

ISSUES AND PROGRESS WITH PROTEIN KINASE INHIBITORS FOR CANCER TREATMENT

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Identification of the key roles of protein kinases in cancer has led to extensive efforts to develop kinase inhibitors for the treatment of a wide range of cancers, and more than 30 such agents are now in clinical trials. Here, we consider the crucial issues in the development of kinase inhibitors for cancer, and discuss strategies to address the challenges raised by these issues in the light of preclinical and clinical experiences so far.

METASTASIS

The dissemination of cancer cells via the bloodstream or lymphatic system to other parts of the body, where they produce further tissue damage.

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Protein kinases have emerged as key regulators of all aspects of neoplasia, including proliferation, invasion, angiogenesis and METASTASIS. Not surprisingly, sequencing of the human genome has revealed at least 500 distinct kinases, which can be grouped into ~20 known families¹ on the basis of structural relatedness. Initial concerns, which all previously argued against protein kinases as suitable drug targets — high intracellular ATP concentrations versus ATP site-directed inhibitors; a common catalytic mechanism across the many families of kinases; structural similarity of other features of kinase enzyme active centres; the importance of kinase activities to many physiological processes unrelated to proliferation — have given way to a stampede of drug discovery and development research in this area (TABLE 1). This is in part related to the remarkable success of STI571 (imatinib mesylate, Gleevec, Glivec; Novartis) in the treatment of chronic myelogenous leukaemia (CML), gastrointestinal stromal cell tumours (GIST) and metastatic dermatofibrosarcoma protuberans, afflictions that are dependent on the expression and activity of the p210^{BCR-ABL}, c-kit, and platelet-derived growth factor receptor kinases, respectively^{2–4}.

Is this enthusiasm for protein kinases as drug targets justified, or premature and ‘out of synch’ with the preclinical and clinical science that is necessary to assure success in these endeavours? This review will seek to define the crucial issues in considering the array of protein kinase targets and drug discovery opportunities. Then, we will discuss the strategies that would most

efficiently address the development challenges raised by these issues. We will highlight specific drugs in clinical development as case studies exemplifying these issues, and attempt to indicate where both problems and opportunities might be perceived. We will consider how strategies used heretofore in deriving protein kinase antagonists might be biasing the types of molecules available for study in the clinic, and thereby perhaps inadvertently limiting opportunities for clinical use. This overview will not focus, therefore, on the molecular taxonomy, biology or biochemistry, or clinical relevance to particular diseases of the various protein kinases (except to explain important strategic issues), as excellent reviews that explore these matters in some detail have been published^{1,5}. Likewise, this review will not comprehensively consider the diverse agents reported in the medicinal chemistry and preclinical literature that have not entered clinical trials. Nor will we consider the complex and diverse agents that specifically target kinases involved with stromal processes, including angiogenesis, as these have also been covered in recent reviews^{6–8}. However, many of the issues raised in this review could be applicable to the development of such agents.

Which kinase to target with what type of drug?

The criteria that qualify a particular kinase as a drug target are not straightforward. One useful concept derived from recent considerations of kinase biology⁵ is that some kinases might be pivotal in neoplastic

pathophysiology by virtue of mutational activation, whereas other kinases might have an amplifying or permissive role in deregulated growth. By this way of thinking, focus should be given to the former group. Indeed, an important criterion to indicate which kinases have pivotal instructional roles might be the occurrence of mutations either in the kinase itself, or in the pathway that is either regulated by or that regulates the kinase. In this way, nature provides *prima facie* evidence of a particular kinase's pivotal importance to the neoplastic state. Recalling the STI571 experience, p210^{BCR-ABL} could be taken to represent an example supporting the probable success of targeting a kinase that is pivotal to the malignant phenotype. Other examples of kinases and their pathways that could be regarded as instructional because of the presence of pathogenic mutations include the kinases that are collectively regulated by the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) system (in relation to mutation of the PTEN (phosphatase and tensin homologue deleted on chromosome 10) lipid phosphatase locus⁹); the c-MET, proto-oncogene products¹⁰; and the recently described B-Raf mutations¹¹. FIGURE 1 illustrates prominent kinase signalling pathways that are targeted by drugs that are now in clinical development (TABLE 1).

Although identifying potential therapeutic targets on the basis of the occurrence of pathogenic mutations seems to be a sound strategy, two questions immediately arise: how does the choice of target bias the basis for perceived success in the clinical trials, and how should disease states that 'depend' on the activated kinase for their progression be defined? Really important 'instructional' kinases feed not only into proliferation pathways, but also into those governing cell survival, apoptosis or the sculpting of stroma through the elaboration of growth factors. Therefore, inhibiting these kinases might be expected, and indeed has been shown in model systems, to lead to cell death and a decrease in neoplastic mass. The most appropriate end point for Phase II studies that assess agents that target these kinases would, therefore, be the occurrence of objective 'responses' that are defined by conventional oncological criteria.

Defining the appropriate patient population in which to test these agents is more problematic. This diagnostic problem was not an issue for CML, as the presence of the KARYOTYPICALLY defined Philadelphia chromosome is a signature for the presence of the activated p210^{BCR-ABL} oncoprotein, which feeds into several proliferation and survival pathways. Facile and readily applied diagnostic strategies to define the dependence of a particular tumour on a kinase pathway's activation state or on the presence of activating mutations in the kinase or kinase pathway are less clear for common solid tumours, which lack consistent karyotypic indicators. Indeed, the development of a diagnostic approach to define the deregulated kinase state in the patient population to be treated becomes as important a developmental issue as the pharmaceutical features of the drug lead. However, few drug companies have had synchronous pathways for target diagnostic and pharmaceutical development activities for their agent

of interest. Rather, the identification of a tolerated dose of the agent in Phase I trials is followed by the delineation of its influence on tumour response or patient survival — alone and in combination with standard cytotoxic regimens — among a restricted number of 'pivotal' histologically defined diseases. As common solid tumours rarely have one defined genetic alteration driving the neoplastic process, this method of proceeding might be very inefficient and potentially dangerous, as drugs useful in a subset of patients' tumours might be discarded. Indeed, the most successful example so far that illustrates this issue — the definition of HER2 overexpression for selection of breast cancer patients for study with trastuzumab (Herceptin; Genentech) — was accomplished by an assay actually validated for use *post hoc* and for which inter-study variability remains a significant concern¹².

A different, but perhaps more cogent, demonstration of this problem arises when one considers that blast phase CML also expresses the target of STI571, p210^{BCR-ABL} kinase. However, patients with the LYMPHOID SUBTYPE of blast crisis derive very little benefit from STI571, and those with its MYELOID subtype derive more limited benefit from STI571 in comparison with chronic phase patients¹³. This outcome reinforces the possibility that it is not merely the expression of a mutated kinase or kinase pathway in a tumour that determines success for protein kinase-directed therapies; the context in which the mutation occurs is also important. Blast phase CML has far more heterogeneous karyotypes compared to chronic phase CML. This signifies the occurrence of further mutations that undoubtedly diminish the likelihood that blast phase CML is driven solely by p210^{BCR-ABL}, even though it is clear that the p210^{BCR-ABL} kinase is probably responsible for the inception of the leukaemogenic process. So, a considerably greater understanding of tumour biology is required to optimally exploit kinase-directed therapies. One implication of these thoughts is that, in the absence of a clear method for defining the importance of a particular kinase target in the molecular milieu of a tumour, initial efficacy trials would concomitantly characterize the studied patient populations at a molecular level. Microarray approaches have delineated different prognoses and, in some cases, differential efficacy of present treatments among subsets of patients with microscopically similar tumour histologies, but with distinct sets of gene expression^{14–18}. At the very least, the interpretation of trials evaluating the activity of a new kinase inhibitor in patients with tumours of a given histology would be greatly assisted by knowing the genetic 'mix' of the tumours of the patients entering the study, so that the activity of the agent being tested could be correlated to the molecular profiles of the tumours. Future technologies that might identify disease subgroupings that are independent of histology include proteomic approaches based on the expression patterns of phosphorylated substrates, which might reveal different phosphoprotein patterns in response to the tumour's microenvironment despite similar levels of expression of unphosphorylated proteins.

LYMPHOID

A term that describes the type of tissue found in the lymph nodes, tonsils, spleen, and thymus. It is responsible for producing lymphocytes and therefore contributes to the body's defence against infection.

MYELOID

A term that describes tissue within red bone marrow that produces the blood cells.

KARYOTYPE

A complete description of the chromosomes present in a cell. It is characterized by numerical and structural abnormalities in most cancers.

Facilitating or permissive kinases can be defined as those not known to be mutated, but known to regulate important cellular pathways that are crucial to the coordination of neoplastic growth or survival. Examples would then include Jun N-terminal kinase mediating

stress responses from a variety of stimuli including chemotherapeutic agents¹⁹, p38 and CHK1 kinases modulating the DNA-damage checkpoint in the G2 phase of the cell cycle²⁰, or protein kinase C influencing the apoptotic threshold after cytotoxic treatment²¹. In

Table 1 | **Selected kinase inhibitors in clinical development**

Target	Agent	Structure	Development stage
Growth-factor-receptor inhibitors (a)			
EGFR	IMC-C225 cetuximab (Erbix; Imclone)	Monoclonal antibody	Phase III
	ABX-EGF (Abgenix)	Monoclonal antibody	Phase II
	EMD 72000 (Merck KgaA Darmstadt)	Monoclonal antibody	Phase I
	RH3 (York Medical Bioscience Inc.)	Monoclonal antibody	Phase II
	MDX-447 (Medarex/Merck KgaA)	Monoclonal antibody bivalent	Phase I
	ZD1839 gefitinib (Iressa; AstraZeneca)	Small-molecule kinase inhibitor	Phase III
	OSI-774 erlotinib (Tarceva; OSI-Pharmaceuticals)	Small-molecule kinase inhibitor	Phase III
	CI-1033/PD183805 (Pfizer)	Small-molecule kinase inhibitor	Phase II
	EKB-569 (Wyeth Ayerst)	Small-molecule kinase inhibitor	Phase I
	GW2016/572016 (GlaxoSmithKline)	Small-molecule kinase inhibitor	Phase I
HER-2/neu	Trastuzumab (Herceptin; Genentech)	Monoclonal antibody	Registered
	MDX-210 (Medarex/Novartis)	Monoclonal antibody	Phase I
	2C4 (Genentech)	Monoclonal antibody	Phase I
	17-AAG (Kosan)	Geldanamycin derivative inhibits HSP90	Phase I
PDGFR/c-Kit/BCR-ABL	Imatinib (STI571/Gleevec; Novartis)	Small-molecule kinase inhibitor	Registered
Ras inhibitors (b)			
Ras	ISIS 2503 (Isis Pharmaceuticals)	Antisense oligonucleotide	Phase II
	R115777 (Johnson and Johnson)	Farnesyl transferase inhibitor	Phase II/III
	SCH66336 (Schering-Plough)	Farnesyl transferase inhibitor	Phase II
	BMS214662 (Bristol-Myers Squibb)	Farnesyl transferase inhibitor	Phase I
Raf inhibitors (c)			
Raf	ISIS 5132/CGP69846A (ISIS Pharmaceuticals)	Antisense oligonucleotide	Phase II
	L-779,450 (Merck)	Small-molecule kinase inhibitor	
	BAY 43-9006 (Onyx/Bayer)	Small-molecule kinase inhibitor	Phase II
MEK inhibitors (d)			
MEK	PD 184352/CI-1040 (Pfizer)	Small-molecule kinase inhibitor	Phase II
	U-0126 (Promega)	Small-molecule kinase inhibitor	Phase I
mTOR inhibitors (e)			
mTOR	CCI-779 (Wyeth)	Inhibits mTOR kinase by binding to FKBP12	Phase II
	RAD001 (Novartis)	Inhibits mTOR kinase by binding to FKBP12	Phase I as a cancer therapeutic
			Phase II/III as an immunosuppressant
	Rapamycin/sirolimus (Wyeth)	Inhibits mTOR kinase by binding to FKBP12	Registered as an immunosuppressant
Cyclin-dependent-kinase inhibitors (f)			
CDK	Flavopirodol/HMR-1275 (Aventis)	Small-molecule kinase inhibitor	Phase II
	E7070 (EISAI)	Small-molecule kinase inhibitor	Phase I
	CYC202 (Cyclacel)	Small-molecule kinase inhibitor	Phase I
	BMS-387032 (Bristol-Myers Squibb)	Small-molecule kinase inhibitor	Phase I
Other targets and agents (g)			
PKC	ISIS 3521/LY900003 Affinitak (ISIS Pharmaceuticals)	Antisense oligonucleotide	Phase III
	CGP41251/PKC412 (Novartis)	Staurosporine analogue	Phase II
	Bryostatin-1 (GPC Biotech)	Small-molecule kinase inhibitor	Phase II
	UCN-01 (Kyowa Hakko Kogyo)	Staurosporine analogue	Phase I/II
PKC-β	LY333531 (Eli Lilly)	Small-molecule kinase inhibitor	Phase I oncology
			Phase II/III diabetic neuropathy
PDK1	UCN-01 (Kyowa Hakko Kogyo)	Staurosporine analogue	Phase I/II

CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; MEK, mitogen-activated protein kinase kinase; mTOR, mammalian target of rapamycin; PKC, protein kinase C; PDK, 3-phosphoinositide-dependent protein kinase-1.

each case, there is little evidence to indicate that the inhibition of one of these kinases alone leads to useful antitumour effects. However, agents directed at these kinase targets in combination with either conventional cytotoxic agents, or with other signal transduction-related agents, might markedly augment antineoplastic activity, as seen in a variety of model systems. Such drugs would ideally be developed as potential modulators, possibly in fixed combinations with other agents. The clinical development challenge for a 'modulator' is to delineate the correct dose with which to modulate the target and, once that is established, to rapidly move to combination studies. This approach would differ from conventional drug development, which seeks to define the antitumour activity of single agents before undertaking combination studies.

Most of the protein kinase antagonists that are now in clinical development are directed towards the ATP-binding site. These antagonists are frequently detected by screening for effects on kinase activity in biochemical assays. Recent insights into the biochemistry of kinase action have led to the recognition of the fact that certain kinases have distinct active and inactive conformations. Therefore, an alternative strategy for identifying kinase inhibitors is to select antagonists that would bind to the inactive form of the kinase, so as to sequester the molecule in a state that cannot participate in signal transduction. Evidence that certain classes of BCR-ABL inhibitors bind differentially to kinases in active and inactive states has already been reported²². Numerous other approaches for deriving kinase inhibitors remain to be exploited, despite the ATP-binding site of kinases having acquired the status of a 'druggable' target. These other approaches are illustrated in FIG. 2. This position is based on the wealth of accumulated structural information on kinases, and on evidence that the application of this structural information can produce structures with markedly different spectra of kinase inhibitory capacity. Alternatives to ATP mimetics include pseudo-substrate approaches (as suggested by the original tyrphostin strategy^{23–25}), alteration of protein kinase stability (for example, by agents that target molecular chaperones such as heat shock protein 90 (REF. 26)), or drugs that affect the docking of scaffold molecules with protein kinases that allow their novel associations²⁷.

Which disease to target, and when?

Reference has been made in the previous sections of this review to the potential need to diagnose the activation state of the kinase target in the tumours of patients entering clinical trials if the drug is to receive a fair assessment of its capabilities. A related issue is the consideration of when in the course of a tumour's evolution kinase-directed drugs are most likely to have an impact. One view of the activity of STI571 in CML is that the drug is most effective in the 'pre'-fully malignant chronic phase of the disease, during which there is little evidence of karyotypic abnormalities other than the presence of p210^{BCR-ABL}. By this reasoning, kinase inhibitors might be expected to have the greatest impact on preventing the evolution of tumours to a fully malignant state

(that is, as chemopreventive agents), or in the treatment of 'small' volume disease after surgical or chemotherapy reduction to prevent the development of clinically overt metastatic disease.

The problem with this approach is that initiating trials of drugs in patients without clinical evidence of disease has generally required evidence of activity as a single agent in patients with advanced disease. The strategic leap that will need to be taken is: given the importance of these pathways in malignant progression, if initial clinical data demonstrate that a defined dose and schedule efficiently modulate the target kinase, then trials in minimal residual disease, or adjuvant trials, are reasonable, despite a lack of evidence supporting single-agent efficacy in advanced disease. This strategic leap is not so great if the agent under investigation has limited toxicity, and, in fact, has already been made for certain clinical situations. For example, celecoxib have been approved for the reduction of colonic polyps in FAMILIAL ADENOMATOUS POLYPOSIS, and trials demonstrating this benefit were carried out despite the fact that this class of drugs are not beneficial to patients with advanced cancer. Even though trials in patients with early-stage disease might be larger and longer in duration than trials in patients with metastatic disease, they would allow a clinically significant impact of an agent to be defined in a population of patients and, therefore, afford a clear registration-directed strategy. Such trials might be justified when there are compelling preclinical data supporting the activity of the agent against micrometastatic disease, and when the immediate and long-term toxicity of the agent are acceptable for patients at significant risk for cancer recurrence. With primary prevention trials, in addition to the practical considerations of time and appropriate sizing of the patient population to detect benefit, the drug would have to be nearly devoid of side effects for persistent dosing to be tenable in patients who are otherwise well.

How to build combinations with standard agents?

Numerous preclinical experiments have indicated the supra-additive effects of kinase antagonists of various sorts in combination with standard cytotoxic agents^{28–31}. Yet the recent apparent failure of certain kinase antagonists to confer benefit when combined with chemotherapy — as compared with chemotherapy alone³² — raises the question of how best to intelligently explore combinations of cytotoxic agents and kinase antagonists in the clinic. In many cases, the basis for augmentation of the effects of chemotherapy by the protein kinase antagonist in preclinical models is not known. This is of more than academic interest, because to implement a strategy for selecting a dose of a kinase inhibitor that modulates the crucial target, and thereby augments the chemotherapy effect, diagnostic strategies for assessing the effect of the drug on the target would ideally be available before the clinical trial of the combination is started. For example, recent preclinical studies have identified survivin phosphorylation by cyclin-dependent kinase 1 (CDK1) as an important mechanism that modulates cellular sensitivity to taxane and

FAMILIAL ADENOMATOUS POLYPOSIS

A genetic disorder characterized by the development of multiple intestinal polyps that are precursor lesions for colon carcinoma.

CDK inhibitor combinations³³, and, perhaps, to susceptibility to CDK-inhibitor-induced apoptosis³⁴. If this combination strategy is to be knowledgeably explored in the clinic, assessment of the phosphorylation state

of survivin in tumours following exposure to such drugs will be necessary. In the absence of data on the effect of a drug on its target, the meaning of the trial might be uninterpretable, as there might be pharmacological

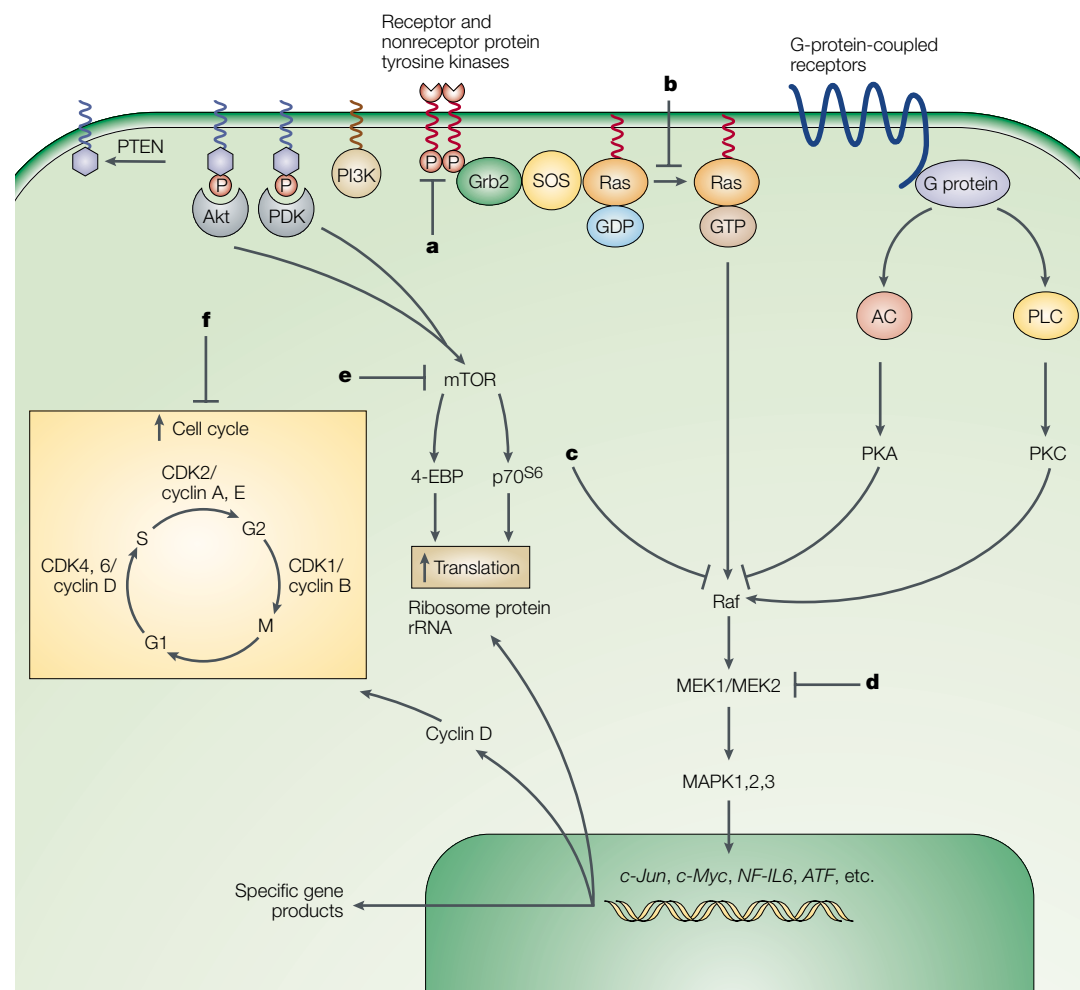


Figure 1 | Kinase targets and agents. A schematic illustration of the principal pathways that are affected by the agents that are discussed in this review. Growth factors induce, or membrane-associated molecules dimerize spontaneously to effect, increased tyrosine kinase activity. Downstream events include the recruitment of adaptor molecules such as growth factor receptor bound (Grb), which binds to phosphorylated tyrosines to recruit effectors such as SOS to form a multi-protein scaffold. In the case of son of sevenless (SOS), this multi-protein scaffold alters the affinity of Ras isoforms for guanosine diphosphate (GDP), allowing exchange for guanosine triphosphate (GTP), which activates Ras. Activated GTP-bound Ras is a potent activating influence for Raf, which in turn activates mitogen-activated protein kinase kinases (MEKs) to phosphorylate mitogen-activated protein kinases (MAPKs) that then influence gene expression (among other targets). Activated tyrosine kinase receptors can also activate phosphatidylinositol 3-kinase (PI3K), which phosphorylates inositol-containing lipids in the plane of the membrane. These in turn recruit pleckstrin-homology (PH) domain-containing molecules such as phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (Akt), which by virtue of their PH domains have affinity for the products of PI3K action. PDK1 contributes to the activation of Akt, which then phosphorylates numerous substrates leading to the activation of the mammalian target of rapamycin (mTOR). mTOR in turn increases the translation efficiency of growth-regulatory gene products through its effects on the 4E-RNA binding proteins (4E-BPs) and the p70^{S6} kinase (p70^{S6}K). Increased translational efficiency is necessary to complement the output from MAPK-influenced gene transcription, which includes elaboration of increased ribosomal RNA (rRNA) and ribonucleoprotein synthesis, and elaboration of cyclin D homologues. The latter cause an increase in cell proliferation through the activation of cyclin-dependent kinases (CDKs). Seven transmembrane G-protein-coupled receptors can activate protein kinases A (PKA) and C (PKC), which modulate the activity of Raf and hence input into the MAPK pathway. The letters **a–f** in the figure refer to the sites of action in this cascade of the classes of signalling agents referred to in TABLE 1, and include epidermal growth factor (EGF)-receptor directed agents (**a**), Ras antagonists including farnesyl transferase antagonists (**b**), Raf antagonists (**c**), MEK-directed approaches (**d**), the mTOR-directed rapamycins (**e**), and CDK antagonists (**f**). It is apparent that opportunities for multiple input into the same final common pathways might be sources of redundancy, and allow variability in the degree to which a particular antagonist might affect a crucially activated pathway in a manner that would convey an adequate therapeutic index. AC, adenylate cyclase; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

interactions between the chemotherapeutic agents and the modulating agent, and interference with, or alteration of, kinase inhibitor uptake or target sensitivity in tumour cells after exposure to the chemotherapeutic regimen. Further complicating this approach, there might be unexpected interactions between kinase antagonists and chemotherapeutic agents that might not necessarily be related to the mechanism of action of the protein kinase antagonist. For example, vascular endothelial growth factor (VEGF)-receptor, alone or in combination with cytotoxic agents, has been associated with THROMBOTIC or haemorrhagic side effects^{35,36}. In this situation, target assessment might determine whether these toxicities are related to the target's action, or whether the toxicities are an adventitious effect of the interacting drugs.

Several strategies for combining protein kinase inhibitors might be formulated on the basis of our present knowledge of cellular signalling pathways (FIG. 1). One approach to constructing combinations of signal transduction-related therapies is to select agents and subsequently demonstrate that the proposed agents affect different pathways that impinge on the regulation of a key target that is necessary for the continued maintenance of the neoplasm. For example, recent preclinical studies have indicated that both trastuzumab and flavopiridol might synergistically affect cyclin D1 elaboration and, therefore, breast cancer cell growth³⁷. Another strategy would be to combine agents that target components in parallel signal transduction pathways, such as a mitogen-activated protein kinase (MEK) inhibitor and an Akt inhibitor. UCN-01, which inhibits the activation of Akt, and the MEK antagonist CI-1044 (PD184352), seem to synergistically interact following UCN-01 activation of mitogen-activated protein kinase (MAPK) signalling³⁸. So, clinical studies in tumours with activated MAPK and/or activated Akt signalling might be the most relevant to explore with these combinations. The surrogate markers of target modulation that could aid in these assessments in lieu of tissue biopsy samples might be downstream readouts of kinase action. For example, glucose uptake measured by POSITRON EMISSION TOMOGRAPHY of fluorodeoxyglucose at short intervals after drug exposure seems to be emerging as an important correlate of drug effect with tyrosine kinase antagonists such as STI571³⁹, and might be generally relevant to molecules that perturb the PI3K/Akt pathway owing to the participation of Akt in the normal regulation of glucose uptake. Similarly, protease elaboration might be considered a surrogate for effects of MET-kinase signalling stimulated by hepatocyte growth factor⁴⁰, and ways to functionally image protease activity are under active consideration⁴¹.

Issues with selected agents in clinical trials

Traditionally, the development of oncological agents has progressed through a well-defined sequence of clinical trials. Phase I studies are the first-in-human studies, and define a tolerable dose for further development based on the occurrence of toxicity and pharmacokinetics. Phase II studies screen for antitumour activity and

Phase III studies are comparative studies designed to delineate patient benefit. Many of the protein kinase inhibitors that are now in clinical development have been evaluated in Phase I and II studies using strategies to circumvent perceived limitations in traditional end points of Phase I and II studies of oncological agents. Because targeted agents are assumed to be less toxic and to induce cytostasis, end points such as maximum tolerated dose (MTD) — based on occurrence of dose-limiting toxicity — and objective tumour response are viewed by some as being inappropriate end points for determining dose and for evaluating antitumour activity for this class of agents. Although much has been made of the need to demonstrate target expression for determining the optimal patient population and dose-related target modulation for evaluation of these agents, as will be apparent from the following examples, very few agents have been evaluated in trials that incorporate these specific design issues.

Epidermal growth factor receptor inhibitors

Overexpression of epidermal growth factor (EGF) ligands and receptors (EGFR) has been implicated in promoting the hallmark neoplastic traits of mitogenesis⁴², inhibition of apoptosis, cell migration⁴³, metastases⁴⁴, angiogenesis^{45,46} and resistance to standard cytotoxic therapies^{47,48}. Experimental evidence indicates that EGFR inhibitors can simultaneously suppress many of these properties while inducing tumour stasis or regression. Several selective compounds that target either the EGFR extracellular ligand-binding region or the intracellular tyrosine kinase region are being developed. At present, the most advanced of the newer therapies in clinical development are anti-receptor monoclonal antibodies IMC-C225 (cetuximab, Erbitux; Imclone), and the reversible small-molecule inhibitors of EGFR, ZD1839 (gefitinib, Iressa; AstraZeneca) and OSI-774 (erlotinib, Tarceva; OSI Pharmaceuticals).

In general, antibodies target EGFR by inhibiting ligand binding and receptor dimerization, whereas small molecules competitively inhibit ATP binding to the receptor, thereby hindering autophosphorylation and kinase activation. Antibodies with intact Fc binding domains, such as IMC-C225, might also induce antibody-mediated cellular cytotoxicity (ADCC). Both classes of molecules induce dose-dependent tumour stasis or even tumour regression in some TUMOUR XENOGRAFT models, and antiproliferative effects seem to be correlated with dose/concentration-dependent inhibition of EGFR phosphorylation^{49,50}. Preclinical models indicate that EGFR expression is required, although the degree of expression above an undefined threshold does not predict sensitivity to EGFR inhibitors^{29,49,51–53}. However, surprisingly little is understood of predictors of sensitivity to EGFR inhibitors, beyond the expression of EGFR and inhibition of EGFR phosphorylation⁵⁴. This circumstance raises the possibility that other, or further, targets to EGFR mediate susceptibility to these drugs. Antitumour effects are associated with induction of G1 arrest and p27, and inhibition of angiogenesis by decreased production of

THROMBOTIC

A term that describes the obstruction of a blood vessel by a mass of blood cells and fibrin (thrombus), which can result from excessive blood clotting.

POSITRON EMISSION TOMOGRAPHY

An imaging technique that is used to detect decaying nuclides, such as ¹⁵O, ¹³N, ¹¹C, ¹⁸F, ¹²⁴I and ^{94m}Tc.

TUMOUR XENOGRAFT

Generally refers to the growth of human tumour cells as tumours in immuno-compromised mice.

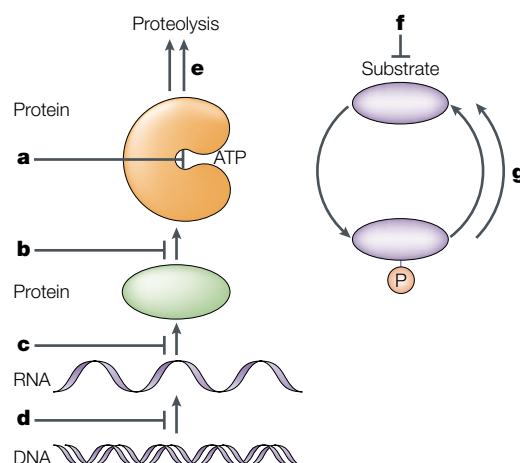


Figure 2 | Several mechanisms can inhibit kinase signalling. The most prevalent approach to the design of inhibitors of kinase signalling has addressed the readily 'druggable' ATP binding site (**a**), which is responsible for transferring phosphate to the substrate. However, in the preclinical literature, signalling inhibitor classes have been described that affect the maturation of the newly synthesized protein to the mature, correctly folded kinase (**b**; for example, benzoquinoid ansamycins), the efficiency of translation of the kinase itself or of its activating regulators (**c**; for example, antisense strategies), the transcription of kinase genes or of their regulators (**d**; for example, the effect of flavopiridol on cyclin D1 elaboration through the effects of the cyclin-dependent kinases that regulate the efficiency of transcription), and the stability of the kinase itself or competition with its substrate (**e**, **f**; for example, certain tyrosinases). Phosphatase activation, leading to accelerated removal of substrate phosphates (**g**) as a means of countering kinase signalling, is a theoretically possible mechanism that has not been clearly exploited in drug candidates studied so far.

VEGF (reviewed in REFS 45, 55). In addition to their shared mechanism of action, these EGFR inhibitors require continuous administration, as interruptions lead to tumour regrowth in xenograft models.

Although single-agent antitumour effects have been observed in preclinical studies, the most promising laboratory data have been generated by combining these targeted inhibitors with standard chemotherapy or radiation^{29,46}. In laboratory xenograft experiments, additivity or synergy between EGFR inhibitors and standard cytotoxic therapies resulted in improved response rates and survival. These preclinical results, and the objective responses of patients in Phase I studies, supported the rapid leap from Phase I trials to Phase III trials evaluating the addition of ZD1839 and IMC-C225 to chemotherapy. One concern raised by this rapid development strategy was the lack of understanding of the basis of the supra-additive effect of anti-EGFR therapies and cytotoxic agents, and whether this effect would be applicable to cancers in patients.

Phase I trials of EGFR inhibitors were designed to evaluate toxicity and pharmacokinetics as primary end points. Pharmacodynamic studies, if included, were carried out on surrogate tissue such as skin, or were evaluated in tumour biopsies from a limited number of

patients. Initial results from clinical trials are notable for the lack of significant toxicity compared with standard chemotherapeutic agents, with certain shared features across agents. All EGFR-targeting agents studied so far induce a characteristic acneiform skin rash that is thought to reflect EGFR inhibition in the skin. In addition to rash, transfusion-reaction-like symptoms have been reported with IMC-C225 and are probably a toxicity that is common to antibodies. The pharmacokinetics of the antibody are predictable, with volume of distribution correlating with intravascular fluid volume and half-life allowing for once-weekly dosing^{56,57}. Notably, the saturation of clearance — which is assumed to correspond to saturation of receptors — occurred at higher doses, and this phenomenon was used to select the dose for Phase II evaluations. The Phase I clinical trials of the EGFR tyrosine kinase inhibitors ZD1839 and OSI-774 indicated that small-molecule inhibitors might have slightly different toxicity profiles and more variable pharmacokinetics than antibodies. In addition to rash, small-molecule EGFR ATP-mimetics consistently cause diarrhoea, which might be dose-limiting, and, less commonly, nausea and vomiting. Whether gastrointestinal side effects reflect the enterohepatic circulation of drug or metabolites, or effects of the kinase inhibitors on other targets in intestinal epithelial cells (as has been described with other kinase inhibitors⁵⁸), remains to be clarified. Results from Phase I trials of OSI-774 and ZD1839 indicate that both of these small molecules have plasma half-lives that allow once-daily dosing. However, interpatient plasma concentrations at steady state and exposures can vary tenfold at a given dose, which reflects the variability in bioavailability and metabolism⁵⁹ of these agents between patients^{60–62}.

The recommended doses of ZD1839 and IMC-C225 have been based on tolerability, pharmacokinetic parameters, the achievement of plasma concentrations that are thought to be biologically relevant in preclinical models^{56,57} and, with ZD1839, the demonstration of modulation of the target and biochemical pathway in skin tissue⁶³. Unfortunately, there is a dearth of data about the corresponding inhibition in tumour tissue. This is concerning, as neither agent is administered at its MTD. Again, this concern is of more than academic interest, as blood flow to and within tumours is increasingly regarded as being very heterogeneous. In contrast to many conventional cytotoxic agents that interact with their targets in a truly covalent fashion or in a very poorly reversible fashion, kinase inhibitors must be present and indefinitely above a certain K_i to effectively compete with ATP. It is possible that effects in well-vascularized surrogate tissue, such as skin, only poorly predict the ability of these agents to have these effects in poorly-vascularized tumours. Clearly, antitumour effects will be determined by either the intratumoural peak concentration of the drug or by the area under the concentration–time curve. Plasma concentrations or pharmacodynamic effects in normal tissue might not reflect intratumour pharmacology. Doses should, therefore, preferably be optimized according to intratumoural effects. However, this strategy has not been

implemented in any of the development campaigns of which we are aware. Although obtaining such information might be tedious, and certainly would not be needed in later-phase trials with the agent, important data to support dose selection for later-phase trials would otherwise be lost if intratumoural pharmacodynamic effects were not evaluated. In the absence of such data, evaluating the MTD in Phase II and III trials at least ensures that patients will not be underdosed, although they might experience unnecessary toxicity.

Preliminary results from Phase II clinical trials that screened for antitumour activity of both anti-EGFR monoclonal antibodies and EGFR small-molecule tyrosine kinase inhibitors are promising. Modest single-agent activity has been observed in uncontrolled trials of IMC-C225 in patients with previously treated colorectal carcinoma (CRC)⁶⁴, and with the small-molecule inhibitors ZD1839 and/or OSI-774 in patients with previously treated non-small-cell lung carcinoma (NSCLC)^{65–67}, squamous cell carcinoma of the head and neck (SCCHN)^{68,69} and ovarian carcinoma⁷⁰. Although objective tumour response rates have been low (5–20%), these results were considered to be encouraging evidence of antitumour activity of agents predicted to be cytostatic from preclinical studies. The results indicate that, in a subset of these common solid tumours, EGFR functions as an important determinant of cell survival. Of note is that randomized Phase II trials of ZD1839 to evaluate daily doses of 250 and 500 mg (doses below the MTD) have yielded similar response rates with significantly more toxicity at the higher dose^{66,67}. These results indicate that the 250 mg daily dose is preferable to the 500 mg daily dose on the continuous schedule. If these results are due to the doses causing plasma concentrations/exposures that are sufficiently different to induce different frequencies and severities of toxicity, but similar intratumoural concentrations, it is possible that a higher dose — that is, the MTD — might lead to higher concentrations and a greater antitumour effect due to higher intratumoural delivery of the agent, albeit at the cost of inducing greater systemic toxicity. Intermittent administration might be required to ameliorate the toxicity caused by higher doses. To our knowledge, there have been no trials evaluating the safety, tolerability and antitumour activity of intermittent dosing schedules — that is, daily doses for 21 days out of every 28 days — of ZD1839.

Several trials have evaluated IMC-C225 in combination with chemotherapy in patients previously treated with a chemotherapeutic alone. The design of these trials was based on the assumptions that disease in this patient population would be resistant to the cytotoxic agent, and that IMC-C225 would be unlikely to induce tumour regressions. So, objective responses in these trials would be evidence of the supra-additive effect of the combination, as predicted by the preclinical models. Although the rationale supporting such trials seems straightforward, they are difficult to conduct, as there should be clear documentation of progressive disease occurring during or shortly after completion of chemotherapy to ensure that disease is truly resistant to

the cytotoxic agent. Ideally, there should also be documentation of the lack of single-agent activity of the targeted agent. Using this design — albeit without the rigorously controlled preconditions required to allow the unambiguous interpretation of results — objective tumour responses were observed in 22% of patients with CRC that received irinotecan with IMC-C225, who had previously been treated with irinotecan only⁷¹. Of patients with SCCHN treated previously with cisplatin only, 13% showed objective tumour responses after treatment with IMC-C225 and cisplatin⁷² in combination. Although these results are encouraging, the 95% confidence intervals around the response rates reported from the trials of irinotecan and IMC-C225, and single-agent IMC-C225, overlap. Although there is, as yet, no reported Phase II study of IMC-C225 alone in patients with SCCHN, the small-molecule inhibitors ZD1839 and OSI-774 produced objective response rates in the range of 6–10%. So, it is not clear whether the results from the combination studies reflect the single-agent activity of IMC-C225 or an additive effect of the combination in patients with chemotherapy-resistant disease.

Despite promising, albeit modest, hints of activity in early clinical trials, the results from the initial Phase III studies that evaluated the addition of EGFR inhibitors to chemotherapy, compared with chemotherapy alone, have been disappointing. ZD1839, at doses of 250 and 500 mg, combined with standard first-line chemotherapy in patients with NSCLC, failed to improve objective response rate, time to progression or overall survival in two large, well-conducted clinical trials³². These results indicate that ZD1839 at each of these doses is ineffective when administered in addition to chemotherapy. Similarly, the addition of IMC-C225 to cisplatin did not improve progression-free survival, despite improving the response rate from 10 to 20% (REF. 73) in a relatively small — and so possibly underpowered — study in patients with SCCHN. These results are surprising given the evidence of antitumour activity from preclinical models and Phase I clinical trials. The absence of demonstrable benefit of the combinations might reflect suboptimal target modulation due to inadequate dosing, antagonism between EGFR inhibitors and chemotherapy that was not anticipated given the results of preclinical combination studies, the same fraction of tumour cells being sensitive to both chemotherapy and the receptor antagonist, the dilution of benefit derived by a small cohort of patients with tumours sensitive to EGFR inhibitors by a larger cohort with insensitive tumours, or the inadequate simultaneous delivery of the EGFR antagonist and chemotherapy to all regions of the tumour(s). Certainly, sensitivity to EGFR inhibition probably requires not only the presence and activation of the EGFR and its pathway, but also the absence of signalling through parallel pathways and downstream components that circumvent the effects of EGFR inhibition on cell-cycle arrest and apoptosis. Without understanding the biological features that determine sensitivity to EGFR inhibitors in some tumours and the mechanism(s) of interactions with cytotoxic drugs, and without diagnostic assays to select patients with such

tumours for clinical trials, it is impossible to determine which of the possible explanations for the failed trials is/are correct. Regrettably, the lack of systematic collection of tumour specimens from patients enrolled in the studies precludes a comprehensive evaluation of molecular markers in tumours that might retrospectively correlate with patient outcome.

A systematic evaluation of tumour tissue for markers that are predictive of sensitivity to EGFR inhibitors was not performed as part of the Phase III trials because such markers have not yet been identified. Although inhibition of EGFR activation would be expected to be necessary for EGFR inhibitors to exert antitumour effects, other factors — such as signalling through other EGFR family members⁷⁴ (HER2, HER3 and HER4), crosstalk with other growth factor pathways, 'activation state' of kinase activity in response to microenvironmental stimuli, allelic polymorphisms of pathway components, alterations of substrate function downstream of the EGFR signalling pathway, or differential intratumoural drug uptake or systemic metabolism — might all influence tumour response to EGFR inhibition. Perhaps the EGFR family, rather than individual family members, should be regarded as the appropriate target for the development of cancer therapeutics. Molecules that inhibit several members of the EGFR family, or strategies that combine selective inhibitors of family members, might be more effective than inhibition of EGFR alone. This hypothesis is supported by results from studies that show that the combination of ZD1839 and trastuzumab is more active than each agent administered singly in laboratory models⁷⁵. Similarly, signalling through alternative growth factor pathways might abrogate the action of EGFR inhibition. Activation of the insulin-like growth factor pathway has been shown to be a mechanism of resistance to both trastuzumab⁷⁶ and the small-molecule EGFR inhibitor AG1478 (REF. 77).

Anti-EGFR antibodies and small-molecule inhibitors of EGFR have different mechanistic and pharmacological properties, and are being evaluated in different clinical situations that might ultimately lead to appreciable differences in clinical outcomes. For example, the agents now under development differ in their affinities for EGFR and other EGFR family members. Among the small molecules, GW2016 inhibits EGFR and HER2, and CI1033 inhibits EGFR, HER2 and HER4 with similar IC_{50} values. Antibodies such as IMC-C225 with an intact Fc portion might also induce ADCC, which might prove to be therapeutically advantageous. The small molecules ZD1839 and OSI-774 are being evaluated at a biologically active dose with limited toxicity and at an MTD, respectively. However, preclinical and clinical studies so far are striking for the similarities shown between the antitumour effects of these various agents, rather than for the differences. Of the agents now in Phase III trials, OSI-774 is being evaluated by comparing it with placebo in NSCLC patients with progressive disease following standard chemotherapy, as well as by comparing OSI-774 in combination with standard first-line

chemotherapy with chemotherapy alone in NSCLC patients that have not been treated previously. The results from these trials might answer the question of whether there is a negative interaction between EGFR inhibitors and chemotherapy. Continuing randomized trials that evaluate IMC-C225 in CRC and OSI-774 in pancreatic carcinoma patients will address the question of whether there are tumour histologies that are more sensitive to EGFR inhibition. Although the results from these Phase III trials might identify an agent, dose and schedule that will benefit patients with a particular disease, it is possible that these trials will be negative, not because EGFR inhibition is invalid as a therapeutic strategy, but because of flaws in the development of these agents. Unfortunately, clinical trials so far have not systematically evaluated pharmacodynamic effects within tumour specimens, so it is not clear that optimal target inhibition is occurring in tumours with any agent. It is possible that higher concentrations, such as those that might be achieved by administering agents at an MTD, might lead to more effective inhibition of EGFR in tumour cells. It might be more feasible to maximize EGFR inhibition in tumours by evaluating increasing doses, using intravenously administered antibodies rather than orally administered small molecules, as escalating doses of the latter might be limited by the bioavailability of the molecules and the occurrence of diarrhoea. Of equal concern is the lack of information on molecular markers that are predictive of the antitumour activity of these agents beyond the presence of EGFR/phosphorylated EGFR. By failing to design trials that optimally define and include subset(s) of patients with tumours most likely to be sensitive to EGFR inhibitors, the beneficial effects of treatment might be diluted. Markers to identify patients who might benefit from treatment are needed. Research goals presently include defining the optimal dose and schedule of agents in combinations with conventional chemotherapeutic agents and with radiation therapy, and determining predictive factors that identify the optimal patient population for treatment with these agents. Future trials will evaluate the safety and efficacy of EGFR inhibitors in combination with other molecularly targeted agents.

Ras/Raf/MEK pathway inhibitors

The Ras family of small G proteins relay signals from activated growth factor receptors to downstream intracellular partners (reviewed in REF. 78). Prominent targets recruited by active membrane-bound Ras are the Raf family kinases, which, in turn, trigger the MEK/extracellular signal-regulated kinase (ERK1/ERK2) pathway. Likewise, Ras can directly activate the PI3K pathway^{79,80}. Ras activation through the Raf/MEK/ERK pathway modulates the activity of nuclear factors such as **Fos**, **Jun** and **AP-1**, which regulate the transcription of genes that are required for proliferation⁸¹.

In human malignancies, Ras mutations are common, having been identified in about 30% of cancers^{11,82,83}. Mutated Ras oncogenes that encode proteins that are constitutively active can induce malignancies in a variety of laboratory models. Mutations in proteins

IC_{50}
The half-maximal inhibitory concentration.

'downstream' of Ras have recently been described. B-Raf somatic mutations in the kinase domain occur in 66% of malignant melanomas, and at a lower frequency in a wide range of human cancers^{11,83}. Mutated B-Raf proteins have elevated kinase activity and are TRANSFORMING in NIH3T3 cells. Given the importance of Ras, and its downstream targets Raf and MEK, in the development of malignancies, and the frequent expression of these proteins in human cancers, it is not surprising that a variety of agents that disrupt signalling through Ras and downstream proteins are under development. These agents can, broadly, be structurally classified as small molecules and antisense oligonucleotides. They can be functionally characterized as those agents that inhibit Ras protein expression (such as the oligodeoxynucleotide ISIS 2503), those that inhibit Ras processing (in particular, the farnesyl transferase inhibitors R115777, SCH 66336 and BMS 214662), and those that inhibit the downstream effectors Raf (such as the oligonucleotide ISIS 5132 and the small molecule BAY 43-9006) and MEK, which is inhibited by CI-1040. For the purposes of illustrating drug development issues, we will focus on agents that target Raf and MEK.

c-Raf kinase inhibitor ISIS 5132. The Raf family is composed of three related serine/threonine protein kinases — Raf-1, A-Raf and B-Raf — that act, in part, as downstream effectors of the Ras pathway⁸¹. Activated Ras interacts directly with the amino-terminal regulatory domain of the Raf kinase, resulting in a cascade of reactions that include direct activation of MEK⁸⁴. Like mutated Ras, constitutively active mutated Raf can transform cells *in vitro*⁸⁵. However, Raf might play a broader role in tumorigenesis, as it can be activated independently of Ras by protein kinase C- α ⁸⁶ and promotes the expression of the multidrug resistance gene *MDR1*⁸⁷.

ISIS 5132 (CGP 69846A), a 20-mer phosphorothioate antisense oligodeoxynucleotide that targets the 3'-untranslated region of c-Raf messenger RNA (mRNA), inhibits both the expression of c-Raf mRNA and the proliferation of lung, colon, cervical and prostate, and ovarian carcinoma cell lines^{88–90}. In addition, ISIS 5132 augments the cytotoxic effects of standard cytotoxic agents^{91,92}.

Three Phase I studies have been reported that evaluated the safety of escalating doses of ISIS 5132 on the following three schedules: two-hour intravenous infusion three times per week for three consecutive weeks⁹³; continuous intravenous infusion for three weeks in each four week period⁹⁴; and weekly 24-hour infusion⁹⁵. Both the two-hour and three-week infusion schedules were well tolerated, with the most common toxicities being fever, fatigue and transient prolongation of activated PARTIAL THROMBOPLASTIN TIME. Reductions of c-Raf-1 expression relative to baseline in peripheral blood mononuclear cells were observed in some patients⁹⁶. The toxicity profile of ISIS 5132 administered as a weekly 24-hour CIV infusion was less favourable. The MTD of ISIS 5132 on this schedule was 24 mg kg⁻¹ week⁻¹, which is considerably higher than the other two schedules, with acute haemolytic anaemia, acute renal failure and ANASARCA

reported as limiting toxicities. There were no major responses reported from the Phase I studies or from a Phase II study in patients with small-cell lung carcinoma (SCLC) or NSCLC⁹⁷, hormone-refractory prostate carcinoma⁹⁸ or metastatic CRC⁹⁹.

Raf inhibitor BAY 43-9006. BAY 43-9006 is a small-molecule inhibitor of Raf with significant dose-dependent antitumour antiproliferative activity in human colon, pancreatic, lung and ovarian carcinoma cell lines (reviewed in REF 100). This agent is active in cell lines with Ras activation either through mutation or through overexpression of growth factor receptors. However, cytostasis is dependent on maintaining continuous dosing. The addition of BAY 43-9006 to irinotecan, vinorelbine or gemcitabine produced at least additive antitumour effects in xenograft models¹⁰¹. Preliminary results from Phase I trials have been reported^{102,103}. With continuous oral dosing, dose-limiting diarrhoea and fatigue occurred at a twice-daily dose of 800 mg. One patient with hepatocellular carcinoma experienced an objective tumour response. In addition, patients have developed ERYTHEMA and SKIN DESQUAMATION reminiscent of hand-foot syndrome induced by infusion of 5-fluorouracil. Attempts to evaluate pharmacodynamic effects in peripheral blood lymphocytes have been incorporated into the Phase I trials¹⁰⁴. Inhibition of ERK phosphorylation in stimulated peripheral blood mononuclear cells was observed in two out of six patients following continuous treatment for 10–14 days with doses starting at 200 mg twice daily, and four out of four patients treated with 400 mg twice daily showed stable suppression of ERK phosphorylation. Unfortunately, the correlation between ERK inhibition in circulating blood cells and tumours has not been evaluated.

It is noteworthy that the limiting toxicities of these two compounds, both of which are purported to target Raf, are quite different. The constellation of skin and gastrointestinal toxicity observed with BAY 43-9006 is common to several small-molecule kinase inhibitors, but not to antisense oligonucleotides, and might reflect differences in either target inhibition achieved intracellularly or in target specificity between the oligonucleotide and small molecule. Certainly, COAGULOPATHY has been observed with antisense oligonucleotides and so its occurrence with ISIS 5132 might not be due to inhibition of Raf kinase. So, it is unclear whether the reported toxicities for these agents are related to Raf inhibition.

MEK inhibitor CI-1040. In the mitogen-activated Ras/Raf/MEK/ERK cascade, Raf usually activates the dual-specific serine/threonine and tyrosine kinases MEK1 and MEK2, which then activate ERK1 and ERK2¹⁰⁵. MEK has not been identified as an oncogene product in human malignancies¹⁰⁶. However, it is a crucial point of convergence that integrates input from a variety of protein kinases through Ras. In addition, MEK is very restricted in its substrate specificity, with the MAPKs being the sole known substrates of importance. So, MEK is a target of great interest for the development of oncological therapeutics.

TRANSFORMING
A term that describes the processes through which normal cells acquire malignant character.

PARTIAL THROMBOPLASTIN TIME
A test to assess the function of specific proteins required to form blood clots.

ANASARCA
Generalized oedema.

ERYTHEMA
Abnormal redness of skin.

SKIN DESQUAMATION
Sloughing of skin layer.

CI-1040 (PD184352) is an orally administered, selective small-molecule inhibitor of MEK^{106,107}. This agent significantly inhibited growth of the colon carcinoma cell lines colon 26, HT-29 and colo205 in both *in vitro* and *in vivo* models. In addition to impairing tumour cell proliferation, CI-1040 blocked cell motility, disrupted the cell–cell contact inhibition that is required for invasion, and induced dose-dependent arrest of G1. Importantly, antitumour activity was achieved without evidence of toxicity, and was correlated with a reduction in the levels of activated MAPK in excised tumours¹⁰⁷. Tumours with low levels of MAPK activation seemed to be less responsive to CI-1040 in preclinical models.

CI-1040 is now undergoing Phase I evaluation in cancer patients¹⁰⁸. This agent seems to be well tolerated with continuous administration of doses of up to 800 mg twice daily with food. Common toxicities reported in the Phase I trial included fatigue, rash and diarrhoea. Pharmacokinetic results showed that CI-1040 achieved target plasma concentrations of 100–300 ng ml⁻¹ — concentrations that are expected to be biologically active on the basis of preclinical *in vivo* tumour models — following a single 800 mg dose. Both western blot and immunohistochemical analyses of blood and tumour specimens for phosphorylated MAPK (pMAPK) expression showed consistent decreases in pMAPK levels after treatment with CI-1040. One patient with pancreatic cancer achieved a confirmed partial response lasting more than six months. On the basis of this study, the dose regimen selected for Phase II testing is 800 mg twice daily with food.

To summarize this subtopic, the Ras/Raf/MEK/ERK pathway represents one of the best-characterized signalling pathways involved in the development and propagation of human cancers and, consequently, Ras, Raf and MEK have emerged as key protein kinases to target for anticancer drug design. Results from clinical trials so far indicate that both antisense oligodeoxynucleotides and small molecules that target the component proteins of this pathway are well tolerated as single agents. The early clinical trials of these relatively non-toxic agents benefited from ‘proof of principle’ biological studies, which showed that the modulation of the purported target in tumour and/or surrogate tissues from patients can be achieved in at least some patients.

Assuming that the agents that target the Ras/Raf/MEK pathway are active in some patients, further research should focus on identifying tumour characteristics that predict antitumour activity with these agents. In particular, sensitive and reliable methods to determine the molecular phenotype of tumours that are likely to be sensitive to agents that target components of the Ras/Raf/MEK pathway need to be developed and validated in clinical trials. One potential diagnostic approach to evaluating agents purported to inhibit this pathway is to assess changes in ERK activation, perhaps by measuring alterations in ERK protein phosphorylation. Preclinical studies to determine the relationship between drug concentrations in the plasma and the effects on ERK phosphorylation in surrogate tissues, such as peripheral mononuclear

blood cells, and tumour, are urgently needed to evaluate the feasibility of this approach as a pharmacodynamic assay of the effect of a drug on its target. Ultimately, it will be important to evaluate combinations of signal transduction pathway inhibitors with standard cancer therapies and with other signalling antagonists to determine the true value of these signal transduction inhibitors as cancer therapeutics.

PI3K/Akt/PTEN pathway inhibitors

PI3Ks phosphorylate phosphoinositides at the 3-hydroxyl of the inositol ring. The 3-phosphorylated phospholipids (PI3Ps) generated by PI3Ks act as membrane tethers for proteins with pleckstrin homology (PH) regions, such as Akt and phosphoinositide-dependent kinase 1 (PDK1). Binding of the PH domain of Akt to membrane PI3Ps causes the translocation of Akt to the plasma membrane, bringing Akt into contact with PDK1, which is responsible for at least one of the two phosphorylation events that are necessary to activate Akt. The tumour-suppressor phosphatase PTEN dephosphorylates phosphoinositide-3,4,5-triphosphate at the D-3 position of the inositol ring and, therefore, is a negative regulator of Akt activation. The PI3Ks, Akt and PDK1 are important in the regulation of many cellular processes including proliferation, survival, carbohydrate metabolism and motility, and there is emerging evidence that these kinases are important components of the molecular mechanisms of diseases such as cancer, diabetes and chronic inflammation^{109,110}.

Several components of the PI3K/Akt/PTEN pathway are involved in oncogenesis (reviewed in REFS 111, 112). Growth-factor-receptor protein tyrosine kinases, integrin-dependent cell adhesion, and G-protein-coupled receptors activate PI3K, both directly, and indirectly through adaptor molecules. Loss of PTEN, amplification of PI3K and overexpression of Akt have been described in many malignancies¹¹¹. In addition, persistent signalling through the PI3K/Akt pathway by stimulation of the insulin-like growth factor receptor is a mechanism of resistance to the EGFR inhibitor AG1478 (REF. 77) and trastuzumab⁷⁶. So, the PI3K/Akt/PTEN pathway is an attractive target for drug development, as such agents might inhibit proliferation, and reverse the repression of apoptosis and the resistance to cytotoxic therapy in cancer cells. Although specific inhibitors of PI3K, PDK1 and Akt have not yet reached the clinic, the rapamycin derivatives CCI-779 and RAD001 — which inhibit the downstream kinase mammalian target of rapamycin (mTOR) — are undergoing clinical evaluation.

Rapamycin and derivatives CCI-779 and RAD001. The macrolide rapamycin (sirolimus, Rapamune; Wyeth), and its derivatives CCI-779 and RAD001 (SDZ RAD, everolimus, Certican; Novartis) inhibit mTOR. In mammalian cells, mTOR is a large polypeptide kinase of 290 kDa¹¹³ that acts as a nutrient sensor and regulator of translation (reviewed in REFS 114, 115). In the presence of mitogen stimulation of the PI3K/Akt pathway and

PEARSON CORRELATION COEFFICIENT

Pearson's correlation coefficient (*r*) expresses the degree of linear relationship. Pearson's *r* values can range between −1.00 to +1.00. A correlation coefficient of +1.00 signifies a perfect positive relationship, whereas −1.00 denotes a perfect negative relationship. The smallest correlation is zero.

THROMBOCYTOPENIA

A reduction in the number of platelets.

NEUTROPENIA

A reduction in the number of neutrophils.

MUCOSITIS

Inflammation of the mucosa.

ASTHENIA

Generalized weakness and debility.

DYSPNOEA

Shortness of breath and discomfort of breathing.

URTICARIA

Red itchy skin lesions.

PRURITIS

Itchiness.

FOLLICULITIS

Inflammation around hair follicles.

LEUKOPENIA

Low white blood cell (leukocyte) count.

STOMATITIS

Inflammation of the lining of the mouth.

MYELOSUPPRESSION

Depressed production of blood cells deriving from the myeloid lineage, including platelets, some leukocytes and erythrocytes. Because many anticancer drugs suppress the growth or proliferation of rapidly dividing cells, myelosuppression is a common side effect.

PNEUMONITIS

Inflammation of the lung tissues.

sufficient nutrients, mTOR participates in the activation of p70^{S6} kinase (p70^{S6}K) and the inactivation of 4E-binding protein-1 (4E-BP1). These events, and possibly signals to other kinases, result in the activation of the translation of specific mRNA subpopulations that are important for cell proliferation and survival. Rapamycin and its derivatives bind to a member of the ubiquitous immunophilin family of FK-506-binding proteins — FKBP-12 — to inhibit mTOR. This complex interacts with mTOR, inhibiting the activation of the phosphoprotein kinase, and, subsequently, the phosphorylation of downstream targets. Preclinically, mTOR inhibitors potently suppress growth and proliferation of lymphocytes and certain tumour cell lines¹¹⁶. At present, rapamycin is approved as an immunosuppressive drug for renal transplant recipients. Related compounds are CCI-779, which is being developed as a cancer therapeutic, and RAD001, which is being developed for both indications.

All of the rapamycins under clinical development have antiproliferative activity as single agents in a variety of haematological and solid tumour systems^{117–125}. Rapamycin also augmented cisplatin-induced apoptosis in murine T-cell lines, the human promyelocytic cell line HL-60, and the human ovarian cancer cell line SKOV3¹²⁶. Interestingly, and of relevance to its use as an anti-organ-rejection agent, RAD001 inhibits growth of post-transplant lymphoproliferative disorder (PTLD)-like Epstein–Barr virus-positive lymphoblastoid B-cell line xenografts in mice¹²⁴. These results indicate that the incidence of PTLD might be reduced in organ-transplant patients that receive rapamycin or RAD001 as immunosuppressants.

CCI-779 is a soluble ester of rapamycin with impressive *in vitro* and *in vivo* cytostatic activity. Results from the National Cancer Institute's human tumour cell line screen showed that CCI-779 and rapamycin share a distinct mechanism of action, and the PEARSON CORRELATION COEFFICIENT of the *in vitro* antiproliferative activities and potencies of the two agents across the 60-cell-line screen is 0.86. *In vitro*, human T-cell leukaemia, prostate, breast and SCLC, as well as glioma and melanoma cell lines, were among the most sensitive to CCI-779, with IC₅₀ < 10^{−8} M (REF. 125). In most *in vivo* human tumour xenograft studies, CCI-779 given on an intermittent schedule caused significant inhibition of tumour growth rather than tumour regression^{125,127,128}. Preliminary results from two Phase I studies that are evaluating increasing doses of CCI-779 on different schedules have been reported^{129–132}. The first study evaluated the pharmacokinetics and biological effects of escalating doses of CCI-779 administered as a daily 30-minute intravenous infusion for five days every two weeks to patients with solid neoplasms^{129,130}. In this trial, patients received doses ranging from 0.75–19.1 mg m^{−2} d^{−1}. Severe (grade 3) toxicities included hypocalcaemia, elevation of hepatic transaminases, vomiting and THROMBOCYTOPENIA. Other toxicities generally ranged from mild to moderate and included NEUTROPENIA, rash, MUCOSITIS, diarrhoea, ASTHENIA, fever and hyperlipidaemia. Hypersensitivity phenomena including chest discomfort, DYSPNOEA, flushing and URTICARIA during CCI-779

infusions were also observed. In heavily pretreated patients, the recommended Phase II dose was 15 mg m^{−2} d^{−1}, as thrombocytopenia limited repeated dosing to 19.1 mg m^{−2} d^{−1}. The MTD in minimally pretreated patients has not been reported. One patient with NSCLC achieved a partial response, and minor antitumour responses and/or prolonged (> 4 months) stable disease were noted in patients with soft-tissue sarcoma and cervical, uterine and renal-cell carcinomas.

In the second study, CCI-779 was given as a weekly 30-minute infusion over a dose range of 7.5 to 220 mg m^{−2} week^{−1} (REFS 131, 132). Of note is that the MTD of CCI-779 had not been defined. Mild to moderate toxicities reported in this trial included skin toxicity, variously described as dryness with mild PRURITIS, eczema-like lesions, urticaria and aseptic FOLLICULITIS. Mild to moderate mucositis, nail changes, thrombocytopenia, LEUKOPENIA and anaemia, asymptomatic hyperlipidaemia, and decreased serum testosterone were also reported. Three patients had partial responses (one each with renal cell, neuro-endocrine and breast carcinomas). Although the frequency of infections was not noted to be high, five patients experienced reactivation of peri-oral herpes lesions. However, immunological analysis of blood cells did not show evidence of immunosuppression. This is consistent with the results of preclinical models, which showed that intermittent dosing schedules of the rapamycins were effective in inducing delays in tumour growth without causing prolonged immunosuppression¹²⁷.

On the basis of these Phase I studies, it seems that CCI-779 is well tolerated and has antitumour activity over a broad dose range. The most common toxicities of CCI-779 — skin reactions and STOMATITIS, hyperlipidaemia, and MYELOSUPPRESSION — are transient, generally mild to moderate in severity, and are similar to those reported for rapamycin. Of note is that rapamycin has been reported to cause PNEUMONITIS, and this toxicity might be observed with CCI-779 treatment as this agent enters broader clinical development^{133,134}. Preliminary results from Phase II trials in breast¹³⁵ and renal-cell carcinoma¹³⁶ indicated that CCI-779 is able to induce objective responses and prolong progression-free survival compared with historical data.

The clinical development pathway of CCI-779 has been fairly traditional. Phase I trials were designed to determine an MTD based on the toxicity and pharmacokinetics of the agent. However, evidence of antitumour activity was apparent over a wide dose range without evidence of limiting toxicity. So, Phase II studies have evaluated weekly intravenous doses of 25, 75 and 250 mg in randomized designs, necessitating larger studies to evaluate the antitumour activity of the different doses. Had assays to determine the degree of inhibition of mTOR by assessing the phosphorylation state of its downstream targets 4E-BP1 and/or p70^{S6}K been available and incorporated into the Phase I studies, they might have been helpful in defining a pharmacologically active dose based on optimal target inhibition. For example, assays for measuring decreases in the phosphorylation of threonine-70 of 4E-BP1 in tumour

tissue¹¹⁵, and p70^{S6}K activity¹³⁷ and ³H-thymidine incorporation^{138,139} in peripheral blood mononuclear cells, might be useful surrogates for determining the inhibition of mTOR activity by CCI-779. Although potentially useful for determining a biologically active dose, drug-induced hypophosphorylation of these molecular targets might not predict antiproliferative effects, as there is evidence that cell-cycle progression and translation can proceed despite hypophosphorylation of 4E-BP1 and p70^{S6}K by rapamycin^{121,140}. So, assessing drug effects using these targets might assist in the determination of a pharmacologically active dose, but might not predict antitumour activity of CCI-779, either because the assays are assessing targets that are not related to the effects of the drug on proliferation or, more likely, because signalling pathways parallel to, or downstream of, mTOR are rendering the cells resistant to the agent.

An alternative strategy to assess the effect of a drug on the cellular pathway would be to evaluate alterations in the expression of proteins that are under the translational control of mTOR. Through its effects on 4E-BP1 and p70^{S6}K, mTOR can modulate translation of the subsets of mRNAs that contain regulatory elements located in the 5'-untranslated regions — that is, mRNAs bearing 5'-terminal oligopolypyrimidine tracts. Assays that assess the amounts of some of these gene products might correlate with the antitumour activity of mTOR inhibitors.

Choosing the appropriate efficacy end point for Phase II studies of CCI-779 also requires careful consideration. Preclinical data indicated that CCI-779 would delay the growth of tumours, rather than induce tumour regressions. On the basis of just these preclinical results, efficacy end points other than response would have been appropriate end points for Phase II trials of CCI-779. However, the objective responses seen in the Phase I studies indicate that CCI-779 might induce apoptosis in certain tumours. The molecular profile of the tumour might be predictive of this drug effect.

On the basis of preclinical results in glioma¹²⁷, SCLC¹²⁰ and rhabdomyosarcoma^{121,122}, tumours that rely on PARACRINE OR AUTOCRINE stimulation of receptors that trigger the PI3K/Akt/mTOR pathway, or tumours with mutations that cause constitutive activation of the PI3K/Akt pathway, might depend on rapamycin-sensitive pathways for growth. In fact, *in vitro* and *in vivo* studies of *Pten*^{+/+} and *Pten*^{-/-} mouse embryonal stem cells, as well as human cancer cell lines with defined PTEN status, showed that the growth of PTEN-null cells was preferentially sensitive to CCI-779 (REFS 141–143). Taken together, these data indicate that mTOR might be a good target for cancer therapy in tumours with Akt activation that results from growth-factor dependency or loss of PTEN function. In addition, Huang and colleagues demonstrated that, in wild type, p53 cooperates in enforcing G1 cell-cycle arrest, leading to a cytostatic response to rapamycin. By contrast, rapamycin-treated tumour cells or mouse embryonic fibroblasts with deficient p53 function underwent cell cycle progression followed by apoptosis¹⁴⁴. Whether the mutational status of PTEN or p53 in human tumours will be predictive of susceptibility to rapamycins remains to be established.

PARACRINE

Describing an agent secreted from a cell that acts on other cells in the local environment.

AUTOCRINE

Describing an agent secreted from a cell that acts on the cell in which it is produced.

In summary, agents in clinical development that target the PI3K/Akt pathway are now limited to the rapamycins, which inhibit the downstream kinase mTOR. Whether modulating downstream targets will provide a better therapeutic index than directly inhibiting PI3K/Akt is an unanswered question, pending clinical evaluation of inhibitors specific for these proteins. Certainly, general principles that have been previously elucidated can be applied to the clinical development of agents that specifically inhibit PI3K/Akt. It would be preferable to have a robust method to identify PI3K/Akt/PTEN pathway activation and signalling that can be applied to pathological specimens to select the patient population in which to study these agents. Given the differential cellular responses to mTOR inhibitors, based on the presence of wild-type or mutated p53 reported by Huang, it would be interesting to evaluate both PI3K pathway activation and p53 mutations as predictors of the antitumour activity of rapamycins and other inhibitors of PI3K/Akt in laboratory models. End points for assessing antitumour activity in clinical trials could then be based on whether tumour regression or tumour stasis was seen in the analogous preclinical models.

Conclusions and future directions

The agents discussed in this review illustrate, in different ways, how the success of STI571 might be an isolated case and might not be repeated by other kinase inhibitors that are brought to the clinic, unless we alter the clinical evaluation of these molecules. This follows on from our belief that the scientific and technological investment in identifying protein kinase targets, and lead compounds to modulate them, have not been complemented by a similar investment in diagnostic strategies to ensure the rational clinical development of these leads. In contrast to the well-recognized development pathway of a cytotoxic agent — Phase I studies defining dose on the basis of the occurrence of toxicity, that give rise to screening Phase II studies using the end point of tumour response as an indicator of antitumour activity, followed by Phase III trials that compare the survival or quality of life of patients receiving the new agent with that of patients receiving standard therapy — we propose that the development of a molecularly targeted agent, including the kinase inhibitors discussed here, requires a different path (TABLE 2).

Specifically, when a protein kinase inhibitor is introduced into the clinic, it should be evaluated in a patient population in which the importance of the targeted kinase in the economy of the neoplasm is clear in advance, as was the case with STI571 in CML. Alternatively, diagnostic strategies — including gene-expression array-pattern subsetting and proteomic examination of kinase substrate proteins — could be used to prospectively identify subsets of patients for whose tumours the importance of the kinase is established. If scientific knowledge is limited so that patients with tumours likely to be sensitive to the kinase inhibitor cannot be identified in advance, at the very least such diagnostic assays could be used to retrospectively

Table 2 | **Empirical and rational development of protein kinase inhibitors**

Phase of development	Empirical development	Targeted development
Preclinical evaluation	Lead compound identification and optimization <i>In vitro</i> and <i>in vivo</i> activity Toxicology Pharmacokinetics	Lead compound identification and optimization <i>In vitro</i> and <i>in vivo</i> activity Toxicology Pharmacokinetics Target/pathway modulation seen Predictive factors for activity identified Assays developed for clinical trials
Phase I	Determine dose for Phase II/III based on toxicity and pharmacokinetics Dose escalation and size of cohorts determined by likelihood of toxicity	Determine dose for Phase II/III based on target modulation, toxicity and pharmacokinetics Dose escalation and size of cohorts determined by likelihood of toxicity and target modulation
Phase II evaluation	Determine antitumour activity Clinical endpoint determined by preclinical results Patient population defined by histology and stage of disease Dose(s) based on toxicity and pharmacokinetics	Determine antitumour activity Clinical endpoint determined by preclinical results Patient population defined by histology, stage of disease and presence of target/marker predictive of drug activity Dose(s) based on target modulation
Phase III evaluation	Clinical benefit Patient population defined by histology and stage of disease Dose(s) based on toxicity and pharmacokinetics	Clinical benefit Patient population defined by histology, stage of disease and presence of target/marker predictive of drug activity Dose(s) based on target modulation

investigate tumour samples that have been collected from enrolled patients to generate hypotheses about molecular markers of drug effect that could be definitively tested in subsequent clinical trials. We propose that a Phase I study of a targeted agent, which evaluates escalating doses on a schedule that is concordant with efficacy in animal models, would be designed to define an MTD. We recognize that an MTD might be higher than a 'biologically effective dose', but we propose that the first step in defining a biologically active dose occurs by identifying the 'upper boundary conditions' of an MTD.

In another significant deviation from 'standard operating procedure', a concerted effort to define target modulation by the inhibitor should occur at the proposed recommended Phase II dose, before initiating Phase II studies to evaluate antitumour activity. In what might be defined as 'pre-Phase II' or 'Phase IIA' studies, comparisons between different schedules and doses to induce consistent modulation of the target across different tumour types should be undertaken. It is at this stage that the appreciation of a lower biologically effective dose might become apparent, and allow a de-escalation of dose on certain schedules. This would require that relevant, robust assays be available before reaching that point in the clinical development. Correlating target modulation in conjunction with drug pharmacology, clinical tumour response, time to progression on study, and ancillary imaging studies (such as those using glucose or choline uptake) can facilitate crucial decisions about the optimal design of the *bona fide* Phase II study to evaluate the antitumour activity of the agent. If, on the basis of preclinical models or results from early clinical trials, the agent is

expected to induce tumour regression, then single-arm Phase II trials with tumour response as the primary end point in an appropriately selected patient population would be sufficient. However, if the agent is expected to delay tumour growth, or is being evaluated in combination with an active cytotoxic regimen, it would be preferable to evaluate its activity in a trial that is designed to be appropriately powered to assess the activity of the new agent versus an appropriately matched population treated with a 'standard' approach. A study design with a current control is particularly important if the patients enrolled in the study are a molecularly defined subset for which there is no appropriate historical data for comparison. In such a study, both the clinical end points of tumour regression and time to tumour progression, as well as the effect on relevant surrogate markers, would be formal end points. At this point in clinical development, effects on the target might be less important as a trial end point, if these features were considered in the pre-Phase II period. Dropping the requirement for identifying target modulation might allow more rapid accrual in Phase II studies, as might allowing crossover between treatment arms. This strategy of defining a biologically active dose in a suitable patient population would allow definitive Phase III trials to be designed with greater confidence of favourable effect on a clinical benefit end point that is required for drug approval. In addition, in the United States, accelerated approval could be granted at the end of Phase II if the agent is shown to induce durable responses in a significant proportion of patients in the Phase II study for whom no effective therapy has previously been identified, as was the case with STI571 in GIST.

Few would argue that it would be preferable to develop targeted agents that are predicted to be relatively non-toxic by identifying a dose on the basis of optimal target modulation and testing activity in patients with tumours that are most likely to be sensitive to the agent on the basis of molecular phenotype. The reasons such approaches have not been taken are related to the limitations in current scientific knowledge — as regards the therapeutic relevance of the expression of many molecular targets in cancer cells and the clinical significance of target modulation by a purported inhibitor — and in technologies to measure the molecular and cellular effects in tumours/surrogate tissue. Given these limitations in science and technology, as well as the extra resources and time required to develop assays to assess target effects and to identify predictive markers, it is not surprising that the efforts to develop assays for use in clinical trials to select dose and patient population have been minimal. Certainly, such efforts might not be considered worthwhile if trials conducted with clinically defined end points in unselected patient populations can yield favourable results. We would argue that the likelihood of a favourable result is greater if assays to select dose and patients for trials were incorporated into the clinical development of these agents, and were conscientiously investigated before larger Phase II/III studies were undertaken.

As described previously, most Phase I trials of kinase inhibitors have been designed to identify the MTD on the basis of the occurrence of toxicity. In the absence of limiting toxicity, trials have been designed to define dose on the basis of pharmacokinetic parameters, such as the achievement of a plasma concentration or exposure that is predicted to be biologically active on the basis of pre-clinical models. In this regard, they do not differ greatly from our proposal, with the exception that, in most cases, conscientious effort to build a 'database' of pre- and post-treatment tissues for genomic or proteomic investigation has not occurred. Phase II/III studies have been designed to evaluate antitumour activity using objective response or time to disease progression/progression-free survival, without the effort to match drug to the biologically relevant patient population to elicit tumour effect. In these respects, the current drug development approach differs greatly from what we propose.

When questions about the appropriate dose have remained at the end of Phase I evaluation, Phase II/III trials have been designed to randomize patients with tumours that are histologically defined to different doses, necessitating larger sample sizes to adequately address both the dose and activity questions. Such studies might not be necessary, or might require fewer patients, if the dose that optimally inhibits the target is determined by evaluating the effects of the agent directly on the target pathway, rather than assessing clinical outcome and if the study population is enriched with patients with tumours that have the molecular phenotype likely to respond favourably to the kinase inhibitor. Similarly, the development of a kinase inhibitor as a modulator of chemotherapy-induced cytotoxicity would ideally proceed only if the presence and function of the kinase target

could clearly be related to the efficacy of the cytotoxic activity. In addition, there should be clear evidence that the target, or its downstream effector molecules, are modulated at the doses and schedules used. If care is not taken to do this, then the use of a design that combines the new agent with a cytotoxic agent, versus the cytotoxic agent alone, might miss modest but clinically relevant augmentation of antitumour effect achieved by addition of the kinase inhibitor. In the future, study populations that are enrolled in Phase II and III trials of kinase inhibitors might actually cut across histologies and define study populations on the basis of the presence and function of a particular target. By selecting patients with tumours that are most likely to be sensitive to a targeted agent, and by having a clear understanding of the drug's effects on its target(s), Phase II trials to screen for antitumour effects and Phase III trials that are designed to show definitive evidence of benefit might actually require fewer patients than the number used by current, biologically uninformed clinical development strategies.

A sceptic might posit that although identifying dose and selecting patients based on target expression/modulation is very interesting from an academic perspective, corporate disinterest will dictate the demise of an agent that does not show single-agent activity in neoplasms of sufficient prevalence to constitute an economically viable market. This narrow perspective ignores emerging data that show that combinations of signalling agents, each at concentrations that are relatively non-toxic, have promising activity in preclinical systems^{145–148}. If it were clear that individual kinase inhibitors were hitting their targets on a given dose and schedule, then a rational basis for constructing combination regimes with other anti-signalling agents would emerge. Whereas combination chemotherapy regimens were devised on the basis of non-overlapping toxicity, combinations of targeted agents would be designed on the basis of the elimination of complementary signalling pathway(s), which could potentially result in greater therapeutic effect than would be predicted by the results of studies that evaluate single-agent activity. It is to be hoped that colleagues in the pharmaceutical industry are aware of this possibility, which can be rationally pursued only with the evaluation of targeted therapies in combination.

The most straightforward development pathway for targeted combinations and modulators of cytotoxic agents would involve the following: first, preclinical evidence from several model systems that demonstrated the inactivity of the agents singly and significant activity of the combination; second, clinical evidence of the safety of each of the agents and of the combination; and third, clinical evidence of the lack of activity/less activity of each of the agents singly, and greater activity of the combination. The requisite clinical data could be generated from single-agent and combination Phase I studies — demonstrating the safety and tolerability of the agents alone and in combination at doses/schedules that modulate purported target(s) — and from single-agent and combination Phase II studies. If well-designed, single-agent Phase II studies do not show

significant antitumour effects compared with the combination, then Phase III could be designed to evaluate the combination or the addition of the combination to standard therapy. There are no regulatory restrictions on the development of combinations of agents if the safety/tolerability and activity of the single agents and the combination are appropriately defined. Arguably, the development of leucovorin — a drug without intrinsic antitumour effect — in combination with 5-fluorouracil for the treatment of patients with CRC, is an example of the successful development of a molecularly targeted bio-modulating agent in combination with a cytotoxic agent.

With the notable exception of STI571 and trastuzumab — the only kinase inhibitors that have clearly been shown to provide benefit so far — Phase III trials evaluating the clinical benefit of targeted agents in cancer patients have not been designed to evaluate the effects of the targeted agent in a patient population that has been selected for target expression. We recognize that selecting patients on the basis of the presence of a target or another marker that is thought to be predictive of the antitumour activity of the agent might miss important activity in patients with tumours that do not have measurable expression of the purported predictive marker. This concern is reasonable given the limitations of our understanding of cancer biology and drug activity, and the dearth of reliable, sensitive assays to assess clinical specimens for marker expression. However, trials of targeted therapies must have adequate numbers of patients to account for the dilution of benefit that might occur if patients with tumours that express the target are included with patients whose tumours do not express the target. With standard cytotoxic treatments that target DNA synthesis or the mitotic spindle, 'target' expression is ubiquitous, and the targeted process — cell proliferation — is clearly required for cancer progression. By contrast, targeted therapies are likely to have activity in only a fraction of patients, either due to a lack of target expression or to the irrelevance of the targeted signalling pathways to tumour growth/proliferation.

This molecular heterogeneity can have a profound effect on the sample size needed to ensure adequate power to exclude a false-negative result, depending on

the prevalence of target expression among the patients enrolled in the study and on the magnitude of effect in patients with tumours that are 'sensitive' to the agent¹⁴⁹. For example, the effect of the prevalence of target expression on sample size and power is such that the Phase III trial evaluating the addition of trastuzumab to chemotherapy, which demonstrated a 22.5% improvement in overall survival among 469 metastatic breast cancer patients with HER2-overexpressing tumours (a molecular abnormality that occurs in 25–30% of breast cancers¹⁵⁰), would have been negative had the agent been evaluated in breast cancer patients without regard to HER2 expression.

In summary, at each stage in the development of kinase inhibitors, and perhaps the development of any 'molecularly targeted' agent, we need to conscientiously incorporate assays to assess the suitability of the patient population, the target and the effects of the target. We believe that attention to this need could improve the efficiency of the evaluation of an agent and the probability of success. We recognize that this approach might differ substantially from the approach that is ingrained in oncological drug development, and will require a 'culture change' on the part of clinical trialists and their sponsoring organizations. Phase I trials that evaluate the effects of an agent on a target could identify a biologically active dose, which might be below the toxic dose, and thereby require fewer dose escalations. Phase II trials that evaluate effects in patients with molecularly defined tumours could assess activity within subsets, which could clearly inform the design of definitive Phase III trials. The alternative to these carefully constructed efforts to define value in these newer agents is the risk that potentially useful approaches will be inefficiently evaluated or perhaps prematurely discarded. These improvements argue for the investment of different types of resources during both the preclinical and clinical development of an agent, and call for a partnering between academic, corporate and scientific funding organizations to ensure that the very best clinical and scientific questions are asked and appropriately answered with these potentially important new additions to the therapeutic armamentarium.

- Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298**, 1912–1934 (2002).
- Druker, B. J. Inhibition of the Bcr-Abl tyrosine kinase as a therapeutic strategy for CML. *Oncogene* **21**, 8541–8546 (2002).
- Joensuu, H. *et al.* Management of malignant gastrointestinal stromal tumours. *Lancet Oncol.* **3**, 655–664 (2002).
- Rubin, B. P. *et al.* Molecular targeting of platelet-derived growth factor B by imatinib mesylate in a patient with metastatic dermatofibrosarcoma protuberans. *J. Clin. Oncol.* **20**, 3586–3591 (2002).
- Blume-Jensen, P. & Hunter, T. Oncogenic kinase signalling. *Nature* **411**, 355–365 (2001).
- Scappaticci, F. A. Mechanisms and future directions for angiogenesis-based cancer therapies. *J. Clin. Oncol.* **20**, 3906–3927 (2002).
- Kerbel, R. & Folkman, J. Clinical translation of angiogenesis inhibitors. *Nat. Rev. Cancer* **2**, 727–739 (2002).
- Madhusudan, S. & Harris, A. L. Drug inhibition of angiogenesis. *Curr. Opin. Pharmacol.* **2**, 403–414 (2002).
- Yamada, K. M. & Araki, M. Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. *J. Cell Sci.* **114**, 2375–2382 (2001).
- Trusolino, L. & Comoglio, P. M. Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat. Rev. Cancer* **2**, 289–300 (2002).
- Davies, H. *et al.* Mutations of the *BRAF* gene in human cancer. *Nature* **417**, 949–954 (2002).
- A comprehensive evaluation of cell lines and tumour histologies for a specific activating mutation that has led to the identification of a subset of tumours that might be evaluated with Raf-targeting agents.**
- van de Vijver, M. Emerging technologies for HER2 testing. *Oncology* **63** (Suppl. 1), 33–38 (2002).
- Druker, B. J. *et al.* Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N. Engl. J. Med.* **344**, 1038–1042 (2001).
- Seminal paper demonstrating that the activity of a small-molecule tyrosine kinase inhibitor in a molecularly defined disease might be reduced in the presence of additional molecular abnormalities.**
- Davis, R. E. & Staudt, L. M. Molecular diagnosis of lymphoid malignancies by gene expression profiling. *Curr. Opin. Hematol.* **9**, 333–338 (2002).
- Shipp, M. A. *et al.* Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. *Nat. Med.* **8**, 68–74 (2002).
- van 't Veer, L. J. *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530–536 (2002).
- Bhattacharjee, A. *et al.* Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc. Natl Acad. Sci. USA* **98**, 13790–13795 (2001).
- Singh, D. *et al.* Gene expression correlates of clinical prostate cancer behavior. *Cancer Cell* **1**, 203–209 (2002).
- References 14–18 demonstrate that there are subsets of tumours within histologies with different clinical outcomes that can be defined by gene expression.**
- Seimiya, H., Mashima, T., Toho, M. & Tsunuro, T. c-Jun NH2-terminal kinase-mediated activation of interleukin-1? converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. *J. Biol. Chem.* **272**, 4631–4636 (1997).
- Bulavin, D. V., Amundson, S. A. & Fornace, A. J. p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphony. *Curr. Opin. Genet. Dev.* **12**, 92–97 (2002).

21. Grant, S. & Jarvis, W. D. Modulation of drug-induced apoptosis by interruption of the protein kinase C signal transduction pathway: a new therapeutic strategy. *Clin. Cancer Res.* **2**, 1915–1920 (1996).
22. Nagar, B. *et al.* Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res.* **62**, 4236–4243 (2002).
23. Levitzki, A. The selectivity of small molecules towards protein tyrosine kinases. *Ernst Schering Res. Found. Workshop* **34**, 71–80 (2001).
24. Levitzki, A. & Bohmer, F. D. Altered efficacy and selectivity of tyrosine kinase inhibitors of the activated states of protein tyrosine kinases. *Anticancer Drug Des.* **13**, 731–734 (1998).
25. Levitzki, A., Gazit, A., Osherov, N., Posner, I. & Gilon, C. Inhibition of protein-tyrosine kinases by tyrphostins. *Methods Enzymol.* **201**, 347–361 (1991).
26. Neckers, L. Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol. Med.* **8**, S55–S61 (2002).
27. Kerkhoff, E. & Rapp, U. R. The Ras-Raf relationship: an unfinished puzzle. *Adv. Enzyme Regul.* **41**, 261–267 (2001).
28. Fan, Z., Baselga, J., Masui, H. & Mendelsohn, J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus cis-diamminedichloroplatinum on well established A431 cell xenografts. *Cancer Res.* **53**, 4637–4642 (1993).
29. Sirotnak, F. M., Zakowski, M. F., Miller, V. A., Scher, H. I. & Kris, M. G. Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin. Cancer Res.* **6**, 4885–4892 (2000).
30. Monks, A. *et al.* UCN-01 enhances the *in vitro* toxicity of clinical agents in human tumor cell lines. *Invest. New Drugs* **18**, 95–107 (2000).
31. Schwartz, G. K., Farsi, K., Maslak, P., Kelsen, D. P. & Spriggs, D. Potentiation of apoptosis by flavopiridol in mitomycin-C-treated gastric and breast cancer cells. *Clin. Cancer Res.* **3**, 1467–1472 (1997).
32. Wilkinson, E. Surprise phase III failure for ZD1839. *Lancet Oncol.* **3**, 583 (2002).
33. O'Connor, D. S., Wall, N. R., Porter, A. C. & Altieri, D. C. A p34/cdc2 survival checkpoint in cancer. *Cancer Cell* **2**, 43–54 (2002).
34. Wall, N. R., O'Connor, D. S., Plescia, J., Pommier, Y. & Altieri, D. C. Suppression of survivin phosphorylation on Thr34 by flavopiridol enhances tumor cell apoptosis. *Cancer Res.* **63**, 230–235 (2003).
35. Kuenen, B. C. *et al.* Dose-finding and pharmacokinetic study of cisplatin, gemcitabine, and SU5416 in patients with solid tumors. *J. Clin. Oncol.* **20**, 1657–1667 (2002).
36. Manley, P. W., Martiny-Baron, G., Schlaeppli, J. M. & Wood, J. M. Therapies directed at vascular endothelial growth factor. *Expert Opin. Investig. Drugs* **11**, 1715–1736 (2002).
37. Nahta, R., Iglehart, J. D., Kempkes, B. & Schmidt, E. V. Rate-limiting effects of Cyclin D1 in transformation by ErbB2 predicts synergy between hereceptin and flavopiridol. *Cancer Res.* **62**, 2267–2271 (2002).
38. Dai, Y. *et al.* Pharmacological inhibitors of the mitogen-activated protein kinase (MAPK) kinase/MAPK cascade interact synergistically with UCN-01 to induce mitochondrial dysfunction and apoptosis in human leukemia cells. *Cancer Res.* **61**, 5106–5115 (2001).
39. Joensuu, H. *et al.* Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N. Engl. J. Med.* **344**, 1052–1056 (2001).
- Seminal paper demonstrating the remarkable activity of small-molecule tyrosine kinase inhibitor in a malignancy defined by a specific activating mutation and for which previously there was no effective treatment.**
40. Webb, C. P. *et al.* The geldanamycins are potent inhibitors of the hepatocyte growth factor/scatter factor-met-urokinase plasminogen activator-plasmin proteolytic network. *Cancer Res.* **60**, 342–349 (2000).
41. Ntziachristos, V., Tung, C. H., Bremer, C. & Weissleder, R. Fluorescence molecular tomography resolves protease activity *in vivo*. *Nat. Med.* **8**, 757–760 (2002).
42. Gallardo, G. *et al.* Regulation by ceramide of epidermal growth factor signal transduction and mitogenesis in cell lines overexpressing the growth factor receptor. *Cell Mol. Biol. (Noisy-le-grand)* **46**, 1305–1312 (2000).
43. Eccles, S. A. Cell biology of lymphatic metastasis. The potential role of c-erbB oncogene signalling. *Recent Results Cancer Res.* **157**, 41–54. (2000).
44. Sieg, D. J. *et al.* FAK integrates growth-factor and integrin signals to promote cell migration. *Nat. Cell Biol.* **2**, 249–256 (2000).
45. Ciardiello, F. & Tortora, G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin. Cancer Res.* **7**, 2958–2970 (2001).
46. Ciardiello, F. *et al.* Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin. Cancer Res.* **6**, 2053–2063 (2000).
47. Milas, L. *et al.* *In vivo* enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clin. Cancer Res.* **6**, 701–708 (2000).
48. Huang, S. M. & Harari, P. M. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin. Cancer Res.* **6**, 2166–2174 (2000).
49. Pollack, V. A. *et al.* Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition *in situ* and antitumor effects in athymic mice. *J. Pharmacol. Exp. Ther.* **291**, 739–748 (1999).
50. Yang, X.-D., Wang, P., Fredlin, C. & Davis, C. G. ABX-EGF, a fully human anti-EGF receptor monoclonal antibody: inhibition of prostate cancer *in vitro* and *in vivo*. *Proc. Am. Soc. Clin. Oncol.* **21**, 2454 (2002).
51. Lynch, D. H. & Yang, X. D. Therapeutic potential of ABX-EGF: a fully human anti-epidermal growth factor receptor monoclonal antibody for cancer treatment. *Semin. Oncol.* **29**, 47–50 (2002).
52. Yang, X. D., Jia, X. C., Corvalan, J. R., Wang, P. & Davis, C. G. Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy. *Crit. Rev. Oncol. Hematol.* **38**, 17–23 (2001).
53. Hambek, M. *et al.* Tumor necrosis factor α sensitizes low epidermal growth factor receptor (EGFR)-expressing carcinomas for anti-EGFR therapy. *Cancer Res.* **61**, 1045–1049 (2001).
54. Bishop, P. C. *et al.* Differential sensitivity of cancer cells to inhibitors of the epidermal growth factor receptor family. *Oncogene* **21**, 119–127 (2002).
- Interesting attempt to correlate sensitivity to different EGFR inhibitors of the NCI 60 cell line screen and molecular profile.**
55. Mendelsohn, J. Targeting the epidermal growth factor receptor for cancer therapy. *J. Clin. Oncol.* **20**, S1–S13 (2002).
56. Baselga, J. *et al.* Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. *J. Clin. Oncol.* **18**, 904–914 (2000).
57. Figlin, R. A. *et al.* ABX-EGF, a fully human anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) in patients with advanced cancer: phase 1 clinical results. *Proc. Am. Soc. Clin. Oncol.* **21**, 35 (2002).
58. Kahn, M. E., Senderowicz, A., Sausville, E. A. & Barrett, K. E. Possible mechanisms of diarrhea side effects associated with the use of a novel chemotherapeutic agent, flavopiridol. *Clin. Cancer Res.* **7**, 343–349 (2001).
59. Swaisland, H., Smith, R. P., Farebrother, J. & Laight, A. The effect of the induction and inhibition of CYP3A4 on the pharmacokinetics of single oral doses of ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), in healthy male volunteers. *Proc. Am. Soc. Clin. Oncol.* **21**, 328 (2002).
60. Baselga, J. *et al.* Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. *J. Clin. Oncol.* **20**, 4292–4302 (2002).
61. Herbst, R. S. *et al.* Selective oral epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 is generally well-tolerated and has activity in non-small-cell lung cancer and other solid tumors: results of a phase I trial. *J. Clin. Oncol.* **20**, 3815–3825 (2002).
62. Hidalgo, M. *et al.* Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *J. Clin. Oncol.* **19**, 3267–3279 (2001).
63. Albanell, J. *et al.* Pharmacodynamic studies of the epidermal growth factor receptor inhibitor ZD1839 in skin from cancer patients: histopathologic and molecular consequences of receptor inhibition. *J. Clin. Oncol.* **20**, 110–124 (2002).
- One of the few papers to incorporate a number of pharmacodynamic assays into**
- a Phase I trial of a tyrosine kinase inhibitor**
64. Saltz, L. *et al.* Single agent IMC-C225 (Erlotinib) has activity in CPT-11-refractory colorectal cancer (CRC) that expresses the epidermal growth factor receptor (EGFR). *Proc. Am. Soc. Clin. Oncol.* **21**, 504 (2002).
65. Perez-Soler, R. *et al.* A phase II trial of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor OSI-774, following platinum-based chemotherapy, in patients (pts) with advanced, EGFR-expressing, non-small cell lung cancer (NSCLC). *Proc. Am. Soc. Clin. Oncol.* **20**, 1235 (2001).
66. Kris, M. G. *et al.* A phase II trial of ZD1839 (Iressa) in advanced non-small cell lung cancer (NSCLC) patients who had failed platinum- and docetaxel-based regimens (IDEAL 2). *Proc. Am. Soc. Clin. Oncol.* **21**, 1166 (2002).
67. Fukuoka, M. *et al.* Final results from a phase II trial of ZD1839 (Iressa) for patients with advanced non-small-cell lung cancer (IDEAL 1). *Proc. Am. Soc. Clin. Oncol.* **21**, 1188 (2002).
68. Senzer, N. *et al.* Phase 2 evaluation of OSI-774, a potent oral antagonist of the EGFR-TK in patients with advanced squamous cell carcinoma of the head and neck. *Proc. Am. Soc. Clin. Oncol.* **20**, 6 (2001).
69. Cohen, E. E. *et al.* Phase II study of ZD1839 (Iressa) in recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN). *Proc. Am. Soc. Clin. Oncol.* **21**, 899 (2002).
70. Finkler, N. *et al.* Phase 2 evaluation of OSI-774, a potent oral antagonist of the EGFR-TK in patients with advanced ovarian carcinoma. *Proc. Am. Soc. Clin. Oncol.* **20**, 831 (2001).
71. Saltz, L. *et al.* Cetuximab (IMC-C225) plus Irinotecan (CPT-11) is active in CPT-11-refractory colorectal cancer (CRC) that expresses epidermal growth factor receptor (EGFR). *Proc. Am. Soc. Clin. Oncol.* **20**, 7 (2001).
72. Baselga, J. *et al.* Cetuximab (C225) plus cisplatin/carboplatin is active in patients (pts) with recurrent/metastatic squamous cell carcinoma of the head and neck (SCCHN) progressing on a same dose and schedule platinum-based regimen. *Proc. Am. Soc. Clin. Oncol.* **21**, 900 (2002).
73. Burtness, B. A., Li, Y., Flood, W., Mattar, B. I. & Forastiere, A. A. Phase III trial comparing cisplatin (C) + placebo (P) to C + anti-epidermal growth factor antibody (EGF-R) in patients (pts) with metastatic/recurrent head and neck cancer (HNC). *Proc. Am. Soc. Clin. Oncol.* **21**, 901 (2002).
74. Yarden, Y. & Slivkowsky, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell Biol.* **2**, 127–137 (2001).
75. Normanno, N. *et al.* Cooperative inhibitory effect of ZD1839 (Iressa) in combination with trastuzumab (Herceptin) on human breast cancer cell growth. *Ann. Oncol.* **13**, 65–72 (2002).
76. Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D. & Pollak, M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J. Natl Cancer Inst.* **93**, 1852–1857 (2001).
77. Chakravarti, A., Loeffler, J. S. & Dyson, N. J. Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling. *Cancer Res.* **62**, 200–207 (2002).
78. Grunicke, H. H. & Maly, K. Role of GTPases and GTPase regulatory proteins in oncogenesis. *Crit. Rev. Oncog.* **4**, 389–402 (1993).
79. Magee, T. & Marshall, C. New insights into the interaction of Ras with the plasma membrane. *Cell* **98**, 9–12 (1999).
80. Lopez-Iasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S. & Wetzker, R. Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science* **275**, 394–397 (1997).
81. Kerkhoff, E. & Rapp, U. R. Cell cycle targets of Ras/Raf signalling. *Oncogene* **17**, 1457–1462 (1998).
82. Pruitt, K. & Der, C. J. Ras and Rho regulation of the cell cycle and oncogenesis. *Cancer Lett.* **171**, 1–10 (2001).
83. Pollock, P. M. & Meltzer, P. S. Lucky draw in the gene raffle. *Nature* **417**, 906–907 (2002).
84. Sekido, Y., Fong, K. M. & Minna, J. D. Progress in understanding the molecular pathogenesis of human lung cancer. *Biochim. Biophys. Acta* **1378**, F21–59 (1998).
85. Stanton, V. P. Jr. & Cooper, G. M. Activation of human raf transforming genes by deletion of normal amino-terminal coding sequences. *Mol. Cell Biol.* **7**, 1171–1179 (1987).
86. Kolch, W. *et al.* Protein kinase C α activates RAF-1 by direct phosphorylation. *Nature* **364**, 249–252 (1993).
87. Cornwell, M. M. & Smith, D. E. A signal transduction pathway for activation of the mdm1 promoter involves the proto-oncogene C-raf kinase. *J. Biol. Chem.* **268**, 15347–15350 (1993).
88. Monia, B. P. First- and second-generation antisense oligonucleotide inhibitors targeted against human C-raf kinase. *Ciba Found. Symp.* **209**, 107–119 (1997).
89. McPhillips, F. *et al.* Association of c-Raf expression with survival and its targeting with antisense oligonucleotides in ovarian cancer. *Br. J. Cancer* **85**, 1753–1758 (2001).
90. Lau, Q. C., Brusselbach, S. & Muller, R. Abrogation of c-Raf expression induces apoptosis in tumor cells. *Oncogene* **16**, 1899–1902 (1998).
91. Langdon, S. *et al.* Antisense oligonucleotide (ISIS 5132) targeting of C-raf kinase in ovarian cancer models. *Proc. Am. Soc. Clin. Oncol.* **20**, 833 (2001).

92. Geiger, T., Muller, M., Monia, B. P. & Fabbro, D. Antitumor activity of a C-raf antisense oligonucleotide in combination with standard chemotherapeutic agents against various human tumors transplanted subcutaneously into nude mice. *Clin. Cancer Res.* **3**, 1179–1185 (1997).
 93. Stevenson, J. P. *et al.* Phase I clinical/pharmacokinetic and pharmacodynamic trial of the c-raf-1 antisense oligonucleotide ISIS 5132 (CGP 69846A). *J. Clin. Oncol.* **17**, 2227–2236 (1999).
 94. Cunningham, C. C. *et al.* A phase I trial of C-raf kinase antisense oligonucleotide ISIS 5132 administered as a continuous intravenous infusion in patients with advanced cancer. *Clin. Cancer Res.* **6**, 1626–1631 (2000).
 95. Rubin, E. *et al.* Phase I trial of the farnesyl protein transferase (FPTase) inhibitor L-778123 on a 14- or 28-day dosing schedule. *Proc. Am. Soc. Clin. Oncol.* **21**, 689 (2000).
 96. O'Dwyer, P. J. *et al.* c-Raf-1 depletion and tumor responses in patients treated with the c-raf-1 antisense oligodeoxynucleotide ISIS 5132 (CGP 69846A). *Clin. Cancer Res.* **5**, 3977–3982 (1999).
 97. Couderc, B. *et al.* Phase II trial with ISIS 5132 in patients with small-cell (SCLC) and non-small cell (NSCLC) lung cancer. A European Organization for Research and Treatment of Cancer (EORTC) early clinical studies group report. *Eur. J. Cancer* **37**, 2194–2198 (2001).
 98. Tolcher, A. W. *et al.* A randomized phase II and pharmacokinetic study of the antisense oligonucleotides ISIS 3521 and ISIS 5132 in patients with hormone-refractory prostate cancer. *Clin. Cancer Res.* **8**, 2530–2535 (2002).
 99. Cripps, M. C. *et al.* Phase II randomized study of ISIS 3521 and ISIS 5132 in patients with locally advanced or metastatic colorectal cancer: a National Cancer Institute of Canada clinical trials group study. *Clin. Cancer Res.* **8**, 2188–2192 (2002).
 100. Wilhelm, S. & Chien, D.-S. BAY 43-9006. *Curr. Pharm. Des.* **8**, 2255–2257 (2002).
 101. Vincent, P. *et al.* Chemotherapy with the raf kinase inhibitor BAY 43-9006 in combination with irinotecan, vinorelbine, or gemcitabine is well tolerated and efficacious in preclinical xenograft models. *Proc. Am. Soc. Clin. Oncol.* **21**, 1900 (2002).
 102. Strumberg, D. *et al.* Final results of a phase I pharmacokinetic and pharmacodynamic study of the raf kinase inhibitor BAY 43-9006 in patients with solid tumors. *Proc. Am. Soc. Clin. Oncol.* **21**, 121 (2002).
 103. Moore, M. *et al.* Phase I study of the Raf-1 kinase inhibitor BAY 43-9006 in patients with advanced refractory solid tumors. *Proc. Am. Soc. Clin. Oncol.* **21**, 1816 (2002).
 104. Hilger, R. A. *et al.* Inhibition of ERK phosphorylation and clinical outcome in patients treated with the Raf kinase inhibitor BAY 43-9006. *Proc. Am. Soc. Clin. Oncol.* **21**, 1916 (2002).
 105. Catling, A. D., Schaeffer, H. J., Reuter, C. W., Reddy, G. R. & Weber, M. J. A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function. *Mol. Cell Biol.* **15**, 5214–5225 (1995).
 106. Sebolt-Leopold, J. S. Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene* **19**, 6594–6599 (2000).
 107. Sebolt-Leopold, J. S. *et al.* Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. *Nat. Med.* **5**, 810–816 (1999).
 108. LoRusso, P. M. *et al.* A phase I clinical and pharmacokinetic evaluation of the oral MEK inhibitor, CI-1040, administered for 21 consecutive days, repeated every 4 weeks in patients with advanced cancer. *Proc. Am. Soc. Clin. Oncol.* **21**, 321 (2002).
 109. Stein, R. C. Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment. *Endocr. Relat. Cancer* **8**, 237–248 (2001).
 110. Stein, R. C. & Waterfield, M. D. PI3-kinase inhibition: a target for drug development? *Mol. Med. Today* **6**, 347–357 (2000).
 111. Vivanco, I. & Sawyers, C. L. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat. Rev. Cancer* **2**, 489–501 (2002).
 112. Cantley, L. C. The phosphoinositide 3-kinase pathway. *Science* **296**, 1655–1657 (2002).
 113. Sabers, C. J. *et al.* Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* **270**, 815–822 (1995).
 114. Schmelzle, T. & Hall, M. N. TOR, a central controller of cell growth. *Cell* **103**, 253–262. (2000).
 115. Rohde, J., Heitman, J. & Cardenas, M. E. The TOR kinases link nutrient sensing to cell growth. *J. Biol. Chem.* **276**, 9583–9586 (2001).
 116. Proud, C. G. & Denton, R. M. Molecular mechanisms for the control of translation by insulin. *Biochem. J.* **328**, 329–341 (1997).
 117. Douros, J. & Suffness, M. New antitumor substances of natural origin. *Cancer Treat. Rev.* **8**, 63–87 (1981).
 118. Eng, C. P., Sehgal, S. N. & Vezina, C. Activity of rapamycin (AY-22,989) against transplanted tumors. *J. Antibiot. (Tokyo)* **37**, 1231–1237 (1984).
 119. Muthukkumar, S., Ramesh, T. M. & Bondada, S. Rapamycin, a potent immunosuppressive drug, causes programmed cell death in B lymphoma cells. *Transplantation* **60**, 264–270 (1995).
 120. Seufferlein, T. & Rozengurt, E. Rapamycin inhibits constitutive p70s6k phosphorylation, cell proliferation, and colony formation in small cell lung cancer cells. *Cancer Res.* **56**, 3895–3897 (1996).
 121. Hosoi, H. *et al.* Studies on the mechanism of resistance to rapamycin in human cancer cells. *Mol. Pharmacol.* **54**, 815–824 (1998).
 122. Hosoi, H. *et al.* Rapamycin causes poorly reversible inhibition of mTOR and induces p53- independent apoptosis in human rhabdomyosarcoma cells. *Cancer Res.* **59**, 886–894 (1999).
 123. Grewe, M., Gansauge, F., Schmid, R. M., Adler, G. & Seufferlein, T. Regulation of cell growth and cyclin D1 expression by the constitutively active FRAP-p70s6K pathway in human pancreatic cancer cells. *Cancer Res.* **59**, 3581–3587 (1999).
 124. Majewski, M. *et al.* The immunosuppressive macrolide RAD inhibits growth of human Epstein-Barr virus-transformed B lymphocytes *in vitro* and *in vivo*: A potential approach to prevention and treatment of posttransplant lymphoproliferative disorders. *Proc. Natl Acad. Sci. USA* **97**, 4285–4290 (2000).
 125. Dudkin, L. *et al.* Biochemical correlates of mTOR inhibition by the rapamycin ester CCI-779 and tumor growth inhibition. *Clin. Cancer Res.* **7**, 1758–1764 (2001).
 126. Shi, Y. *et al.* Rapamycin enhances apoptosis and increases sensitivity to cisplatin *in vitro*. *Cancer Res.* **55**, 1982–1988 (1995).
 127. Gibbons, J. J. *et al.* The effect of CCI-779, a novel macrolide anti-tumor agent, on the growth of human tumor cells *in vitro* and in nude mouse xenografts *in vivo*. *Proc. Am. Assoc. Cancer Res.* **40**, 2000 (1999).
 128. Geoerger, B. *et al.* Antitumor activity of the rapamycin analog CCI-779 in human primitive neuroectodermal tumor/medulloblastoma models as single agent and in combination chemotherapy. *Cancer Res.* **61**, 1527–1532 (2001).
 129. Hidalgo, M. *et al.* Phase I and pharmacological study of CCI-779, a cell cycle inhibitor. *Clin. Cancer Res. (suppl)* **6**, 413 (2000).
 130. Hidalgo, M. *et al.* CCI-779, a rapamycin analog and multifaceted inhibitor of signal transduction: a phase I study. *Proc. Am. Soc. Clin. Oncol.* **19**, 726 (2000).
 131. Raymond, E. *et al.* CCI-779, an ester analogue of rapamycin that interacts with PTEN/PI3 kinase pathways: A phase I study utilizing a weekly intravenous schedule. *Clin. Cancer Res. (suppl)* **6**, 414 (2000).
 132. Raymond, E. *et al.* CCI-779, a rapamycin analog with antitumor activity: A phase I study utilizing a weekly schedule. *Proc. Am. Soc. Clin. Oncol.* **19**, 728 (2000).
 133. Oz, H. S., Hughes, W. T., Varlek, G., McAlister, V. C. & MacDonald, A. S. Provocative effects of the immunosuppressants rapamycin, tacrolimus, and dexamethasone on pneumonitis in contrast to the anti-pneumonitis effects of mycophenolate mofetil. *Transplantation* **72**, 1464–1465 (2001).
 134. Lennon, A., Finan, K., Fitzgerald, M. X. & McCormick, P. A. Interstitial pneumonitis associated with sirolimus (rapamycin) therapy after liver transplantation. *Transplantation* **72**, 1166–1167 (2001).
 135. Chan, S. *et al.* First report: a phase 2 study of the safety and activity of CCI-779 for patients with locally advanced or metastatic breast cancer failing prior chemotherapy. *Proc. Am. Soc. Clin. Oncol.* **21**, 175 (2002).
 136. Atkins, M. B. *et al.* A randomized double-blind phase 2 study of intravenous CCI-779 administered weekly to patients with advanced renal cell carcinoma. *Proc. Am. Soc. Clin. Oncol.* **21**, 36 (2002).
 137. Gallant, H. L. & Yatscoff, R. W. P70 S6 kinase assay: a pharmacodynamic monitoring strategy for rapamycin; assay development. *Transplant. Proc.* **28**, 3058–3061 (1996).
 138. Ferron, G. M., Pyszczyński, N. A. & Jusko, W. J. Gender-related assessment of cyclosporine/prednisolone/sirolimus interactions in three human lymphocyte proliferation assays. *Transplantation* **65**, 1203–1209 (1998).
 139. Ferron, G. M. & Jusko, W. J. Species- and gender-related differences in cyclosporine/prednisolone/sirolimus interactions in whole blood lymphocyte proliferation assays. *J. Pharmacol. Exp. Ther.* **286**, 191–200 (1998).
 140. Marx, S. O. & Marks, A. R. Cell cycle progression and proliferation despite 4BP-1 dephosphorylation. *Mol. Cell Biol.* **19**, 6041–6047 (1999).
 141. Neshat, M. S. *et al.* Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. *Proc. Natl Acad. Sci. USA* **98**, 10314–10319 (2001).
 142. Podsypanina, K. *et al.* An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/- mice. *Proc. Natl Acad. Sci. USA* **98**, 10320–10325 (2001).
 143. Yu, K. *et al.* mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. *Endocr. Relat. Cancer* **8**, 249–258 (2001).
 144. Huang, S. *et al.* p53/p21(CIP1) cooperate in enforcing rapamycin-induced G(1) arrest and determine the cellular response to rapamycin. *Cancer Res.* **61**, 3373–3381 (2001).
 145. Wang, S., Wang, Z. & Grant, S. Bryostatin 1 and UCN-01 potentiate 1-β-d-arabinofuranosylcytosine-induced apoptosis in human myeloid leukemia cells through disparate mechanisms. *Mol. Pharmacol.* **63**, 232–242 (2003).
 146. Cartee, L. *et al.* Protein kinase C-dependent activation of the tumor necrosis factor receptor-mediated extrinsic cell death pathway underlies enhanced apoptosis in human myeloid leukemia cells exposed to bryostatin 1 and flavopiridol. *Mol. Cancer Ther.* **2**, 83–93 (2003).
 147. Rosato, R. R. *et al.* The cyclin-dependent kinase inhibitor flavopiridol disrupts sodium butyrate-induced p21WAF1/CIP1 expression and maturation while reciprocally potentiating apoptosis in human leukemia cells. *Mol. Cancer Ther.* **1**, 253–266 (2002).
 148. McKinstry, R. *et al.* Inhibitors of MEK1/2 interact with UCN-01 to induce apoptosis and reduce colony formation in mammary and prostate carcinoma cells. *Cancer Biol. Ther.* **1**, 243–253 (2002).
 149. Betensky, R. A., Louis, D. N. & Cairncross, J. G. Influence of unrecognized molecular heterogeneity on randomized clinical trials. *J. Clin. Oncol.* **20**, 2495–2499 (2002).
- One of the first papers to highlight the effects of molecular heterogeneity within tumours of patients in a clinical trial can have on sample size and power if molecular phenotype determines the magnitude of response to treatment.**
150. Slamon, D. J. *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **344**, 783–792 (2001).
- Seminal paper demonstrating the benefit of an antibody directed against a receptor tyrosine kinase in a selected patient population.**

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