-Cys-containing peptide inhibitors [16–18] have been explored. Relative to peptide-substrate structure-based design efforts, peptidomimetics incorporating Psi-[CH_2NH]-substitutions [16] or a benzodiazepine replacement of the central dipeptide moiety [17] have yielded high affinity inhibitors. In another series of inhibitors, the central dipeptide is replaced with various isomeric and/or substituted derivatives of amino-benzoic acid [19], including the biphenyl derivative which is particularly effective [20]. Thus, peptide scaffolds that are conformationally flexible or constrained as well as nonpeptide template replacements can be used to link the Cys and Met substructures. Although some of these compounds have free sulfhydryl groups, there is no evidence that they become farnesylated, suggesting that the substituted peptide inhibitors may bind the target enzyme in the same way as their peptide substrate counterparts. Although preliminary results look promising, it is unclear whether the modification of other prenylated proteins will also be affected. Moreover, since normal Ras also requires prenylation for activation, inhibition of prenylation should also block normal Ras functions.

The complexity of the activation of Ras by tyrosine kinases pales in comparison to its downstream pathway. Again, much of what we have learned about the downstream signals has emerged from studies on yeast, flies and worms [14]. Upon its activation, GTP-bound Ras recruits protein serine kinases to the membrane, where they can be activated. The best characterized of these interactions involves the proto-oncogenic serine kinase Raf [21]. Although the mechanism by which Raf is activated remains unknown, its recruitment by Ras to the plasma membrane is essential. Once activated, Raf can in turn phosphorylate another protein kinase, called MEK or MAP kinase kinase. MEK exhibits sequence homology to the *byrl* and *ste7* gene products of *Schizosaccharomyces pombe* and *S. cerevisiae* [22]. This enzyme, which represents a site of integration from other signaling pathways, is a dual specificity kinase that phosphorylates MAP kinase on both tyrosine and threonine residues, resulting in its activation. Upon activation, MAP kinase [23] can translocate into the nucleus, where it catalyzes the phosphorylation of transcription factors such as $p62^{TCF}$, initiating a transcriptional program that leads the cell to commit to proliferation or differentiation (Fig. 4). MAP kinase can also phosphorylate a number of other proteins involved in cellular signaling, including other kinases and phospholipases.

At first glance, this phosphorylation cascade, known as the MAP kinase pathway, appears to be a linear trail connecting Ras to the nucleus. In reality, however, the circuitry is quite complex, and this pathway interacts extensively with other phosphorylation cascades that are used in a diverse set

Figure 4



Regulation of the MAP kinase pathway by growth factors. A scheme is depicted for the molecular interactions involved in the activation of MAP kinase. MEK (MAP kinase kinase) is activated by Raf once Raf has been recruited to the plasma membrance by activated Ras. MEK phosphorylates MAP kinase, activating it and allowing it in turn to phosphorylate a number of downstream targets such as: $pp90^{rsk}$; $p62^{TCF}$, which leads to commitment to differentiation or proliferation; phospholipase A_2 (PLA_2) or PHAS-1; PKA, protein kinase A, PKC, protein kinase C, SYP.

of responses to extracellular stimuli, ranging from mating responses in yeast to stress and mitogenic responses in higher eukaryotes. These complex pathways offer numerous opportunities for integration of diverse stimuli into a single response, yet also allow for the specificity, feedback and crosstalk that are the hallmarks of signal transduction.

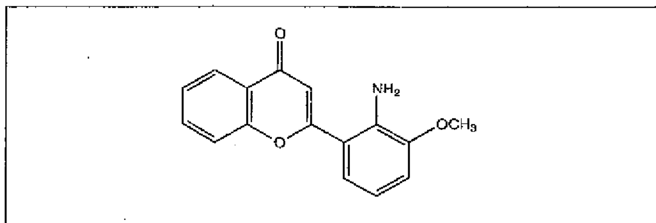
MAP kinase itself absolutely requires both threonine and tyrosine phosphorylation for activation, making it one of the most tightly regulated enzymes in the signaling process. It can be activated only by MEK, but can be inactivated by serine phosphatases, tyrosine phosphatases, and a dual specificity phosphatase called MKPP, or MAP kinase phosphatase [24]. MEK is one of the unusual enzymes in the signaling pathway. This dual specificity kinase exhibits astonishing substrate specificity for a protein kinase, phosphorylating only members of the MAP kinase family. MEK itself is activated by phosphorylation on serine residues. Although Raf is the best characterized of the MEK kinases, there are others that may serve to integrate growth signals from different stimuli [25].

A number of sites in the MAP kinase pathway are amenable to therapeutic intervention. Agents that inhibit these enzymes might be especially useful in targeting tumors that contain activating Ras mutations, since Ras is directly upstream of these enzymes and they are essential for the propagation of the Ras pathway. One example of a compound that affects the MAP kinase pathway is PD98059 (Fig. 5). This is a kinase inhibitor which is non-competetitive for ATP and blocks MEK activity and the subsequent stimulation of MAP kinase without inhibiting any other protein or lipid kinases [26]. The precise mechanism by which this compound blocks MEK activity is still unknown, but it appears to be an allosteric regulator of MEK, preventing its phosphorylation and subsequent activation through a novel regulatory site on the enzyme [27].

PD98059 blocks growth-factor-dependent activation of MAP kinase, inhibits DNA synthesis in a variety of cell types and prevents neurotrophin-induced differentiation of PC-12 cells. It can revert Ras-transformed fibroblasts to a normal phenotype, suggesting that blockade of this pathway might be useful in preventing the progression of tumorigenesis [26]. In contrast to the effects of the MEK inhibitor on growth, it has no effect on metabolism, for example the regulation of carbohydrate and lipid metabolism by insulin [28]. As insulin signals through a tyrosine kinase receptor, it is clear that, although many hormones can activate the MAP kinase pathway, it is not always used for transduction of the relevant signal.

In addition to its antiproliferative effects [26], PD98059 also prevents the MAP kinase-mediated phosphorylation of other cytoplasmic substrates in some cells, including other protein kinases, phospholipase A_2, and even the upstream signaling proteins known to be targets of desensitization.

Figure 5



Structure of PD98059, an inhibitor of MEK.

For example, PD98059 blocked the growth-factor-induced phosphorylation of SOS, causing it to uncouple from Grb2, reducing the further activation of Ras [29]. Thus, in normal cells, the activation of the MAP kinase pathway leads to a desensitizing retrophosphorylation that prevents the persistent activation of the Ras protein, perhaps explaining in part why growth factor activation does not generally lead to tumors.

There may also be a second way in which the consequences of MAP kinase pathway activation are controlled. In the case of neuronal differentiation, the kinetics of MAP kinase activation appear important in determining whether a cell commits to differentiate. In general, prolonged activation and nuclear translocation of MAP kinase is associated with neurite outgrowth in PC-12 cells; mitogenic stimuli that activate MAP kinase transiently do not produce neuronal differentiation. Similarly, when 3T3 cells are engineered to respond to NGF by causing them to express the trkA NGF receptor they show growth arrest and differentiation in the presence of NGF. These responses are associated with induction of the cyclin-dependent kinase inhibitor $p21^{cip1/Waf1}$, and the subsequent down-regulation of CDK4 activity (K Pumiglia and SJ Decker, personal communication). The effect can be blocked with PD98059, suggesting that it is the sustained activation of MAP kinase induced by NGF that is responsible for the dramatic change in the phenotype of the cell. Presumably MAP kinase acts via the phosphorylation of proteins, perhaps the products of immediate early genes.

Development of signal transduction inhibitors

Signal transduction is a particularly attractive target for research aimed at discovering antiproliferative agents because it is the site of the fundamental lesion leading to uncontrolled cell growth. Such agents should be highly specific, avoiding the toxicities associated with standard cytotoxic agents, which, in general, kill proliferating cells without distinguishing between normal and pathological growth. Thus, specificity must be constantly monitored throughout development. Traditionally, agents progress along a developmental path that increases in complexity, cost and potential pitfalls over time. It may be possible to streamline much of this process when developing inhibitors of signal transduction. All of the pathways described above involve fairly well-characterized proteins for which the genes have been cloned. These proteins have already been the focus of a great deal of structural and functional analysis, and can usually be expressed in large quantity. They are therefore amenable to *in vitro* biochemical assays *via* rational, structure-based design, or random screening of libraries of low molecular weight peptidomimetics or other organic molecules. Agents active in such screens can be evaluated in counter-screens devised to detect undesirable activity. For example, inhibitors of the Grb2 SH2 domain would be counter screened with a series of irrelevant SH2 domains, to evaluate the specificity of the interaction.

Active and highly specific compounds that emerge from biochemical assays are usually evaluated further in cellular assays in tissue culture cells. For example, a MEK inhibitor can be evaluated for its ability to prevent the growth-factor-dependent activation of MAP kinase in fibroblasts. Such an assay will reveal whether a compound can traverse the cell membrane, and will confirm its efficacy and specificity. Once a compound that specifically blocks a pathway has been found, it is possible to test how important that pathway is in cell proliferation. Initial evaluations are likely to be carried out in a well-established model system, such as a mouse fibroblast cell line, followed by a panel of human tumor cells. Such 'tertiary' assays may include DNA synthesis, cell proliferation, cell survival, colony formation, cell-cycle progression or monitoring of morphological markers in cells treated with growth factors or transformed with viral oncogenes.

Although cellular assays are unlikely to be good predictors of clinical efficacy for antiproliferative agents, it may be possible to resolve a number of issues regarding the biology of a tumor type at this level of investigation. Indeed, there are far more questions than answers, since results are not yet available from experiments exposing tumors to signal transduction inhibitors. Will transformed cells develop resistance to such agents? Will signal transduction inhibitors synergize with or sensitize tumors to traditional cytotoxic drugs? Will the blockade of growth factor/oncogene signaling induce cells to undergo apoptosis or differentiation? Will the blockade of growth factor signaling pathways lead to clinically unacceptable side effects?

Since signal transduction is a novel and untested target for the discovery of antiproliferative drugs, signal transduction inhibitors will require nontraditional approaches to development. The clinical efficacy and toxicity of such compounds remain completely unexplored and are difficult to predict for these compounds, like all chemotherapeutic agents. It is possible, even likely, that the successful use of such mechanism-based therapies will be highly individualized, perhaps dependent not only on tumor cell type, but also on specific determinants of the molecular lesions

present in a given patient's cancer. As for all aspects of drug development, there are many hurdles and uncertainties to overcome. Nevertheless, the fact that cancer is now known to result from derangements in the signal transduction pathways controlling cell growth means that we are now able to focus the attack where the problem arises.

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