Novel Combinations Based on Epidermal Growth Factor Receptor Inhibition

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Abstract
In spite of recent advances in molecular biology leading to the introduction of clinically active novel agents, such as imatinib, erlotinib, and bevacizumab, therapy of the most common epithelial tumors, such as lung cancer, remains unsuccessful. The diversity of molecular abnormalities in these tumors is felt to partly contribute to their resistance to therapy. It is, therefore, widely accepted that one approach to improving the efficacy of cancer therapy is the development of rational, hypothesis-based combinations of anticancer agents that may exhibit synergistic cytotoxic interactions. A number of empirical combination studies with the epidermal growth factor receptor and classic cytotoxic agents were undertaken in clinical trials, with disappointing results. It is, therefore, felt that preclinical combinations of epidermal growth factor receptor inhibitors and other novel agents, based on sound knowledge of complementary signaling pathways whose concerted inhibition would be hypothesized to inhibit growth, is the reasonable approach in the future. A brief overview of some of these pathways (mammalian target of rapamycin, vascular endothelial growth factor receptor, and ras/mitogen-activated protein kinase signaling) is provided in this review.

As the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors progress through clinical trials, there is increasing interest in examining the effects of these agents in combination with other anticancer drugs. A number of different approaches have been taken in the development of clinical combinations. The first approach to combination therapy has historically been to use agents with nonoverlapping toxicities; the second approach has used agents with broad activity in different tumor types; and the third approach uses agents with activity in a specific tumor type. The final approach, which is hypothesis based rather than empirical, revolves around the determination of preclinical synergy (1).

Determination of Preclinical Synergy
Although controversy persists, and new methodologies continue in development, two methods for the determination of synergy/additivity have emerged over the past 10 years as the main functional systems for the pharmacologist and investigator in cancer. These are the median effect/combination index method and the isobologram method, which are based on formal mathematical formulae to determine whether the interaction between two agents in specific model systems are additive, synergistic, or antagonistic (reviewed in ref. 2). The median effect principle was obtained from the derivation of enzyme reaction rate equations and dose-effect relationship equations and has been popularized by the work of Chou and Talalay (3) who described this approach and also provided a computer program for analyzing data by this method (4). The isobologram method is a generally valid procedure for analyzing interactions between agents irrespective of their mechanisms of action or the nature of their concentration-response or dose-response relations (5). Clearly, anticancer agents are tested in a few preclinical models, whereas clinical studies are done in hundreds of patients whose heterogeneous tumor properties may not mimic the molecular abnormalities found in the preclinical models. Thus, the preclinical information has to be looked at as data that represent guidelines allowing for more rational study designs. However, the preclinical data may not be predictive of clinical response. When used in the following sections, the terminology of synergy and additivity are based on formal median effect analyses.

EGFR Signaling Pathways
EGFR (ErbB1) is a trans-membrane receptor tyrosine kinase belonging to the family of ErbB-related kinases whose activity is normally modulated by ligand binding and dimerization. Other members of this family include HER-2/neu (ErbB2), ErbB3, and ErbB4. Occupancy of the extracellular ligand–binding domain of EGFR, ErbB3, or ErbB4 results in homodimerization or heterodimerization and subsequent trans-phosphorylation of multiple tyrosine residues in the COOH-terminal region of the receptors (6). These activating...
phosphorylations provide docking sites for phospho-tyrosine–binding proteins that initiate signaling through multiple downstream pathways (Fig. 1). In particular, Ras activation stimulates downstream signaling to extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase. Phosphatidylinositol 3’-kinase (PI3K) pathway activity can result either from EGFR activation or from EGFR-dependent signaling via Ras (7). The PI3K/Akt pathway then activates key molecules, such as BAD, implicated in the regulation of apoptosis (8). Other notable EGFR signaling targets include the transcription factor signal transducers and activators of transcription 3 and phospholipase C-1 (9, 10). Other mitogenic signaling effectors influence the activity of these pathways, and, collectively, these effectors form a complex signaling network that regulates a diverse array of cellular functions, including proliferation, angiogenesis, invasion, and apoptotic response. The complexity of downstream signals and parallel signaling pathways would predict that combinations of EGFR tyrosine kinase inhibitors, and a number of other signal transduction inhibitors may be synergistically cytotoxic. A number of such combinations have been tested preclinically and clinically.

**Inhibition of Signaling from EGFR and Mammalian Target of Rapamycin**

A serine/threonine downstream mediator in the PI3K/Akt signaling pathway, mammalian target of rapamycin (mTOR), plays a critical role in regulating important cellular functions. These include cell proliferation, growth, survival, mobility, and angiogenesis. Rapamycin and its analogues (temsirolimus (CCI-779), everolimus (RAD001), and AP23573) have specific antagonistic action on the function of mTOR. This leads to inhibition of downstream signaling elements and results in cell cycle arrest in the G1 phase. All three agents have shown promising activity in early clinical trials (11).

Because EGFR and mTOR function as key modulators in interconnected signaling pathways, we and others (11, 12) have hypothesized that a synergistic dual inhibition may arise from the combined inhibition of downstream pathways that may collectively control tumor cell proliferation and cell cycle progression. Previous studies (13) have placed mTOR downstream from EGFR in a linear signaling cascade. However, at least in U251 cells, inhibition of EGFR did not affect mTOR signaling to p70S6 kinase, which suggests that these two signaling mediators function in parallel signaling pathways that modulate tumor proliferation. In support of this idea, recent data have described non-PI3K/Akt pathways that regulate mTOR activity. For example, mTOR signaling is known to be inhibited in a low nutrient (glucose, amino acid, and ATP) milieu. mTOR was presumed to serve as the direct cellular sensor for ATP levels. However, current data have implicated AMP-activated protein kinase, a well-characterized sensor of intracellular ATP/AMP ratios, in the regulation of mTOR activity. The LKB1 tumor suppressor gene encodes a serine/threonine kinase that activates AMP-activated protein kinase, leading to negative regulation on mTOR. Thus, LKB1 is required for repression of mTOR under low ATP conditions, with the LKB1/AMP-activated protein kinase/TSC2 axis emerging as an alternate pathway regulating mTOR activity (14). In addition, Nobukumi et al. have shown that a major pathway by which amino acids control mTOR signaling is distinct from that of insulin. Amino acids mediate mTOR activation by signaling through class 3 PI3K, hVps34, whereas insulin and a number of other growth factors signal through the more commonly described class I PI3K/Akt pathway (15). These data support the concept of combined therapy with EGFR and mTOR inhibitors in patients with epithelial malignancies, including lung cancer.

**Clinical Trials with EGFR Inhibitors and mTOR Inhibitors**

Based on biological mechanisms and preclinical evidence of cytotoxic synergy between rapamycin and EGFR inhibitors (16), a phase I trial of the mTOR inhibitor temsirolimus and the dual EGFR/HER2 inhibitor EKB-569 in patients with advanced solid tumors is ongoing. A second study evaluates a different combination of the mTOR inhibitor everolimus (RAD001) with the EGFR tyrosine kinase inhibitor erlotinib. This latter trial is a multi-institutional phase I/II study in patients with advanced non–small cell lung cancer. Results of these studies are awaited with interest.

**Inhibition of Signaling from EGFR and Ras/Raf/ERK**

The Ras pathway plays a critical role in transducing proliferative and antiapoptotic signals from cell surface receptors to the nucleus. Oncogenic Ras mutations have been identified in ~30% of human malignancies. These mutations result in constitutive activation of Ras, producing continuous stimulation of cell proliferation and inhibition of apoptosis.
The Ras signaling pathway, therefore, remains a prime target of novel cancer therapeutic agents.

As previously discussed, upon specific binding of EGF-like ligands to the extracellular domain, ErbB receptors dimerize, either as homodimers or as heterodimers, and undergo autophosphorylation at specific tyrosine residues within the intracellular domain of the receptor. These phosphorylated tyrosines serve as docking sites for adapter molecules, which then transduce signals downstream. One important downstream pathway is the Ras/Raf/ERK cascades. Current data have correlated somatic mutations in the EGFR gene with dramatic clinical response to gefitinib and erlotinib in patients with non–small cell lung cancer. In addition, it has been shown that activation of the Ras/Erk pathway through mutations in the ras gene contributes to resistance of non–small cell lung cancer cells to EGFR inhibitors (18).

Based on these data, we hypothesized that inhibition of Ras/Raf signaling with farnesyl transferase inhibitors may enhance the cytotoxicity of EGFR inhibitors. Preclinical studies in A549 and H622 cell lines indicated that the EGFR tyrosine kinase inhibitor erlotinib showed additive cytotoxic effects in combination with the farnesyl transferase inhibitor tipifarnib. We subsequently undertook a phase I study to determine the maximum tolerated dose, pharmacokinetics, and pharmacodynamics of erlotinib in combination with tipifarnib. Preliminary results have been reported (19). Patients received escalating doses of erlotinib (once daily orally) and tipifarnib (bid orally on days 1-21) on a 28-day cycle. Full doses of both agents have been given without protocol-defined dose-limiting toxicity. Dose escalations of both agents were continued until cumulative grade 3-4 toxicity was encountered (range, 5.9-43.9 weeks; ref. 29). This combination strategy is under further evaluation, with erlotinib being substituted for sorafenib (17). The Ras signaling pathway, therefore, remains a prime target of novel cancer therapeutic agents.

Vascular endothelial growth factor (VEGF) is the prototype heparin-binding peptide in a family of secreted ligands with endothelial-specific mitogenic activity. It exerts its activity by binding to the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed almost exclusively in endothelial cells. Its major physiologic role is to promote corrective angiogenesis in response to hypoxia. VEGFRs are single-pass transmembrane receptors that possess intrinsic receptor tyrosine kinase activity within the cytoplasmic domain of the receptor. Ligand binding results in VEGFR dimerization, which in turn triggers kinase activation and autophosphorylation of specific intracellular VEGFR tyrosine residues. Autophosphorylation at these tyrosine residues further increases the catalytic activity of the tyrosine kinase and provides potential docking sites for cytoplasmic signal transduction molecules, such as phospholipase C-ι. These protein interactions are essential for mediating the intracellular signaling that is required to induce cellular responses to VEGF (20).

It has been shown that expression of proangiogenic molecules (such as VEGF) by tumor cells can be stimulated by EGFR/ErbB2 receptor signaling (21, 22). Furthermore, blockade of EGFR and/or ErbB2 function using specific monoclonal antibodies or small-molecule tyrosine kinase inhibitors has been shown to decrease tumor cell production of proangiogenic molecules and inhibit tumor-associated angiogenesis (23–25). Based on the above data, synergistic inhibition of tumor growth and angiogenesis may be predicted to occur after use of agents that inhibit EGFR and angiogenesis. Preliminary clinical data with a combination of erlotinib and bevazicumab have been reported (26).

An multitarget inhibition approach that potentially combines inhibitors of angiogenesis and the Ras/Raf/ERK pathway and EGFR has been tested. Sorafenib (BAY 43-9006) is an oral multi-kinase inhibitor that targets Raf kinase and the receptor tyrosine kinases of VEGFR and platelet-derived growth factor receptor, which are both implicated in tumor angiogenesis (27). A combination of sorafenib and EGFR inhibitors would theoretically inhibit growth factor signaling upstream at the level of EGFR and downstream at the level of Raf kinase. In addition, antiangiogenic effects through inhibition of VEGFR, platelet-derived growth factor receptor, and Raf in endothelial cells and in tumor cells may be achieved.

In preclinical studies, combining sorafenib with gefitinib resulted in prolonged tumor growth delay compared with either agent alone (28). Based on these considerations, a phase I study was done to assess the combination of sorafenib and gefitinib in patients with advanced non–small cell lung cancer. The most common drug-related adverse events were diarrhea, fatigue, elevated alanine aminotransferase, and elevated aspartate aminotransferase. One partial response lasting for 8 months was seen out of 30 evaluable patients. Disease stabilization, including tumor shrinkage that did not meet the Response Evaluation Criteria in Solid Tumors for response, was achieved in 20 patients (63%) with a median duration of 20.4 weeks (range, 5.9-43.9 weeks; ref. 29). This combination strategy is under further evaluation, with erlotinib being substituted for gefitinib.

### Conclusion

The clinical benefit of the EGFR tyrosine kinase inhibitors in patients with non–small cell lung cancer is now clear. However, a substantial proportion of patients derive minimal benefit from these agents. In addition to defining the molecular characteristics that may predict for response to these agents, rational combinations with agents that inhibit complementary pathways are seen as another approach to improving clinical outcomes. Based on early evidence, mTOR inhibitors, VEGF inhibitors, and agents inhibiting the Ras/Raf/ERK pathway are seen as promising in combination with EGFR inhibitors. Definitive clinical results are awaited with interest.

### Open Discussion

**Dr. Thomas Lynch:** You did a number of very interesting phase I studies with combinations. These trials are small, and they are really designed to show safety and side effect profiles for the combinations. Yet, I certainly look at them and say, "They didn’t see anything there." Might we be giving up on these combinations too quickly because of the less-than-encouraging results of small phase I/II studies?
Dr. Adjei: That is a fair point, and we struggle with that. When you are doing a single-agent phase I study, you see a few patient who stay on for a few months. You’re happy with the results because these are patients with really bad disease and after two cycles everybody progresses. When you do a combination study, especially with an agent that is felt to have some activity, the level of expectation is higher. It can be dangerous to draw these conclusions, especially in a situation where you might have looked at different tumor types. There may have been only three lung cancer patients in the study, but because there was no signal you might give up on the combination. With a combination that we think might be reasonable for lung cancer, when we get to the recommended phase II dose, we expand the cohort and try to put on 15 or 20 lung cancer patients. We do that for 2 weeks. Usually, we will do pharmacokinetics and any correlative studies at the same time. If we accrue an additional 20 lung cancer patients, and we don’t see any effect at all, then maybe it is reasonable to give up. Other people might have a different approach.

Dr. Tim Eisen: Can I ask you a little bit more about the stabilization of disease in combination data with sorafenib? My experience with that drug is in renal cell cancer. The assessment of response has been a real challenge with the anti-vascular agents. You expect a higher investigator-assessed response rate than you do with independent assessment, but that was a particular problem here. There is a lot of central necrosis with these drugs, at least in renal cancer, with swelling of the lesions. A new lesion is progression by definition, but swelling of an existing lesion has been seen in GIST and now renal cell carcinoma. Could you just tell us whether you are seeing any central necrosis, or what is interpreted as central necrosis, and what you felt the importance of the disease stabilization rate was in that setting?

Dr. Adjei: There were two patients with central necrosis of the tumor. With one, the tumor size was unchanged, and with the other patient, the tumor was a bit bigger. Both seemed to be feeling better and to have benefitted. We are not getting a lot of the classical RECIST responses, but we have patients who benefit in the sense that they are feeling better. They are more active, and they are staying on study for a long time. In fact, we have one study closed for interim analysis where we are going to go back and think about the criteria for reopening it. If we go by RECIST criteria, we might decide that the treatment is ineffective, and we will close it; however, there are a lot of patients on study who are doing well.

Dr. Eisen: With sorafenib in renal cell carcinoma, we really have had to rewrite RECIST for that particular use. If you see stabilization of disease and an enlargement of the lesion, that should actually be considered response, not progression.

Dr. Glenwood Goss: Has anybody either done PET or biopsied those rims? If the tumor is necrotic, and you are left with just a rim, you don’t know whether that is just inflammatory or whether there is residual tumor there.

Dr. Adjei: Perhaps, they can do the biopsies with the renal cell cancer. In lung cancer, you are not going to biopsy if it is a perihilar lesion or something like that.

Dr. Alan Sandler: It doesn’t matter so long as it stays the same.

Dr. Goss: It is very important in reporting your results to know whether that is a partial response or not.

Dr. John Heymach: This was a particular problem in studying SU11248 for GIST. The tumors became PET negative within 24 hours, didn’t change in size at all, but stayed like that for 6 months.

Dr. Lynch: Dr. Adjei, can you comment on where you think the imaging revolution will take us? Do you think that we will be using these molecularly targeted imaging agents or is that fantasy?

Dr. Adjei: An image of tumor in situ avoids the problems that Dr. Johnson faced in his pertuzumab trial with doing invasive biopsies in order to look at markers. That is very appealing. The problem we are finding in these ancillary studies is the lack of standardization: different radiologists call things differently. I think we will get to a standard eventually.

Dr. Bruce Johnson: You commented that it took you 2 years of negotiating with the companies to do one of these combination trials; I believe it was with erlotinib and tipifarnib. That has been the rule rather than the exception in attempting to do combination studies, and it makes these studies almost impossible.

Dr. Adjei: Yes, right now, we stay away from those study designs. The trial we are doing now is with two drugs from one company. That study you mentioned, by the time it got approved, we didn’t want to do it. It is a big problem.

Dr. Jeffrey Engelman: A comment on the mTOR pathway. You showed how PI3K/Akt regulates the mTOR pathway. Another pathway that regulates mTOR even more is a protein called LKB, which phosphorylates MAP kinase and tuberin. There are studies from Spain suggesting that LKB is deleted in 20% to 30% in lung cancer [Oncogene 2004;23:3084], so as we go forward with correlative science, it may be interesting to look at that subset.

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