Commentary

Linking molecular therapeutics to molecular diagnostics: Inhibition of the FRAP/RAFT/TOR component of the PI3K pathway preferentially blocks PTEN mutant cells *in vitro* and *in vivo*

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Molecular therapeutics, targeting the underlying defects leading to cancer initiation and progression, are the "holy grail" of cancer research, translation, and therapy. This quest has taken a major leap forward with the demonstration that STI571 (Gleevec) induces clinical remissions in over 90% and molecular remissions in 10–20% of patients with IFN-refractory chronic phase chronic myelogenous leukemia (CML). The efficacy of STI571 in CML has been shown to be caused by the requirement of the bcr/abl fusion protein, unique to CML, for its initiation and progression, requiring molecular diagnostics to identify sensitive patients. A plethora of molecular therapeutics targeting signal transduction pathways are under evaluation. Despite the presence of the target in normal cells, the drugs have, in general, been remarkably nontoxic as compared with conventional chemotherapy or radiation therapy (see Fig. 1). As expected, these agents exhibit little, if any, activity in tumors where the target is not amplified or activated. Thus individualization of therapy driven by effective molecular diagnostic approaches to determine the status of the targets in patients' cancers is as essential a component of a molecular therapeutics program as is the identification and validation of new targets or the development of novel targeted drugs.

CCI-779, a homolog of the macrolide antibiotic, rapamycin, which exhibits better pharmacologic characteristics than rapamycin, has been under evaluation as an anticancer agent (1, 2). Although rapamycin and CCI-779 exhibit activity against multiple tumor cell lines *in vitro* and *in vivo*, it has not previously been possible to predict which tumors will respond to the effects of rapamycin based on cell lineage or the presence of specific genetic abnormalities. In this issue of PNAS, two manuscripts (3, 4) demonstrate that CCI-779 decreases the growth of tumors containing mutations in the PTEN (a.k.a. TEP and MMAC1) tumor suppressor gene *in vitro* and *in vivo*. Thus molecular diagnostics that can identify tumors with abnormalities in PTEN function may predict response to CCI-779, identifying a population of patients likely to benefit from therapy.

The effects of CCI-779 on growth of PTEN$-/-$ embryonic stem cells or on spontaneous tumors in heterozygous PTEN$+/-$ mice were maintained throughout the course of treatment. Tumor growth, however, resumed after cessation of therapy, indicating that CCI-779 was cytostatic. In addition to inducing a G_1 arrest, rapamycin can induce apoptosis, a process that can be linked to the presence of an aberrant G_1 checkpoint caused by loss of p53 or p21 function (2, 5). The effects of rapamycin on apoptosis also can be revealed by concurrent treatment with conventional chemotherapy or radiation (6, 7). It may thus be possible to design therapeutic combinations with CCI-779 that will convert the cytostatic effects of CCI-779 to a cytotoxic effect. An alternative paradigm is long-term therapy, converting cancer from a rapidly lethal condition to a chronic disease.

Rapamycin and CCI-779 also may demonstrate therapeutic efficacy that is independent of direct tumor cytostasis or apoptosis. At least one cell line, which is resistant to rapamycin *in vitro*, is sensitive to rapamycin *in vivo* (7). Further, rapamycin has been demonstrated to inhibit the proliferation of endothelial cells, production of neovascularizing factors, and the production and activation of proteinases, suggesting that rapamycin may inhibit neoangiogenesis or tumor-stromal interactions *in vivo* (2, 8, 9).

Rapamycin and CCI-779 selectively bind FKBP12, inhibiting the activity of the TOR/RAFT/FRAP (TOR, target of rapamycin, ref. 10). Inhibition of TOR interferes with the activation of its downstream targets, p70S6 kinase and 4E-BP, resulting in decreased translation of mRNAs with a 5′ terminal oligopolypyrimidine tract and CAP-dependent translation, respectively. TOR is a member of the phosphatidylinositol 3-kinase (PI3K) protein super family of serine threonine kinases. This family includes the catalytic subunits of PI3K, the ataxia telangiectasia mutated gene, and the related ATR protein, the PAF400 and TRRAP components of the histone acetylase complex, and the DNA-dependent protein kinase (11, 12). PI3K has the unique additional ability to phosphorylate membrane phosphatidylinositols on the 3 site of the inositol ring.

PTEN originally was identified by the late Peter Steck and Ramon Parsons by its localization at a site of loss of heterozygosity and deletions on chromosome 10q23 in human cancers (13, 14). It was simultaneously identified as a transforming growth factor β-regulated gene by Li and Sun (15). PTEN selectively dephosphorylates the same site in membrane phosphatidylinositols phosphorylated by PI3K, an activity that is clearly linked to the tumor suppressor activity of PTEN (11, 12). PTEN also can dephosphorylate proteins and polyphosphoinositols. The roles of the latter processes in the tumor suppressor role of PTEN are less well characterized.

The PI3K pathway is constitutively activated in cells with abnormalities in PTEN. Lack of functional PTEN results in a cell autonomous accumulation of phosphatidylinositol 3,4,5P3, which leads to the recruitment to the membrane and activation of a subset of pleckstrin homology, Phox, and C1 and C2 domain-containing proteins (11, 12). Introduction of a wild-type PTEN gene into cancer cell lines lacking functional PTEN protein decreases signaling through the PI3K pathway as indicated by a decrease in phosphatidylinositol 3,4,5P3 levels and alterations in downstream events including activity, phosphorylation, or localization of

See companion articles on pages 10314 and 10320.

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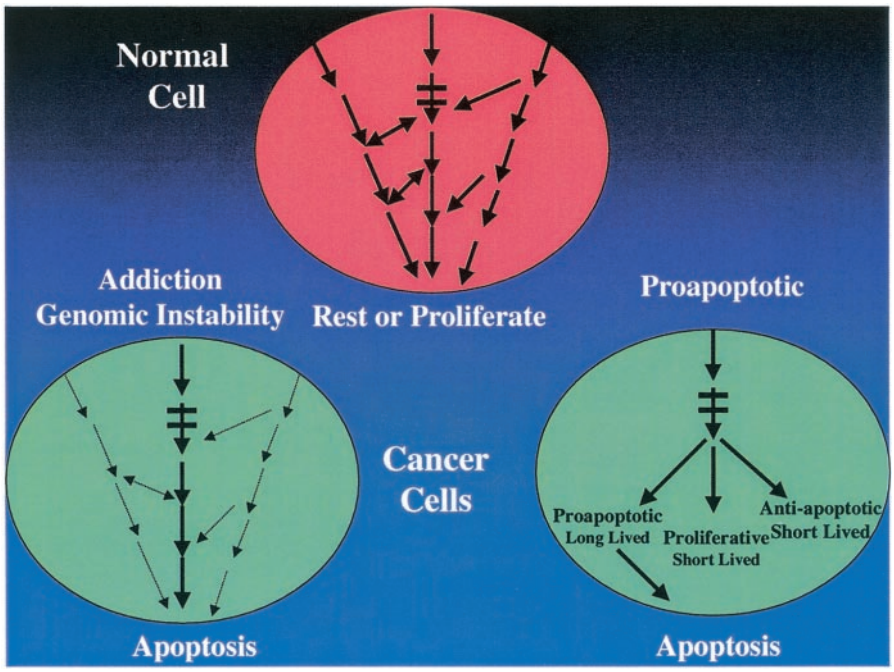

Fig. 1. Normal cells have multiple functional signaling pathways. These signaling pathways exhibit a complex array of cross connections. When a specific signaling event is acutely inhibited, normal cells likely have multiple mechanisms available to bypass the effects of the blockade. Indeed, the lack of phenotype or modest phenotype in many knockout mice is compatible with this model. Alternatively, normal cells may be able to enter a resting state when specific signaling pathways are inhibited. In the addiction or genomic instability model, the genomic instability that is necessary for tumor initiation and progression may contribute to a loss of alternative signaling pathways. When a cell is driven by a specific molecular event, there may be little selective pressure to maintain additional signaling pathways contributing to the loss. Thus acute inhibition of the driving pathway with molecular therapeutics may result in growth inhibition and apoptosis. In the proapoptotic model, growth stimuli may both induce proapoptotic and antiapoptotic signals. This may also explain the counterintuitive increase in proapoptotic molecules in multiple tumors. In the presence of signal transduction inhibitors, production of both antiapoptotic or proapoptotic signals is blocked. If the proapoptotic signal is long-lived or irreversible, the tumor cell would then undergo apoptosis. This model is compatible with the observation that introduction of multiple different oncogenes renders cells more sensitive to inhibitors of those oncogenes than parental cells. Indeed as discussed herein, introduction of activated PI3K or AKT renders cells sensitive to the effects of CCI-779 and in the case of PI3K, LY294002. These differential effects may provide the therapeutic index that allows molecular therapeutics to be successful in cancer management.

AKT, PDKI p70S6K, GSK3-α, GSK3-β, Bad, 4E-BP1, β-catenin, NFκB, and Forkhead, increased expression of p27Kip1, decreased cyclin D1 and myc expression, and HIF1 stabilization (11, 12). Each of these proteins plays a role in the regulation of cell cycle progression, cell differentiation, or apoptosis. Indeed, PTEN can induce growth arrest or apoptosis in cells with aberrant PTEN dependent on the presence of exogenous growth factors or ligation of integrins (16). PTEN efficiently augments anoikis, apoptosis that occurs after detachment from the extracellular matrix, suggesting that PTEN regulates the metastatic cascade (16). Many of the effects of PTEN can be ameliorated by activated AKT, suggesting that AKT may be a major downstream target for the function of PTEN. Importantly, enforced expression of high levels of PTEN in cells with normal PTEN function has only modest effects on cell signaling, proliferation, or viability (11, 12). This finding suggests that drugs targeting the PTEN signaling pathway may have limited effects on normal cells, decreasing their toxicity. In support of this contention, CCI-779, at doses that decreased the proliferation of PTEN mutant tumors in mice, did not demonstrate deleterious side effects (3, 4).

PTEN is functionally compromised in advanced tumors from a number of different cell lineages, suggesting a function in tumor progression. The development of cancers in individuals with germ-line mutations in PTEN, in mice with mutant PTEN, as well as a high frequency of PTEN mutations in endometrial hyperplasia suggests that PTEN also may play a role in tumor initiation (3, 4, 11–15). However, even in lineages where PTEN mutations are relatively frequent, only a portion of tumors exhibit aberrant PTEN function requiring molecular diagnostics to identify the full spectrum of patients whose tumors are likely to be responsive to molecular therapeutics targeting the PTEN pathway. As PTEN function can be compromised by multiple mechanisms, the development of molecular diagnostics to identify the status of PTEN in patient tumors may prove complex. Somatic PTEN mutations are found in approximately half of gliomas, and prostate, endometrial, and endometrioid ovarian cancers (11–15). Loss of heterozygosity of PTEN and silencing of the remaining allele is a frequent event in melanoma, leukemia, prostate, and endometrial cancers (17–19). In addition to methylation-dependent silencing, PTEN is functionally inactivated in a high proportion of leukemia and lymphomas by methylation-independent mechanisms (20). PTEN protein stability and enzyme activity can be regulated by posttranslational modification including serine, threonine, and tyrosine phosphorylation (refs. 21 and 22; data not shown). A global molecular diagnostic approach assessing the function of the PTEN-regulated signaling pathways in cancers isolated directly from the patient will be necessary to identify the complete spectrum of tumors that may respond to CCI-779.

In the studies in this issue of PNAS, mutation in PTEN was associated with phosphorylation, activation, or translocation to the membrane of AKT, p70S6 kinase, TOR, and 4E-BP (3, 4). The increased phosphorylation of p70S6 kinase was reversed *in vitro* and *in vivo* by incubation with CCI-779. However, the degree of inhibition of p70S6 kinase by CCI-779 in tumors *in vivo* was not sufficient to indicate outcome as CCI-779 inhibited p70S6 kinase equally in responsive and unresponsive tumors (3). Nevertheless, inhibition of p70S6 kinase phosphorylation or activity may provide a surrogate marker for efficacy of CCI-779 during therapy. This finding may be particularly important as at effective doses CCI-779 does not exhibit significant toxicity, precluding the use of the minimum tolerated dose as a mechanism to guide phase II and III studies. In ovarian cancer samples directly from the patient, down-regulation of PTEN expression was associated with an increase in AKT1 and AKT2 phosphorylation and activity (23, 24). This finding suggests that analysis of phosphorylation or activation of downstream targets of the PTEN/PI3K pathway may identify a broader spectrum of patients responsive to the effects of CCI-779 than would mutational analysis alone. This hypothesis is supported by the observation that multiple cell lines with normal PTEN are sensitive to rapamycin or CCI-779 (2, 7). We have demonstrated that ovarian cancer cells lines with intact PTEN, but with amplification and activation of PI3K, are as sensitive to rapamycin as are PTEN mutant cells (data not shown). Further, DU145, which has intact PTEN, is sensitive to rapamycin potentially because of amplification of AKT3 and subsequent activation of TOR (25). Inactivation of PTEN function also may not be

sufficient to predict sensitivity to CCI-779 as cells with amplified myc have been reported to be resistant to rapamycin. In support of this contention, MG132, which has a mutation in PTEN, has been reported to be resistant to rapamycin (2, 7). To better evaluate the function of the PTEN signaling cascade, we and others have begun to characterize the PTEN and PI3K-dependent transcriptome in an effort to identify changes in mRNAs or proteins that may be diagnostic of abnormalities at any location in the PTEN pathway.

In addition to functional activation of the PTEN/PI3K pathway by transmembrane signaling and oncogenes, multiple components of the pathway are aberrant in tumor cells. Two of the catalytic subunits of PI3K, p110α and p110β, are located within a large amplicon on chromosome 3q, which is present in ovarian, head and neck, cervix and nonsmall cell lung cancers (12, 26). Some ovarian cancers demonstrate amplification of the p85β regulatory subunit of PI3K. A truncated activated p85 subunit, which has transforming activity, has been identified in tumors of the hematopoietic lineage. The downstream PTEN/PI3K targets, AKT2 and AKT3, frequently are genomically amplified in ovarian and pancreatic cancers as well as in a lesser portion of breast cancers (28) and amplified in hormonally unresponsive breast and prostate cancers (25), respectively. p70S6 kinase is located at a site of amplification and is increased in breast cancers (28). This observation suggests that the spectrum of tumors responsive to therapy with CCI-779 may extend beyond those with aberrant PTEN function. Indeed, rapamycin reverses transformation of chicken embryo fibroblasts induced by activated PI3K or AKT, but not by multiple other oncogenes including *src* and *RAS* (29). In support of this concept, CCI-779 blocked the growth of a prostate cancer cell line expressing activated AKT at concentrations that were without activity on the parental line (3).

Abnormalities in signaling through the PTEN/PI3K pathway can render cells sensitive to the effects of molecular therapeutics in addition to CCI-779 and rapamycin. Inhibition of the integrin-linked kinase, which is regulated by PI3K and a potential regulator of AKT, with either genetic or molecular therapeutic approaches induces a G_1 arrest followed by apoptosis in PTEN negative cells both *in vitro* and *in vivo* (30). The PI3K inhibitors wortmannin and LY294002 have demonstrated marked antitumor activity both *in vitro* and *in vivo* (31, 32). Cells with abnormalities in PTEN or amplification of PI3K are particularly sensitive to the effects of these inhibitors. Supportive of this concept, we have demonstrated that introduction of an activated PI3K into ovarian cancer cells, with intact PTEN and PI3K, increases their sensitivity to apoptosis induced by PI3K inhibitors (data not shown).

The studies in this issue of PNAS with CCI-779 have further established the PTEN/PI3K pathway as a potential target for therapy in human cancer patients. As abnormalities in components of PTEN/PI3K can contribute to tumor initiation, this pathway also may be a target for cancer chemoprevention. Indeed, hexakisinositolphosphate, which inhibits PI3K activity, is being evaluated in chemoprevention models and trials (33). Multiple biotech, pharmaceutical, and academic centers are developing novel agents targeting specific components of the PTEN/PI3K pathway. It is likely that drugs targeting different components of the pathway will exhibit different mechanism-based activities and toxicities. The balance between efficacy and toxicity will likely be the major determinant of validity of particular targets. In addition, different abnormalities present in tumors may alter the sensitivity to molecular therapeutics targeting specific targets. Xenograft, transgenic, or knockout models driven by alterations that mimic changes in components of the PTEN/PI3K pathway observed in human tumors will prove important for the selection and validation of molecular therapeutics targeting specific components of the PTEN/PI3K pathway. Indeed, the utilization of multiple xenograft, transfected cell lines, knockout embryonic stem cells, and knockout murine models was critical to the validation of CCI-779 as a molecular therapeutic with selective activity in cells with abnormalities in PTEN (3, 4).

Over the next several years, further dissection of the molecular pathways driving tumorigenesis and tumor progression will lead to the discovery and credentialing of new molecular targets. For these to be applied optimally in the clinic, molecular diagnostics must be coupled for appropriate patient selection. Continued molecular monitoring will be needed to ensure that resistant clones do not arise during drug exposure. The vision for the future is the design of low-toxicity, molecular-targeted cocktails focused to the specific pathway hierarchy of the patient's cancer.

1. Eng, C. P., Sehgal, S. N. & Vezina, C. (1984) *J. Antibiot.* **37,** 1231–1237.
2. Hidalgo, M. & Rowinsky, E. K. (2000) *Oncogene* **19,** 6680–6686.
3. Neshat, M. S., Mellinghoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H. & Sawyers, C. L. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 10314–10319. (First Published August 14, 2001; 10.1073/pnas.171076798)
4. Podsypanina, K., Lee, R. T., Politis, C., Hennessy, I., Crane, A., Puc, J., Neshat, M., Wang, H., Yang, Y., Gibbons, J., *et al*. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 10320–10325. (First Published August 14, 2001; 10.1073/pnas.171060098)
5. Huang, S., Liu, L. N., Hosoi, H., Dilling, M. B., Shikata, T. & Houghton, P. J. (2001) *Cancer Res.* **61,** 3373–3381.
6. Shi, Y., Frankel, F., Radvanyi, L., Penn, L., Miller, R. G. & Mills, G. B. (1995) *Cancer Res.* **55,** 1982–1988.
7. Geoerger, B., Kerr, K., Tang, C. B., Fung, K. M., Powell, B., Sutton, L. N., Phillips, P. C. & Janss, A. J. (2001) *Cancer Res.* **61,** 1527–1532.
8. Vinals, F., Chambard, J. C. & Pouyssegur, J. (1999) *J. Biol. Chem.* **274,** 26776–26782.
9. Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M. M., Simons, J. W. & Semenza, G. L. (2000) *Cancer Res.* **60,** 1541–1545.
10. Gingras, A. C., Raught, B. & Sonenberg, N. (2001) *Genes Dev.* **15,** 807–826.
11. Simpson, L. & Parsons, R. (2001) *Exp. Cell Res.* **264,** 29–41.
12. Mills, G. B., Lu, Y., Fang, X., Wang, H., Eder, A., Mao, M., Swaby, R., Cheng, K. W., Stokoe, D., Jafe, R., *et al*. (2001) *Semin. Oncol*., in press.
13. Steck, P., Pershouse, M. A., Jasser, S. A., Yung, A. W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., *et al*. (1997) *Nat. Genet.* **15,** 256–362.
14. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., *et al*. (1997) *Science* **275,** 1943–1947.
15. Li, D. M. & Sun, H. (1997) *Cancer Res.* **57,** 2124–2129.
16. Lu, Y., Lin, Y., Lapushin, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M., *et al*. (1999) *Oncogene* **18,** 7034–7045.
17. Whang, Y. E., Wu, X., Suzuki, H., Reiter, R. E., Tran, C., Vessella, R. L., Said, J. W., Isaacs, W. B. & Sawyers, C. L. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5246–5250.
18. Salvesen, H. B., MacDonald, N., Ryan, A., Jacobs, I. J., Lynch, E. D., Akslen, L. A. & Das, A. (2001) *Int. J. Cancer* **91,** 22–26.
19. Zhou, X. P., Gimm, O., Hampel, H., Niemann, T., Walker, M. J. & Eng, C. (2000) *Am. J. Pathol.* **157,** 1123–1128.
20. Dahia, P. L., Aguiar, R. C., Alberta, J., Kum, J. B., Caron, S., Sill, H., Marsh, D. J., Ritz, J., Freedman, A., Stiles, C., *et al*. (1999) *Hum Mol. Genet* **8,** 185–193.
21. Torres, J. & Pulido, R. (2001) *J. Biol. Chem.* **276,** 993–998.
22. Vazquez, F., Ramaswamy, S., Nakamura, N. & Sellers, W. R. (2000) *Mol. Cell. Biol.* **20,** 5010–5018.
23. Kurose, K., Zhou, X. P., Araki, T., Cannistra, S. A., Maher, E. R. & Eng, C. (2001) *Am. J. Pathol*. **158,** 2097–2106.
24. Yuan, Z. Q., Sun, M., Feldman, R. I., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S. V. & Cheng, J. Q. (2000) *Oncogene* **19,** 2324–2330.
25. Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J. & Roth, R. A. (1999) *J. Biol. Chem.* **274,** 21528–21532.
26. Shayesteh, L., Lu, Y., Kuo, W., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B. & Gray, J. W. (1999) *Nat. Genet.* **21,** 99–102.
27. Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G. & Masciullo, V. (1995) *Int. J. Cancer* **64,** 280–285.
28. Barlund, M., Forozan, F., Kononen, J., Bubendorf, L., Chen, Y., Bittner, M. L., Torhorst, J., Haas, P., Bucher, C., Sauter, G., *et al*. (2000) *J. Natl. Cancer Inst.* **9,** 1252–1259.
29. Aoki, M., Blazek, E. & Vogt, P. K. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 136–141. (First Published December 26, 2000; 10.1073/pnas.011528498)
30. Yoganathan, T. N., Costello, P., Chen, X., Jabali, M., Yan, J., Leung, D., Zhang, Z., Yee, A., Dedhar, S. & Sanghera, J. (2000) *Biochem. Pharmacol.* **60,** 1115–1119.
31. Hu, L., Zaloudek, C., Mills, G. B., Gray, J. & Jaffe, R. (2000) *Clin. Cancer Res.* **6,** 880–886.
32. Schultz, R. M., Merriman, R. L., Andis, S. L., Bonjouklian, R., Grindey, G. B., Rutherford, P. G., Gallegos, A., Massey, K. & Powis, G. (1995) *Anticancer Res.* **15,** 1135–1139.
33. Dong, Z., Huang, C. & Ma, W. Y. (1999) *Anticancer Res.* **19,** 3743–3747.