The Biology Behind

Introduction

Protein kinases are currently a major focus for cancer drug discovery because of their integral roles in cellular transformation, growth control, metastasis, and angiogenesis. Indeed, >30 kinase inhibitors are currently in clinical trials (1), and ongoing drug discovery efforts will add significantly to the list of novel targeted therapies for clinical evaluation in the coming years. A successful, efficient, and rational strategy for clinical evaluation of these new agents may benefit from adapting a new paradigm. Initial Phase I studies should incorporate direct “on target” measures of drug action to determine the maximum “biological effective” dose rather than a traditional maximum tolerated dose. Ideally, Phase II patients may then be selected for treatment based on phenotypic characteristics such as expression of the drug target, identification of activating mutations in the signaling pathway, or other markers associated with tumor stage and histology. In this issue, Peralba et al. (2) propose measurement of p70s6 kinase activity in PBMCs from study subjects as one strategy to guide rational development of CCI-779, an ester of rapamycin and inhibitor of the mTOR protein kinase. The rationale for this study, identification of mTOR and elucidation of a complex cellular sensing and growth regulation system, originated >25 years ago. Three natural product molecules, cyclosporin A, a cyclic peptide, and the structurally related macrolides FK506 and rapamycin, are currently approved agents for use in prevention of allograft graft rejection. Their macrolides FK506 and rapamycin, are currently approved cyclosporin A, a cyclic peptide, and the structurally related immunophilins, mTOR, and Pharmacodynamic Strategies for a Targeted Cancer Therapy


Matthew W. Harding
Vertex Pharmaceuticals, Inc., Cambridge, Massachusetts 02139

Immunophillins, Protein Folding, and Immunosuppression

Rapamycin, a natural product macrolide, was isolated in the early 1970s and evaluated initially for antifungal activity (3); however, subsequent studies revealed that rapamycin also blocked proliferation of normal lymphoid cells and tumor cell lines (4). Cyclosporin A was also initially characterized in the mid-1970s (5). The relatively selective immunosuppressive activity of CsA was shown to result from inhibition of Ca²⁺ -dependent events in T-cell activation and transcription of cytokines, including IL-2, granulocyte macrophage colony-stimulating factor, and IFN-γ (6). A report in 1987 described FK506 and its effects on T-cell activation that were identical to CsA (7). Despite its similar structure, the activity of rapamycin appeared to result from inhibiting IL-2-dependent G₁ cell cycle progression (8). Although the mechanisms for CsA, FK506, and rapamycin immunosuppression were initially described at a functional level, the biochemical target molecules for these agents were unknown.

In 1984, Handschumacher et al. (9) reported the identification of cyclophilin as a cytosolic binding protein for CsA. The binding affinity of cyclosporine analogues for cyclophilin correlated with inhibition of T-cell activation in vitro. That same year, Fischer et al. (10) described a Mₛ ~18,000 protein that catalyzed the interconversion of cis- and trans-amide bonds adjacent to proline residues in peptide substrates and termed this activity PPIase. Subsequent studies demonstrated that the PPIase protein and cyclophilin were the same molecular entity, and that CsA inhibited its PPIase activity (11, 12). A possible role for PPIases in immunosuppressive drug action became more intriguing when two groups reported isolation of FKBP12, another cytosolic protein, and demonstrated that both FK506 and rapamycin inhibited its PPIase activity (13, 14).

The observation that CsA, FK506, and rapamycin all bound to and inhibited a PPIase fueled research in this area. At present, the cyclophilins and FK506 binding protein comprise two families of “immunophillins,” and these proteins are highly conserved across species (reviewed in Ref. 15). At least six human proteins exist in each family, and members are present in distinct cellular locations including the cytosol (cyclophilin A and FKBP12), the endoplasmic reticulum (cyclophilin B and FKBP13), nucleus (FKBP25, FKBP52), and mitochondria (cyclophilin D). The immunophillins appear to function as accessory helper enzymes or folding chaperones that provide functional stability to multiprotein macromolecules. PPIase activity and prolyl isomerization may alter protein conformations and the spatial arrangement of protein domains. Specific immunophillins have been identified in association with Ca²⁺ release channels (FKBP12 with ryanodine receptor and inositol 1-phosphate receptor), receptor kinases (FKBP12 with the transforming growth factor β R-1 subunit), and in steroid receptor complexes (cyclophilin 40, FKBP51, and FKBP52).

However, the role of cyclophilin and FKBP12 in immunosuppressive drug action involves more than simply PPIase in-
Hibitation. Studies with CsA, FK506, or rapamycin analogues identified molecules that were potent PPlase inhibitors but weak immunosuppressants, and investigations concluded that PPlase inhibition was necessary but not sufficient for drug action. Studies with one synthetic analogue in particular (506BD, a truncated FK506/rapamycin mimetic, is one example; see Ref. 16) supported the hypothesis that immunosuppressive action resulted from a “gain of function” after immunophilin binding. Indeed, studies using immunophilin fusion protein affinity columns isolated the calmodulin-dependent protein phosphatase calcineurin and identified it as the target of both the cyclophilin-CsA and FKBP12/FK506 protein drug complexes (Ref. 17; Fig. 1). Additional investigation identified NFAT as the primary calcineurin substrate (nuclear factor κB and Oct-1 are others). CsA and FK506 inhibit calcineurin-dependent dephosphorylation and nuclear translocation of NFAT preventing transcription of early T-cell activation genes (18). Rapamycin had no effect on calcineurin function, and identification of a target for cyclophilin-CsA and FKBP12-FK506 complexes suggested the possibility of a similar target for the FKBP12-rapamycin complex.

**Identification and Function of TOR Proteins**

Progress in defining the molecular action of rapamycin began in yeast. Genetic studies identified two genes (TOR1–1 and TOR2–1) with mutations that prevented binding of FKBP12-rapamycin complexes and conferred resistance to growth inhibition (19). Subsequent biochemical studies with FKBP12-rapamycin affinity probes identified a mammalian homologue, mTOR (20, 21). mTOR is a large (Mr ~289,000) multidomain serine/threonine kinase (Fig. 2). It is a member of the PI3K family of protein kinases based on homology within its catalytic domain. The NH3-terminal region of TOR proteins contains multiple repeat HEAT motifs that are proposed to mediate protein-protein interactions. The “toxic” or FAT domain is also common to PI3K family members, but its function has not been defined, and a COOH-terminal repressor domain appears to be unique to mTOR. The FKBP12-rapamycin complex binds within the FAB domain in mTOR adjacent to the COOH-terminal catalytic kinase domain. Signals that activate mTOR have not been clearly elucidated, and this subject is presently controversial. Phosphorylation by Akt has been proposed, whereas interaction of proteins with HEAT motifs is another possible regulatory mechanism, and it may be that mTOR is a constitutively active enzyme.

An understanding of the function of TOR is most advanced in yeast (22, 23). TOR proteins are “sensors” that appear to control cell growth and proliferation based on amino acid and nutrient availability or growth factor stimulation via regulation of translation and transcription (Fig. 3). The p70s6 kinase and the translation inhibitor 4E-BP-1 are the two best-characterized mTOR substrates (24). Growth factor activation of the PI3K

---

**Fig. 1** Model of immunosuppressive drug action in lymphocyte activation. CsA and FK506 interact with the immunophilins cyclophilin A or FKBP12, respectively. The protein-drug complexes inhibit calcineurin-dependent dephosphorylation of p-NFATc in the cytosol, preventing its nuclear translocation and transcription of early T-cell activation genes. In contrast, FKBP12-rapamycin complexes inhibit mTOR kinase preventing IL-2 signaling and cell cycle progression from G1 to S.

**Fig. 2** Domain structure of the mTOR protein kinase.

Downloaded from clincancerres.aacrjournals.org on October 2, 2017. © 2003 American Association for Cancer Research.
pathway results in phosphorylation and activation of p70s6 kinase by mTOR or PDK-1. Two p70s6 kinase isoforms have been identified, and multiple phosphorylation sites appear to regulate enzyme activation (Fig. 4). A series of COOH-terminal Ser/Thr residues are phosphorylated after mitogen stimulation, and phosphorylation of these sites is sensitive to rapamycin. However, at least one site in each isoform (Ser371 or Ser370) appears to be rapamycin insensitive. Specific mTOR sites in the regulatory and catalytic domain include residues Thr389/388 and T229/228, and the later site also appears to be phosphorylated by PDK-1. Although mTOR and rapamycin-sensitive sites have been identified, regulation of p70s6 activity appears to be complex and involve other kinases.

Activation of p70s6 kinase results in phosphorylation of the S6 protein in the 40S ribosome subunit enhancing 40S translation to polysomes and a preferential increase in the translation of TOP mRNAs. Approximately 15–20% of all of the mRNAs have the 5’-TOP structural feature and 5’-TOP mRNAs typically code for ribosomal proteins, initiation, and elongation factors, and other components of protein synthesis machinery. In quiescent cells, 4E-BP-1 is either not or only partially phosphorylated, and in this form, it associates with inactive eIF4E. Growth factor stimulation results in sequential phosphorylation of four sites including mTOR “priming” sites (T37 and T46), and S65 and T70 “release” sites, which may be phosphorylated by mTOR or other mitogen-activated kinases.
Once released, active eIF4E binds to the m7GpppN cap structure of another set of specific mRNAs that have a highly ordered secondary structure (e.g., mRNAs encoding growth factors, cyclin D1, c-myc, and certain receptors) to promote their efficient translation.

CCI-779, Pharmacodynamics, and p70s6 Kinase Activity

CCI-779 is an analogue of rapamycin that incorporates a bis-hydroxymethyl-propanoic acid ester. Like rapamycin, CCI-779 binds to FKBP12 and is an mTOR inhibitor with potent antitumor activity in vitro and in xenograft models (25). Initial Phase I studies evaluated CCI-779 administered as a 30-min infusion weekly (dose range, 7.5–220 mg/m²) or daily for 5-days (daily dose of 0.75–24 mg/m²), with pharmacokinetics and safety as traditional endpoints. Reported toxicities were generally mild, and evidence of antitumor activity was observed over a broad range of the doses examined (summarized in Refs 1 and 26). Phase II studies proceeded testing several doses of CCI-779 (25, 75, and 250 mg/m²), because an maximum tolerated dose had not been clearly established, and early studies did not include a marker to identify a “biologically active” dose.

In this issue, Peralba et al. (2) present initial data evaluating p70s6 kinase activity as a marker of CCI-779 drug action. The rationale and logic are very strong: PBMCs are easy to collect, p70s6 kinase is constitutively active in PBMCs, and it is a direct substrate for mTOR. The assay method uses well-characterized reagents and is feasible for routine analysis including rapid isolation of PBMCs from whole blood, snap freezing of cell pellets, immunoprecipitation of p70s6, and measurement of kinase activity with a p70s6 RSK peptide substrate.

The p70s6 kinase assay was demonstrated to be linear and exceptionally sensitive (IC₅₀ ~0.5 nm) with Raji cell in vitro. Inhibition of p70s6 kinase activity was similar in mouse PBMCs and in MDA-468 breast cancer xenograft tissue extracts at 24 and 72 h after a single 10 mg/kg dose of CCI-779. However, no pharmacokinetics data were presented to correlate with the extent and duration of p70s6 kinase inhibition. In PBMCs isolated from normal humans, p70s6 kinase activity was measurable with adequate reproducibility (40% coefficient of variation and 14% intra-subject variability) over a 1-week interval. Measurement of p70s6 kinase activity was then monitored in PBMCs from RCC patients after single doses of 25, 75, and 250 mg/m² CCI-779 (27). Maximum suppression of p70s6 kinase activity was achieved at the lowest dose tested (25 mg/m²), and suppression of p70s6 kinase activity was sustained for at least 8 days and perhaps longer. Although only nine subjects were evaluated with this method, a linear relationship was observed between the degree of p70s6 kinase suppression in PBMCs and the time to tumor progression among these RCC patients.

These interesting results raise a few questions about CCI-779 and also identify some of the challenges in adapting pharmacodynamic assessments for targeted therapies. First, there are practical questions about CCI-799 and p70s6 kinase with regard to the optimal dose and administration schedule. On the basis of the PBMC p70s6 kinase activity data, the maximum biological effect of CCI-779 may be achieved at ~25 mg/m², and it is possible higher doses may not provide more significant clinical activity. Indeed, the antitumor activity of CCI-799 in the RCC Phase II study (partial, minor responses, stable disease) was similar for the 25, 75, and 250 mg/m² dose groups (27), and other studies have also reported antitumor activity over a broad dose range (1, 26). Secondly, the long duration of p70s6 suppression is consistent with the activity of the weekly administration schedule. However, it is surprising that a normally constitutive and dynamic enzyme such as p70s6 could be inhibited for an extended period without significant toxicities or effects on normal cell types. Perhaps in situ variability or a low level of active p70s6 is sufficient to keep normal quiescent cells viable.

The next question to ask is whether or not p70s6 activity is sufficient as a single “on target” pharmacodynamic marker. Considering the complexity of the mTOR pathway and p70s6 regulation, incorporation of a second target-related marker would be a valuable addition to support the pharmacodynamic analysis. Antibodies against phospho-T70 of 4E-BP-1 have been described (28), and an immunoblot method could be adapted as a confirmatory measure of mTOR inhibition in PBMCs. Development of an immunohistochemical method for phospho-T70 4E-BP-1 would also be useful to assess mTOR activity in tumor tissue. Alternatively, a change in the abundance of 5’TOP or m7GpppN cap mRNA translation products (measured by ELISA or a proteomics analysis) could also provide additional evidence of mTOR inhibition in PBMCs or tumor tissue. Finally, the major question for this or any other method and strategy is whether or not the target activity marker in the surrogate normal tissue is representative of its activity in the tumor. Regulation of p70s6 kinase in resting, normal PBMCs is likely to differ from tumor cells, which by definition do not have normal cell growth control mechanisms. Indeed, there are reports of cell lines that are resistant to mTOR inhibition and proliferate despite reduced p70s6 kinase activity or 4E-BP-1 hypophosphorylation (26, 29, 30). In a clinical trial setting, it is often not possible to obtain biopsies from patients in multiple dose groups; however, data comparing drug action on the target activity marker in surrogate and tumor tissue from a limited set of subjects is necessary to validate the method and strategy.

In conclusion, the PBMC p70s6 kinase assay reported by Peralba et al. (2) is a feasible and sufficiently robust method for pharmacodynamic analysis of CCI-779. The authors note that studies in progress will additionally evaluate dose and schedule relationships, and establish a stronger correlation of p70s6 inhibition in PBMCs with clinical outcome. Therapeutic agents targeting protein kinases will present unique challenges for development of methods and strategies for target-based pharmacodynamic analysis. The new drug candidates are likely to be the best tools for dissection of complex signaling pathways and identification of the key pharmacodynamic markers. Analysis of drug action on target markers will benefit early development and guide selection of the optimal biologically effective dose and schedule for disease-directed Phase II studies. The success of this approach is dependent on the selectivity of the markers for the drug target, and our ability to characterize and validate their functions in surrogate and tumor tissue in preclinical models, and then in normal human cells and tumor specimens.
References


Matthew W. Harding

Cite this article


Updated version

Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/8/2882

Cited articles

This article cites 27 articles, 8 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/8/2882.full#ref-list-1

Citing articles

This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/9/8/2882.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.