Cancer Chemother Pharmacol (1998) 42 (Suppl): S3–S21

REVIEW

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Clinical evaluation of biologically targeted drugs: obstacles and opportunities

Abstract Recent insights into the molecular mechanisms of cancer have indicated that a variety of fundamental cellular processes are dysregulated in malignant cells. These processes include cell cycle control, signal transduction pathways, apoptosis, telomere stability, angiogenesis, and interactions with the extracellular matrix. Remarkable advances in molecular genetics, enzymology, and medicinal chemistry have permitted the design of compounds that modulate some of these processes with specificity that was unimaginable a decade ago. As these novel, biologically targeted compounds enter the clinic, they will require a strategy for clinical evaluation and development different from that used commonly for cytotoxic antineoplastic agents. This review examines the development of cancer drugs directed against angiogenesis, metastasis, signal transduction, telomerase, and molecular message (antisense), outlines strategies for the clinical testing of agents directed at these processes, and contrasts these efforts with traditional approaches to cancer drug testing.

Key words Molecular mechanisms · Apoptosis · Telomerase · Angiogenesis · Antisense · Metastasis

Introduction

The dramatic expansion of knowledge regarding the molecular events that cause cancer and contribute to its fundamental biology has opened new avenues for its treatment and prevention [6]. Traditional approaches to cancer drug discovery, based on largely random screening of chemical libraries or natural products against murine tumors, identified the current arsenal of useful agents, most of which act through direct attack on DNA or inhibit its synthesis or function. Most of the drugs are cytotoxic, have limited specificity for tumor cells compared to host tissues, and are most effective against rapidly proliferating tumors. In an attempt to produce greater specificity of action and to take advantage of the latest discoveries in cancer biology, both commercial and academic investigators have turned their efforts toward specific molecular or biochemical targets known to play a role in cancer etiology and progression. Illustrative of this change in strategy is the National Cancer Institute (NCI) 60 cell line screening program for cancer drug discovery, which was instituted in 1985 and became fully operational in 1991. This has been adapted, through molecular characterization of the cell lines and computer-based analysis of response profiles, to yield information about molecular mechanisms of compound action. This type of analysis will allow NCI investigators to select compounds for development based on their ability to kill cells of a given molecular profile [171]. In general, the biotechnology industry and traditional pharmaceutical companies have eschewed in vivo and cell-based discovery programs; instead they have directed their efforts toward discovery of new entities that act at one of many new molecular targets dysregulated in cancer cells, including cell cycle components, signal transduction pathways, apoptosis, telomerase, adhesion, and motility. Rapid throughput assays have been established to identify lead compounds, which can then be refined through structural analysis and analogue synthesis in an iterative process that yields a drug with the requisite specificity and pharmacologic properties.

Such compounds are now entering clinical trial in large numbers and present a significant challenge to clinical researchers. They differ from traditional cytotoxic chemotherapeutic drugs in significant ways, and they will likely require a strategy for clinical evaluation very different from that employed for traditional cytotoxic drugs.

Work presented at the 13th Bristol-Myers Squibb Nagoya International Cancer Treatment Symposium, "Strategic Cross Talk between Major Oncology Groups/Clinical Pharmacology in Cancer Chemotherapy," 17–18 October 1997, Nagoya, Japan

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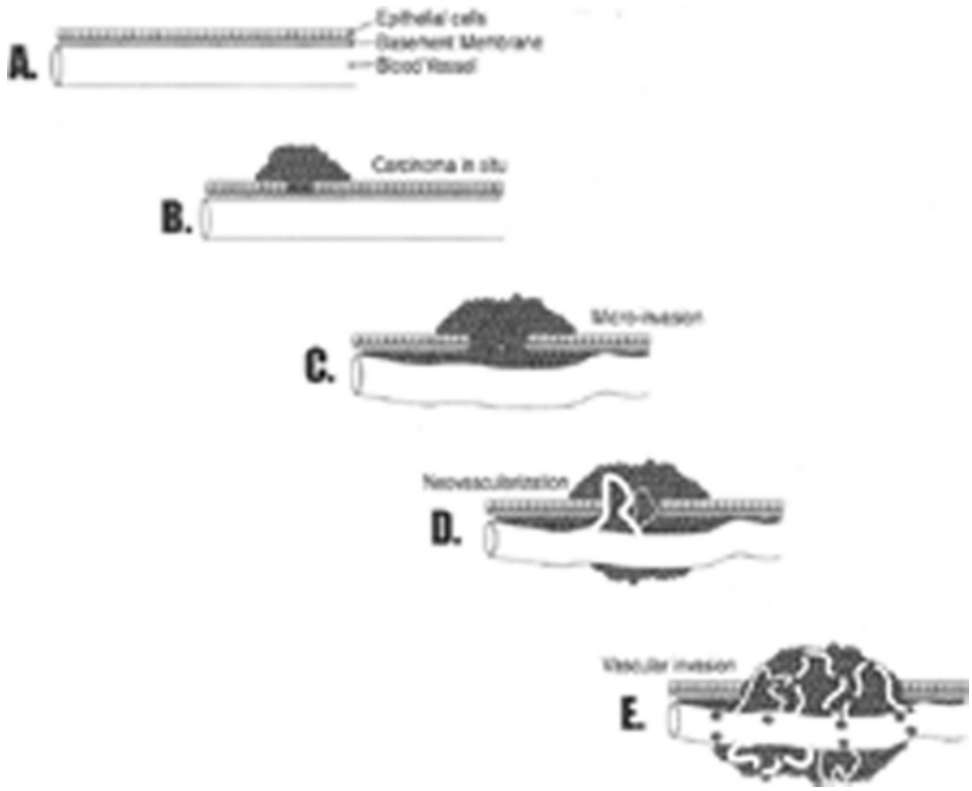

Fig. 1 A model for tumor invasion and metastasis. A normal epithelial tissue (**A**) undergoes neoplastic transformation and clonal proliferation resulting in a carcinoma in situ (**B**). MMPs produced by the tumor cells and by nonneoplastic cells associated with the tumor, such as macrophages, allow the tumor cells to penetrate the basement membrane and invade locally (**C**). Angiogenic factors such as VEGF and bFGF are produced by the invading tumor and stimulate neovascularization of the growing nodule (**D**). Tumor cells and nonneoplastic tumor-associated cells in the growing tumor nodule continue to produce angiogenic factors and MMPs. This allows individual tumor cells to break loose from the nodule and to invade the permeable neovasculature and metastasize hematogenously (**E**)

While many of these compounds, if effective, will inhibit cell proliferation, they are not cytotoxins, and it is uncertain whether established tumor masses will regress. For example, antimetastatic compounds have uncertain potential for inducing apoptosis and tumor shrinkage and it is likely that they will need to be administered continuously and for prolonged periods of time. Since few of these new drugs are completely specific inhibitors of a single protein, their selectivity and specificity of action, and, ultimately, their therapeutic ratio will likely depend on finding a "biologically effective dose" that inhibits the targeted molecule, such as a receptor or tyrosine kinase, but has little effect on related molecules. Since their ultimate effect on time to progression, time to metastasis, or survival may require months or years to determine, the testing of these agents will depend on surrogate measures of efficacy. These may take the form of pharmacokinetic, molecular, or biological endpoints. Even in the absence of definite evidence of antitumor benefit, early trials that demonstrate the safety of a new drug at doses that inhibit a biological target may lead to large, randomized trials aimed at determining efficacy. Thus a successful phase I (dose-seeking) trial that establishes the safety of a biologically effective dose may be followed by a limited phase II evaluation to confirm interaction with the proposed molecular target in a specific patient population, and then a broad phase III trial to establish efficacy.

In this paper we examine several of the most interesting areas of cancer drug development: drugs directed against angiogenesis, metastasis, signal transduction, telomerase, and molecular message (antisense). We outline strategies for their clinical testing and contrast these efforts with the traditional approaches to cancer drug testing.

Inhibitors of angiogenesis

Neovascularization is a critical step in the growth of macroscopic tumors [55]. This work has evolved from observations made in the 1960s that the presence of a primary tumor inhibits the growth of distant metastases [125]. Further work indicated that the progression from the preneoplastic to the invasive, neoplastic stage is associated with angiogenesis and that these early neoplastic tissues produce soluble angiogenic substances [17, 44, 50]. More recently it has been shown that solid tumors do not grow larger than 2–3 mm in size before neovascularization is initiated [44, 55]. Furthermore, increased neovascularization in invasive breast and gastric carcinomas is prognostic for the presence of metastatic disease [14, 67, 91, 157, 169, 170] (Fig. 1).

Many new molecular components of the angiogenic process are now being identified, and this work provides targets for anticancer drug discovery. A complex array of at least 12 activators and a similar number of inhibitors of angiogenesis have been identified. Activators of angiogenesis, including vascular endothelial growth factor (VEGF), angiogenin, angiopoietin 1 and 2, and acidic and basic fibroblast growth factor (aFGF and bFGF), are expressed in many human and mouse tumors, as well as in healthy tissues. Similarly, antiangiogenic factors are produced by

Table 1 Inhibitors of angiogenesis

Compound	Sponsor	Clinical status	Reference
TNP-470	Takeda	Phase I	41
Thalidomide	NCI Entremed	Phase II	40, 42
Interferonα-2a	Schering AG	Phase III	38, 153
Interleukin 12	Roche; Genetics Institute	Phase II	94, 164
Pentosan polysulfate	NCI	Not currently in trial	90, 139
Sandostatin	Novartis	Phase I	178
Platelet factor 4	Repligen	Phase I	92, 144
CM101	Carbomed, Zeneca	Phase I	165
Batimastat	British Biotech	Phase I	176
Marimastat	British Biotech	Phase III	11
Vitaxin (antibody to integrin αV ß3)	IXSYS	Phase I	18, 45
SU5416	Sugen	Phase I	–
CT2584	Celltech Therapeutics	Phase I	–
Antibody to VEGF	Genentech	Phase I	99, 183
Soluble VEGF receptor (sFLT)	GenVec Inc.	Preclinical development	84
Angiostatin	Entremed Bristol-Myers Squibb	Preclinical development	117
Endostatin	Entremed	Preclinical development	116

tumors as well as by healthy tissues [16, 29, 39, 69, 93]. It is becoming clear that the regulation of angiogenesis depends on the relative balance of a complex interaction of positive and negative signals. Disruption of this balance can shift the equilibrium toward angiogenesis [52, 130].

Several elegant experiments have demonstrated that inhibitors of angiogenesis may be valuable as antineoplastic drugs. In the Lewis lung cancer mouse model for metastatic malignancy, resection of the primary tumor implant is followed by rapid growth of multiple lung metastases, together with dense neovascularization of previously quiescent, small nodules [118]. Primary tumors produce proteases that cleave extracellular proteins. The product of one such cleavage reaction is angiostatin, a 38-kD plasminogen fragment that can be purified from the urine and serum of animals with primary tumors and that inhibits the neovascularization and growth of lung metastases. It has no direct effect on the proliferation of various mouse and human tumor cell lines [118], but causes clinical complete remissions of established human breast, colon, and prostate xenografts in severe combined immunodeficiency mice, as well as of primary and metastatic murine tumors in immunocompetent mice. Continued daily dosing of angiostatin is required to maintain the dormancy of residual microsopic tumor nodules; on cessation of therapy, dormant tumors resume growth within weeks. Angiostatin-induced tumor shrinkage is associated with a 4-fold increase in apoptotic cell death, but no decrease in the fraction of cells in S-phase [81, 117]. Endostatin, a 20-kD fragment of collagen XVIII, has similar antiangiogenic, proapoptotic, and antitumor properties, thus defining a new family of potent angiogenesis inhibitors derived from the proteolysis of common extracellular proteins [116]. In earlier studies with angiostatin, continued daily dosing was required to maintain dormancy of residual microscopic tumor nodules [81, 117]. However, it was recently demonstrated that murine tumors did not regrow when combined therapy with angiostatin and endostatin was discontinued [12].

The medical community now has access to a collection of molecules that inhibit angiogenesis, and several are currently in clinical trials (Table 1). These include TNP-470, thalidomide, marimastat (which is also a matrix metalloproteinase inhibitor), anti-VEGF antibody, interferon (INF)α-2a, and others [27, 44, 72, 99, 183]. INFα-2a is the first to have found clinical application, being used in the treatment of refractory, life-threatening hemangiomas in children [38, 174]. The next generation of more potent, endogenous angiogenesis inhibitors, such as angiostatin, endostatin, and soluble VEGF receptor (sFLT), has shown promise in animal studies, but is not yet available for clinical trials [84, 116, 118]. An important step will be to devise effective methods of evaluating the value of these agents as they become available for phase I studies so that useful drugs are not missed and useless drugs do not consume clinical trial resources. Even the very potent angiogenesis inhibitors angiostatin, endostatin, and sFLT may require continued administration for inhibition of angiogenesis and regression of gross tumors in animal models. Therefore these agents will probably need to be administered for prolonged periods, and the development of orally active formulations will be a priority.

Methods to assess angiogenesis activity in man

The assessment of angiogenesis and its inhibitors poses significant challenges in clinical trials. Tumor neovascularization results in increased capillary density, capillary diameter, and capillary permeability. However, changes in rates of blood flow are difficult to predict and may be reduced in highly vascularized tumors due to increased

Table 2 Modalities to assess angiogenesis

Imaging modality	Variable assessed
Histology	Capillary number
	Capillary density
MR	Capillary number
	Capillary density
	Capillary diameter
	Capillary permeability
	Blood volume
PET	Blood flow
	Blood volume
	Glucose utilization
	Nucleic acid metabolism
	Radiolabeled ligands / antibodies bound to specific targets

hydrostatic pressure in the extracellular space within the tumor [4, 15, 113]. The spectrum of vascular changes may be assessed by either invasive or noninvasive approaches including direct evaluation of capillary density in tumor biopsies and indirect evaluation of capillary diameter, density, and permeability, and tumor blood flow using functional imaging methods such as magnetic resonance (MR) or positron emission tomography (PET) (Table 2). Additional molecular studies might evaluate the effect of a drug on its target, either in tumor biopsy specimens or in a healthy tissue surrogate, provided that the target is expressed in an accessible tissue. Finally, changes in serum levels of angiogenic factors, such as VEGF and bFGF, can be followed, but it is difficult to predict how these levels will be affected by angiogenesis inhibitors.

In animal studies, tumor neovascularization can be measured directly by counting capillary loops in thick histological sections stained with antibody to an endothelial antigen such as CD34, CD31, or von Willebrand factor. Similar methods have been applied successfully in human tumors, and as previously mentioned in breast cancer and gastric cancer the degree of tumor neovascularization predicts the occurrence of metastatic disease [91, 157, 170]. However, this method requires generous biopsy samples because the microvessels are heterogeneously distributed and the most accurate predictor of metastatic potential is the capillary density in areas of densest neovascularization [170]. Methods of computer-aided image analysis are in development and will probably improve the speed and reproducibility of capillary counting [5].

Imaging of neovascularization

A noninvasive alternative for assessing tumor vascularity would have the advantage of convenient and repetitive sampling over time and would allow functional measurements such as blood flow, blood volume, and capillary permeability. While the potential for functional measurements using MR and PET has been demonstrated in animals, neither technique has been successfully applied to tumors in humans, although limited studies using PET in patients with brain tumors have been performed [134]. Considerable progress has been made in MR imaging, and it may well become the method of choice for assessing tumor neovascularization in vivo. There are at least three MR-based approaches in active development for use in humans: monitoring of tumor ring enhancement with standard gadolinium contrast MR; microvascular imaging with high-field-strength magnets and surface coils; and dynamic contrast imaging using blood pool contrast agents. A fourth approach, which is further from clinical application, is MR spectroscopy of paramagnetic isotopes such as ^{31}P and ^{13}C to assess tumor metabolic activity, which in turn could be affected by changes in blood supply.

Standard contrast-enhanced MR has some value in detecting areas of neovascularization. Capillary counts and histological evaluation have confirmed that tumor neovascularization is primarily confined to the outer rim of larger tumor masses. This finding correlates with the "ring enhancement" seen with standard contrast-enhanced MR and computed tomography imaging of tumors, and is likely to be related to the increased permeability of the young capillary beds to contrast agents as well as increased capillary density; these changes are mediated by angiogenic factors such as VEGF and bFGF [44, 121]. In cases where easily visualized ring-enhancing lesions are seen, resolution of ring enhancement on standard contrast-enhanced MR may be a useful measure of antiangiogenic activity.

Abramovitch et al. have shown that MR imaging using a high-field-strength magnet (4.7 T) and surface coil can successfully visualize micro-neovascularization of glioma spheroids grown as subcutaneous implants in nude mice [1]. This method takes advantage of the fact that the deoxyhemoglobin in blood potently quenches the signal intensity of the gadopentate (Gd-DTPA) contrast. This type of study evaluates the entire tumor nodule; therefore the sampling bias inherent in histological analysis is avoided and the resulting data can be quantified and reliably reproduced. 4.7-T magnets are too small in diameter to image humans, but 3.0-T magnets are available in research settings and may be adequate. The high-field surface coil technique could be used to assess both the number and diameter of vessels in accessible tumors, both of which increase in tumors in response to angiogenic factors. It might also be possible to monitor systemic angiogenic inhibition following treatment with antiangiogenic drugs by measuring neovascularization in healthy tissue such as that surrounding a small, standardized skin incision or surrounding a subcutaneously implanted pellet containing bFGF or VEGF.

Van Dijke et al. have described the use of a high-molecular-weight, blood pool contrast agent (albumin-[Gd-DTPA]) with a 2.0-T MR imager (a field strength available in newer clinical scanners) to measure neovascularization in mammary tumor implants in rats [162]. This technique takes advantage of the differential permeability of the tumor-associated neovasculature to albumin-[Gd-DTPA] compared to healthy vessels and the increased tumor blood volume compared to that of surrounding tissues. The albumin-[Gd-DTPA] contrast leaks through

the walls of the permeable, young vessels and accumulates in the interstitial space of the tumors. Mature vessels are impermeable to the high-molecular-weight contrast agent and in the absence of neovascularization the contrast agent is confined to the intravascular space. Van Dijke et al. found a correlation between neovascularization measured histologically and MR-derived measures of neovascularization using albumin-[Gd-DTPA] contrast. Rapidly growing tumors demonstrated increased angiogenesis compared to more slowly growing neoplasms as judged by both histological and MR criteria [162]. The observed correlation of MR with capillary counts in murine tumors indicates that the MR method may be useful to assess neovascularization in vivo. Unfortunately, the albumin-[Gd-DTPA] blood pool contrast agent cannot be used clinically due to systemic toxicity [121]. Development of a nontoxic blood pool contrast agent is a high priority.

An alternative imaging technology is PET, which is a sensitive technique that can localize and quantify any compound that can be labeled with one of a number of short-lived, unstable isotopes including ^{18}F, ^{15}O, and ^{11}C. [^{15}O]H_2O is used for accurate measurement of cerebral blood flow [70], [^{18}F]fluorodeoxyglucose (FDG) uptake is commonly used to measure cellular glucose utilization, and ^{18}F-labeled nucleotides and ^{11}C-labeled amino acids are used to measure rates of nucleic acid and protein synthesis, respectively [22, 77, 126]. Tumor metabolic activity as assessed using FDG-PET predicts response to chemotherapy in glioblastomas and predicts tumor grade in lymphomas [85, 134]. It is expected that while antiangiogenic agents may acutely increase blood flow due to decreased intratumoral hydrostatic pressure and decreased vessel permeability, with prolonged treatment angiogenesis inhibitors will cause decreased tumor blood flow as well as decreased tumor metabolic activity. Therefore PET imaging to quantify blood flow in tumor masses will probably be a useful surrogate marker for drug activity.

Perhaps the most promising use of PET is the quantitative detection of molecular entities related to angiogenesis, such as receptors or antigens on vascular endothelium, through the use of labeled antibodies or ligands. Potential targets that are relatively specific for tumor neovasculature include VEGF receptors and the antigen detected by murine monoclonal antibody E-9 [141, 166]. Precedent for this method exists in the detection of melanoma lesions using an ^{18}F-labeled compound (^{18}F-10B-L-BPA) that is taken up by melanin-producing cells [101]. This approach might allow quantitative, noninvasive assessment of the effectiveness of angiogenesis inhibitors during dose-escalation phase I studies. Since any antineoplastic therapy that inhibits tumor growth would also be expected to inhibit further neovascularization and reduce the rate of glucose metabolism, noninvasive measurements of these processes should also be useful for evaluating the effectiveness of antitumor therapies that function through a variety of mechanisms.

PET imaging does have several important limitations. As observed previously, initial treatment with angiogenesis inhibitors may increase tumor blood flow as a result of decreased hydrostatic pressure within the tumor [160, 161]. In addition, angiogenesis inhibitors do not reduce the proliferative rate of the tumor cells; they seem to cause tumor shrinkage by increasing the fraction of apoptotic cells relative to proliferating cells [66]. Therefore measures of tumor blood flow or tumor nucleic acid metabolism and glucose utilization by PET may not initially decrease during treatment, although with time these measures should decrease as the neovasculature regresses and the volume of viable tumor cells decreases. A second problem is that the isotopes used are very unstable, with half-lives of minutes to 1–2 h. This makes it necessary to produce labeled compounds for PET imaging near to the facility where they are to be used. In addition, only a small number of suitable isotopes are available for labeling biological molecules and the spatial resolution of PET imaging remains inferior to that of MR.

Despite these limitations, both the MR- and PET-based methods described above have enormous potential in the evaluation of novel, biological target-directed therapies such as angiogenesis inhibitors. At the current time, PET measurements of blood flow (using [^{15}O] H_2O) and of glucose metabolism (using FDG), and MR measures of ring enhancement are available for clinical use. Both MR and PET may provide methods of assessing relevant surrogate endpoints that are predictive of drug function in animals, and could be used to define minimal effective doses in phase I trials. However, in initial phase I trials it will be essential to validate these new methods by comparing results from the most promising noninvasive imaging methods with capillary counts in biopsies and with traditional measures of tumor response.

Clinical studies to date of antiangiogenic compounds such as thalidomide and TNP-470 have yielded only isolated cases of apparent tumor regression without clear evidence of antiangiogenic effects [11, 40–42, 53]. With more potent agents such as endostatin and angiostatin, it is possible that more dramatic and consistent evidence of tumor regression will be produced during phase I–II trials. However, for the purposes of establishing biological activity in dose-seeking phase I trials and for understanding the time of onset of effects, it will be critical to develop noninvasive, surrogate measures of drug effect.

Inhibitors of the matrix metalloproteinases

The matrix metalloproteinase (MMP) inhibitors (MMPIs) represent a second family of biological target-directed antineoplastic agents that act on a process essential to invasion and metastasis. A number of these agents are now being evaluated in clinical trials (Table 3).

MMPs comprise a large family of Zn-dependent proteases that together hydrolyze the components of the extracellular matrix. They are categorized into collagenases that hydrolyze fibrillar collagen; stromelysins that hydrolyze proteoglycans and glycoproteins; gelatinases that hydrolyze nonfibrillar collagens; and metalloelastases that hydrolyze elastin. The MMPs are critical components

Table 3 Inhibitors of MMPs

Compound	Sponsor	Clinical status	Reference
Batimastat	British Biotech	Phase I	176
Marimastat (oral formulation)	British Biotech	Phase III	11
AG3340	Agouron	Phase I	136
COL-3	Collagenix, NCI	Phase I	–
BAY12-9566	Bayer	Phase I	–
CT1746 (oral formulation)	Celltech Therapeutics	Preclinical development	2

both of the process of invasion and metastasis and that of angiogenesis. The first challenge in invasion by a carcinoma is penetration of the basement membrane by individual malignant cells. To metastasize, these cells must then gain access to the vasculature or the lymphatics, and must exit the vessels and physically invade the target tissue [19] (Fig. 1).

Increased expression of MMPs is associated with increased metastatic potential in several animal tumor models. An endogenous inhibitor of MMPs, tissue inhibitor of metalloproteinases (TIMP-1), inhibits tumor formation by some tumor cell lines when it is injected into mice. Similarly, when various cell lines are engineered to express TIMP-1 or TIMP-2 their ability to invade and metastasize is reduced [19, 80, 138].

Since 1994 a number of small molecules that mimic the structure of collagen and competitively inhibit the MMPs have been synthesized (Table 3). These include batimastat, marimastat, and CT1746. Marimastat and CT1746 are active by the oral route, while batimastat is given intravenously. Batimastat and marimastat are currently in phase I and phase III clinical trials, respectively. These agents have been shown to inhibit the growth and regional invasiveness of human tumor xenografts in nude mice, and to inhibit the growth and metastasis of Lewis lung tumors in immunocompetent mice. Batimastat has induced partial regression (about 50% by volume) of established tumors in nude mice, but not complete remissions [2, 3, 19, 167, 176, 177]. Batimastat and marimastat also have antiangiogenic activity [44]. The mechanism of their antiangiogenic activity is not clear, but it might be a direct result of MMP inhibition because angiogenesis probably requires MMP activity to allow the neovasculature to invade the tissue being vascularized.

As with the angiogenesis inhibitors, the MMPIs are directed against a process essential to tumor invasion and metastasis. Tumor cells themselves secrete MMPs, as do endothelial cells of the neovasculature and macrophages infiltrating the tumor [19, 36]. Like angiostatin and endostatin, the MMPIs are not cytotoxic to tumor cells in vitro, and based on data from animal models it is uncertain whether they will cause tumor shrinkage. Also like the angiogenesis inhibitors, if effective they will need to be administered for long periods of time and, for convenience, by the oral route. Due to the requirement for chronic use and the possibility of late toxicity, the optimal dose will be the minimal biologically effective dose, not the maximum tolerated dose (MTD).

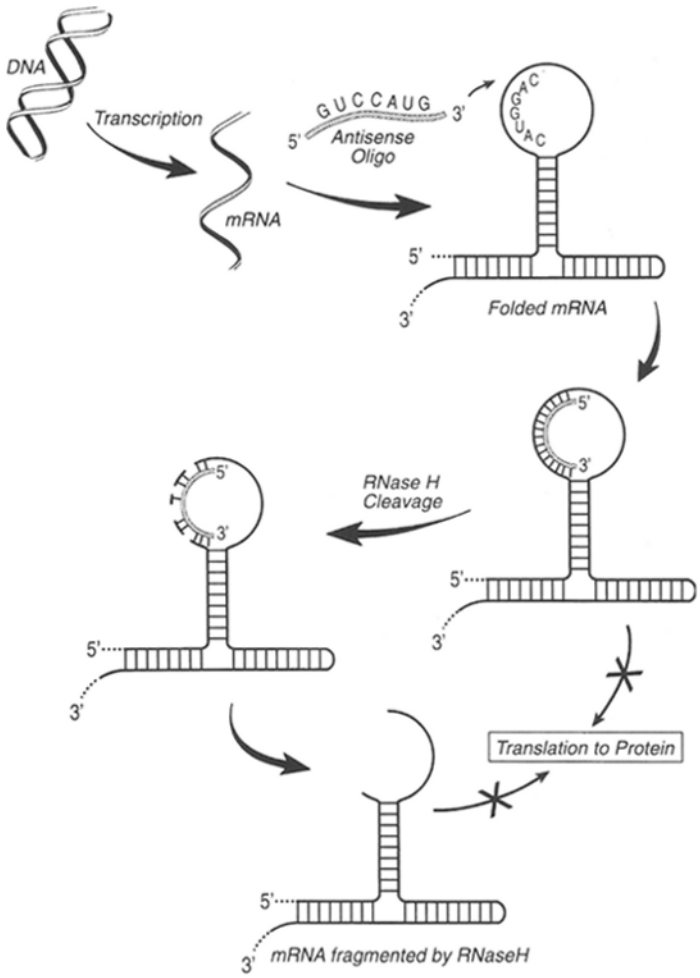

Fig. 2 Antisense mechanisms. A gene encoded by DNA is transcribed as RNA and processed posttranscriptionally to form a mature mRNA molecule with complex secondary structure. After the antisense oligonucleotide drug hybridizes to its complementary sequence in an accessible region of the mRNA molecule, translation of the mRNA to protein is inhibited. This probably occurs by at least two mechanisms: 1) RNase H hydrolyzes the RNA portion of the RNA:oligonucleotide hybrid, which results in destruction of the mRNA molecule; and 2) the hybridized oligonucleotide causes steric inhibition of the translation machinery

The processes of angiogenesis and hydrolysis of the extracellular matrix are intimately connected and interdependent. This interdependence is illustrated by a well-characterized assay for neovascularization in which the growth of capillaries into the cornea under the stimulus of bFGF is associated with dissolution of the corneal matrix, and the demonstration that treatment of endothelial cells with bFGF or VEGF induces expression of MMPs [44, 150]. Therefore MR and PET imaging of neovascular parameters should also be useful as surrogates for MMPI activity in phase I, dose-escalation studies. Other potential secondary endpoints include in vitro assays of MMP activity in lysates of tumor biopsies and in serum, and serum levels of tumor markers, if available.

The appropriate clinical setting for assessing antimetastatic activity may be quite different than that required for determining cytotoxicity in that patients might have to be treated at a time when metastasis has not yet occurred. In

classical settings for adjuvant therapy of breast or colorectal cancer, following resection of the primary tumor it is believed that metastasis has already occurred. However, in the case of breast cancer recent evidence suggests that some micrometastatic disease develops after resection of the primary tumor because adjuvant chest wall radiotherapy after modified radical mastectomy both improves local control and decreases mortality due to metastatic disease [120, 127]. Like radiotherapy, adjuvant treatment with MMPIs might improve survival by inhibiting the development of late micrometastases. There is also a possibility that in the setting of micrometastatic disease, antimetastatic agents such as MMPIs may still be useful. In most organs the growth of micrometastatic disease into symptomatic macroscopic disease requires that the micrometastases stimulate angiogenesis and penetrate adjacent healthy tissue. Most organs except the central nervous system can tolerate the presence of noninvasive mass lesions. Thus inhibition of MMP might produce prolonged periods of stable metastatic disease.

A recent preliminary report of a metaanalysis of dose-escalation trials of marimastat in patients with advanced malignancies reported a statistically significant decrease in the rate of rise of relevant tumor markers (carcinoembryonic antigen [CEA], CA125, and prostate-specific antigen) after 4 weeks of treatment compared to the rate of rise before treatment [129]. There was no significant difference in response rate (as judged by tumor marker levels) over a 5-fold dose range of marimastat (10 mg bid to 50 mg bid), although the lowest dose (5 mg qd) was significantly less active [129]. This study was a metaanalysis; therefore it is difficult to be certain that similar groups were compared. If these observations can be duplicated in a single larger trial, a dose at the lower end of this range should be examined in long-term randomized trials designed to measure time to occurrence of new metastatic lesions and survival. Ideally, the lowest functional dose will have the least risk of causing nonspecific, systemic toxicity.

Inhibitors of telomerase and regulation of telomere length

One of the most exciting and potentially exportable insights into cancer biology in recent years has been the discovery that cancer cells express an unusual enzyme that allows them to undergo unlimited cycles of replication. Normal cells lose small segments of DNA at the ends, or telomeres, of their chromosomes with each cycle of replication. Most cancer cells, as well as various normal stem cell populations, express telomerase, an enzyme that catalyzes the synthesis of telomeres. Fifteen years of investigation suggest that telomerase is an important determinant of cellular immortality and may be responsible for neoplastic behavior in a wide variety of neoplasms [10, 24, 58, 82]. Inhibition of telomerase might restore mortality to malignant cells that express this enzyme. Compounds that inhibit the telomerase enzyme are already in development, and the recent cloning of the human telomerase gene should accelerate the discovery of new inhibitors [100, 110].

Telomeres are composed of multiple copies of short, untranscribed, repetitive sequences of DNA spanning 5–15 kb [10]. They protect the ends of the chromosomes and facilitate chromosomal stability. In vitro and in vivo they have been shown to adopt an unusual quaternary structure known as a G-quartet, which may play a role in telomerase binding or function, or in chromosomal processing during the cell cycle [172].

Since DNA polymerase cannot initiate DNA synthesis at the extreme 3′ end of a chromosome, telomeres shorten by 50–100 bp with each round of cell division [10, 59]. Telomere shortening may be an essential part of the aging process, and has been observed both in vivo and in primary cells in culture [58, 61]. After multiple divisions, cells in primary culture that do not express telomerase arrest in the G_1 phase of the cell cycle and undergo senescence. This sequence of events is overcome when cells are transformed with certain viral oncogenes. The transformed cells continue to grow for about 20 more divisions. They then enter "crisis" and accumulate karyotypic abnormalities consistent with telomere loss, and most of the cells die. However, a small fraction of the cells survive crisis and become immortal cell lines. These cells are found to express telomerase, which stabilizes telomere length and enables the cell to divide indefinitely [24, 59]. Therefore telomerase activation may be a final step in the evolution of the malignant phenotype.

A variety of neoplasms express telomerase activity, including breast, lung (small and non-small cell), ovarian, endometrial, cervical, hepatocellular, pancreatic, gastric, bladder, renal, prostate, and oral squamous cell cancers [82, 145]. Telomerase expression has also been seen in hematopoietic tumors such as acute leukemias, chronic myeloid leukemia (CML) in blast crisis, and non-Hodgkin's lymphomas [25, 115, 158]. However, telomerase expression is only detected in a limited number of nonmalignant tissues, including early hematopoietic cells and activated lymphocytes, as well as embryonic germ cell tissues, early oocytes, keratinocytes, colonic epithelium, and hair follicles [37, 57, 68, 128, 173, 180, 182]. Expression in these tissues is generally at low levels and may be essential to the continuous self-renewal of stem-cell populations. Thus telomerase expression in tumors may represent a necessary component of the cell immortalization machinery.

As an enzyme, telomerase has several unusual features. It contains an RNA molecule and functions as an RNA-dependent DNA polymerase, similar to reverse transcriptase. These are features which can be exploited in targeted drug design [54, 88]. Human immunodeficiency virus reverse transcriptase inhibitors weakly inhibit telomerase and are prototypes for drug discovery efforts [154, 181]. Other compounds found to inhibit telomerase include purine nucleotides modified at the N7 position, phosphorothioate oligonucleotides that mimic the telomere G-quartet structure, and ribozymes directed at the RNA component of telomerase [43, 78, 96, 143]. The telomerase holoenzyme

also includes regulatory subunits that are potential targets in the development of inhibitors [60, 111, 163].

Testing telomerase inhibitors will pose unique clinical challenges and opportunities. The kinetics of cell killing are hard to predict. Since telomeres are not significantly longer in tumor cells than they are in cells in crisis, telomerase inhibition may only be necessary over a few cell divisions before cell death ensues [59]. However, if a greater number of cell divisions is required, clinically apparent tumors may progress to a fatal outcome before telomere shortening induces senescence. Therefore telomerase inhibitors may work best in the adjuvant setting or in premalignant states. A substantial number of cell divisions may be tolerable in a patient who is disease-free on clinical grounds, but who has micrometastases. In addition, telomerase inhibitors could be particularly effective in the chronic phase of such diseases as CML and myelodysplastic syndrome (MDS), where acceleration and development of acute leukemia are associated with acquisition of telomerase activity [25, 119, 158]. Taken during the stable phases of these diseases, telomerase inhibition could neutralize aggressive, immortal cell clones as they arise.

The side effects of telomerase inhibitors may be minimal, but will also be difficult to predict. Most normal cells should be unaffected because they do not express telomerase. Normal cells that express telomerase have telomeres considerably longer than the relatively short telomeres of tumor cells. This may allow telomerase-dependent stem cells to tolerate telomerase inhibition for periods of time that are sufficient to shorten telomeres in malignant cells critically. Toxicity to normal stem cell populations would be expected to cause side effects similar to those of traditional cytotoxic drugs, such as mucosal breakdown, bone marrow suppression or aplasia, alopecia, and infertility. It is possible that these complications might be irreversible with prolonged administration if a large fraction of a stem cell population is affected. Due to the possibility of long-term effects on the bone marrow, it might be necessary to harvest and store bone marrow progenitor cells prior to therapy.

As with angiogenesis inhibitors and MMPIs, telomerase inhibitors may require administration for prolonged periods of time and oral formulations will be most useful. Again, the optimal dose will be the biologically effective dose rather than the MTD. Therefore in vivo surrogate markers of antitumor activity need to be sought. Telomerase activity could be assayed while patients are receiving the inhibitors, with dosing based on laboratory evidence of telomerase inhibition or telomere shortening. In some neoplasms these parameters could be assayed directly. For example, leukemias in peripheral blood, solid tumors in fluid collections such as ascites, cutaneous or head and neck tumors, and malignant cells in cerebrospinal fluid could be sampled repeatedly during dose-finding studies. In solid tumors it would also be important to assess telomerase inhibition in biopsy samples.

Telomerase activity or telomere length could also be sampled in normal tissues to provide information about tissue penetration and to judge the potential effect on a progenitor cell population. Analysis of normal cells may be all that is possible while monitoring drug activity in adjuvant settings, but it may be necessary to use a source of cells such as peripheral blood or bone marrow that can be enriched in stem cells. In this fashion, telomerase activity and telomere length in normal cells could be useful in the effort to correlate drug levels and enzyme inhibition.

Telomerase is potentially an important therapeutic target in the treatment of a broad range of solid and hematologic malignancies. The limited expression of telomerase in normal cells suggests that telomerase inhibitors may have relative specificity for malignant cells. The next challenge is to convert lead compounds into drugs that are orally available and tolerated with prolonged administration, and then to devise effective methods of evaluating these compounds for anticancer activity in vivo.

Antisense oligonucleotides as drugs

Antisense oligonucleotide technology provides a mechanism to inhibit expression of a specific gene based on knowledge of the cDNA sequence. Antisense oligonucleotides have the desired specificity of action to become ideal anticancer drugs because they can inhibit the expression of oncogenes and other molecular targets in cell lines and tumor tissues in a sequence-specific manner. This makes them particularly well suited to target cancer-specific genes such as the *bcr-abl* fusion gene in CML, the *pml-rarα* fusion gene in promyelocytic leukemia, the *bcl2* gene in low-grade lymphomas, and the translocated *myc* gene in Burkitt lymphomas. In these diseases, expression of the specific gene product is characteristic of the malignancy, and in experimental settings inhibition of gene expression reverses the malignant phenotype [28, 30, 114, 159]. These hematologic malignancies that carry unique genetic translocations may represent the most promising clinical opportunities in which to test the therapeutic potential and biological mechanisms of antisense drugs.

The majority of epithelial solid tumors carry complex karyotypic abnormalities and it is likely that a multitude of genetic abnormalities contribute to the malignant phenotype [86], rendering them less susceptible to inhibition of the function of any single mutated cancer gene. However, a large number of genes that are fundamental participants in cell cycle control, initiation of apoptosis, and signal transduction has now been identified, and derangement of these processes is at the heart of the malignant phenotype. Therefore it is possible (and there is supportive experimental evidence) that some solid tumors will be particularly sensitive to antisense compounds directed at one or more of these targets at doses that will not be toxic to normal cells.

Unmodified deoxyribose oligonucleotides (ODNs) are unstable in the circulation, primarily due to attack by ubiquitous 3′–5′ exonucleases. Numerous modifications have been made to the phosphodiester backbone in an attempt to produce antisense compounds that are clinically useful [26, 107, 135]. The phosphorothioate (PS) modifica-

Table 4 Antisense drugs

Drug	Target gene	Sponsor	Clinical status	Reference
Isis 3521	*pkcα*	Isis Pharmaceuticals	Phase I	31, 112
Isis 5132	c-*raf*	Isis Pharmaceuticals	Phase I	105, 108
Isis 2503	Ha-*ras*	Isis Pharmaceuticals	Phase I	20
–	*bcl2*	Genta	Phase I	168
OL(1)p53	*p53*	Lynx Therapeutics	Phase I	9
–	*bcr-abl*	–	Not in active trial	148
GEM 210	*mdr-I*	Hybridon	Preclinical development	8, 89
GEM 220	VEGF gene	Hybridon	Preclinical development	151
GEM 230	*pka*	Hybridon	Preclinical development	21

tion (replacement of a nonbridging oxygen with sulfur) is the best available at this time. It results in ODNs that are resistant to cleavage by 3′–5′ exonucleases, stable in the circulation, and taken up by cells in culture and by normal and tumor tissue in vivo. PS-ODNs hybridize to RNA and DNA with high sequence specificity, although with reduced affinity compared to native DNA. When bound to RNA, they mediate RNase H cleavage of hybrids, which is probably the most important mechanism by which antisense oligonucleotides inhibit gene expression (Fig. 2). They are also easily synthesized in bulk [106–108, 124, 132, 135].

The chemical structure of antisense compounds, including the PS-ODNs, has intrinsically unfavorable features for their use as drugs: they are highly negatively charged; have very large molecular weights (in the order of 6800 for a 20-base oligonucleotide); and are acid labile. These properties require that they be delivered intravenously, and once in the blood they probably do not cross cell membranes efficiently. They are presumed to enter cells by endocytosis, and after intravenous administration much of the drug is found in cytoplasmic vesicles where it is probably not available to the cell as a functional antisense compound [152]. Nevertheless, antisense oligonucleotides have been demonstrated to inhibit expression of a variety of genes in a sequence-specific manner, both in cultured cells and in animal models, and to inhibit the proliferation of some cell lines and animal tumors that express these genes. Targets to date have included *bcl2* [79, 132, 184], *Ha-ras* [135], the *bcr-abl* fusion gene [148, 156], *c-myc* [87, 104], p53 [7], *c-raf* [105], *c-myb* [33, 98], and protein kinase C (*pkc* α) [31, 32]. Antisense oligonucleotides for several of these are currently in clinical trials (Table 4).

A particularly instructive series of experiments has been carried out by Monia and colleagues [105–108]. First they demonstrated that the specific target sequence chosen is critical to antisense activity and that a priori guesses at "important" sequences, such as the translation initiation codon, do not necessarily yield the best drugs. In the case of the *c-raf* gene, they found that an oligonucleotide directed against a small area in the 3′ untranslated region was the most potent inhibitor of *c-raf* expression [105]. Presumably, the most sensitive sequence is accessible to oligonucleotide hybridization and to Rnase H cleavage after the RNA has assumed its secondary structure and after it has bound to associated proteins. Using the PS-ODN with the most potent in vitro effect Monia et al demonstrated antiproliferative activity against an assortment of cell lines and human solid tumor xenografts in nude mice. They correlated tumor shrinkage with declines in both *c-raf* RNA and protein levels, demonstrated that RNA levels for the homologous *A-raf* gene were not affected, and that the effect declined progressively as an increasing number of sequence mismatches were inserted [105, 108]. With continued administration of the c-raf antisense oligonucleotide the tumor xenografts resumed growth, albeit at a reduced rate. Britten's group recently showed that in cultured cervical cancer cell lines antisense inhibition of *c-raf* increases sensitivity to paclitaxel [129a]. These data raise the intriguing possibility that *c-raf* inhibition will be most effective in combination with cytotoxic drugs such as paclitaxel.

As demonstrated by the experiments with c-raf and with *bcl2* by Ziegler et al. [184], the choice of the target sequence in the gene of interest is fundamental to the design of an effective antisense drug. This choice should be made empirically by screening the entire set of 20–25-base oligonucleotides that comprise the target RNA. Since the primary activity of antisense oligonucleotides seems to be mediated by RNase h cleavage of oligonucleotide:RNA hybrids, the oligonucleotides that reduce RNA levels most effectively in cultured cells should be chosen for tests in animal tumors, including tumor xenograft systems.

Clinical testing of antisense molecules poses some special challenges. Once an optimal antisense sequence has been identified, it should be tested in a population of patients whose tumors express the target gene. In initial trials it is important to assess the molecular response by measuring target gene RNA levels and, if possible, protein levels in tumor biopsies and/or other cells, such as peripheral white blood cells, if the gene is expressed in normal tissues. In animal studies with PS-ODNs, many of the dose-related toxicities, such as complement activation, nonspecific immune stimulation, partial thromboplastin time (PTT) increase, and thrombocytopenia, are a result of the general chemical structure of the oligonucleotides and not the specific antisense sequence [62, 109, 137]. Therefore the toxicity appears not to be related to the antineoplastic mechanism. The ideal treatment dose is that which maximally inhibits the target gene without intolerable toxicities and, as with the angiogenesis inhibitors, MMPIs, and telomerase inhibitors, this may be less than the MTD. In some cases it is possible that dose-limiting toxicity will be a result of target gene inhibition. This would seem to be most

likely to occur if the target is ubiquitously expressed. However, it is possible that tumors will be dependent upon higher levels of expression of the target and will therefore be sensitive to doses well tolerated by the patient.

The preclinical data with antisense drugs indicate that these drugs have antiproliferative activity in both cultured cells and tumor xenografts. However, the published studies of in vivo experimental therapy with antisense compounds do not provide evidence of tumor regression; rather the compounds appear to be cytostatic.

The antiproliferative effect requires continued exposure to the antisense drug; therefore antisense drugs will ideally be able to be administered orally, although the compounds presently in trial are not orally bioavailable due to their size, charge, and acid lability. Ideally, antisense drugs should have minimal toxicity when administered for prolonged periods, and should not have bone marrow toxicity because they may be most potent in combination with cytotoxic drugs. For unknown reasons, for the PS-ODNs currently in clinical trials thrombocytopenia is dose limiting in animals (Table 4).

The preliminary results of a clinical trial of an antisense PS-ODN directed against *bcl2* were recently published. Nine patients with Bcl2-positive, relapsed, stage IV non-Hodgkin lymphoma were treated with an 18-base antisense PS-ODN targeted against *bcl2* [168]. This is a particularly interesting setting in which to test an antisense drug. Essentially all low-grade, follicular lymphomas express *bcl2* as a consequence of the t(14;18) translocation and in diffuse, large B-cell lymphomas increased *bcl2* expression is a poor prognostic marker [63, 64, 114, 123]. Increased *bcl2* expression is also known to inhibit apoptosis and to be associated with resistance to multiple chemotherapeutic agents in both cell culture and tumors in vivo [35, 65, 76, 103]. Furthermore, overexpression of *bcl2* is seen in half or more of human malignancies [131].

In the initial trial report, the antisense PS-ODN against *bcl2* was tolerated in doses up to 73.6 mg/m^2/day when given by continuous subcutaneous injection for 14 days, but there was no consistent decrease in Bcl2 expression. It is possible that at higher doses or with more prolonged administration a more significant molecular response would be seen. One patient had a radiological complete response (CR), and four patients (including the patient with the CR) had a decrease in serum lactate dehydrogenase. However, this study raises an important issue regarding the choice of antisense sequence. The antisense sequence used by Webb et al. encompassed the first six codons of the *bcl2* message. Based on other in vitro studies, an antisense PS-ODN targeted to this region of the *bcl2* RNA is only about 25% as active as an antisense sequence targeted to codons 141–147 in assays of viability in a small cell lung cancer cell line [184]. Would antisense to the latter sequence be more active in clinical trials?

A phase I trial with antisense against *p53* in 16 patients with refractory acute myeloid leukemia or MDS produced no responses, but neither were there any toxicities that could be shown to be attributable to the oligonucleotide [9]. The rationale for this approach is unclear in that destruction of wild-type *p53* mRNA should lead to proliferation and should inhibit apoptosis, both undesirable effects. Furthermore, the results of this study are difficult to interpret because in eight patients the *p53* status was unknown, and in seven of the other eight it was wild type.

PS-ODNs directed against *pkc*α and c-raf have entered clinical trials and cause the same prolongation of PTT and decreased platelet count as observed in the *bcl2* antisense trials. *Pkc*α antisense, now in phase II trials, caused partial regression of a pelvic mass in a patient with ovarian cancer and stabilization of a rising CEA in a patient with colon cancer in a phase I trial [112, 147]. No molecular correlates of drug action have been described in data presented to date.

In summary, antisense oligonucleotides offer an attractive but difficult approach to molecular targeting of anticancer therapy. While cell and animal data have confirmed the feasibility of turning off specific genetic messages, the limited clinical experience to date has provided hints of activity but no convincing confirmation of specificity of action at the molecular level in humans. Further improvements in drug design and delivery systems, including orally bioavailable drug, are needed if this interesting approach is to become an effective therapy for human cancer.

Ras farnesylation inhibitors

Alterations in signal transduction pathways play a central role in the process of malignant transformation and have become primary targets for molecular therapies. Among the most frequent mutations in human tumors are those that activate the Ras family of proteins. These small GTP-binding molecules function as intermediates in relays that transmit the signals from activated growth factor receptors (epidermal growth factor [EGF], basic and acidic FGF, insulin-like growth factor 1 [IGF-1], and other receptors) to downstream intracellular partners (Fig. 3). The oncogenic mutations in Ras destroy the protein's ability to hydrolyze GTP and leave the Ras protein in a permanently activated state [71, 179]. Thus the activated Ras protein has become a primary target for anticancer drug development. In choosing Ras as a target it is important to understand that growth factor signaling displays redundancy, ie, some receptors are able to utilize more than one pathway to transmit their signals to the nucleus. Thus the IGF-1 receptor can utilize the Ras protein as an intermediate or may signal through activation of an alternative pathway involving activation of phospholipase C and protein kinase C [95]. Signaling pathways are also degenerate [34, 71], ie, multiple receptors may share common signaling pathways (Fig. 3). This degeneracy raises the concern that inhibition of a single pathway may interrupt the response to a large number of growth factors that are important for the function of normal cells, and thereby cause substantial systemic toxicity.

Numerous oncogenes are mutated versions of growth facor receptors (erbB, erbA, fms, etc) and signal transduc-

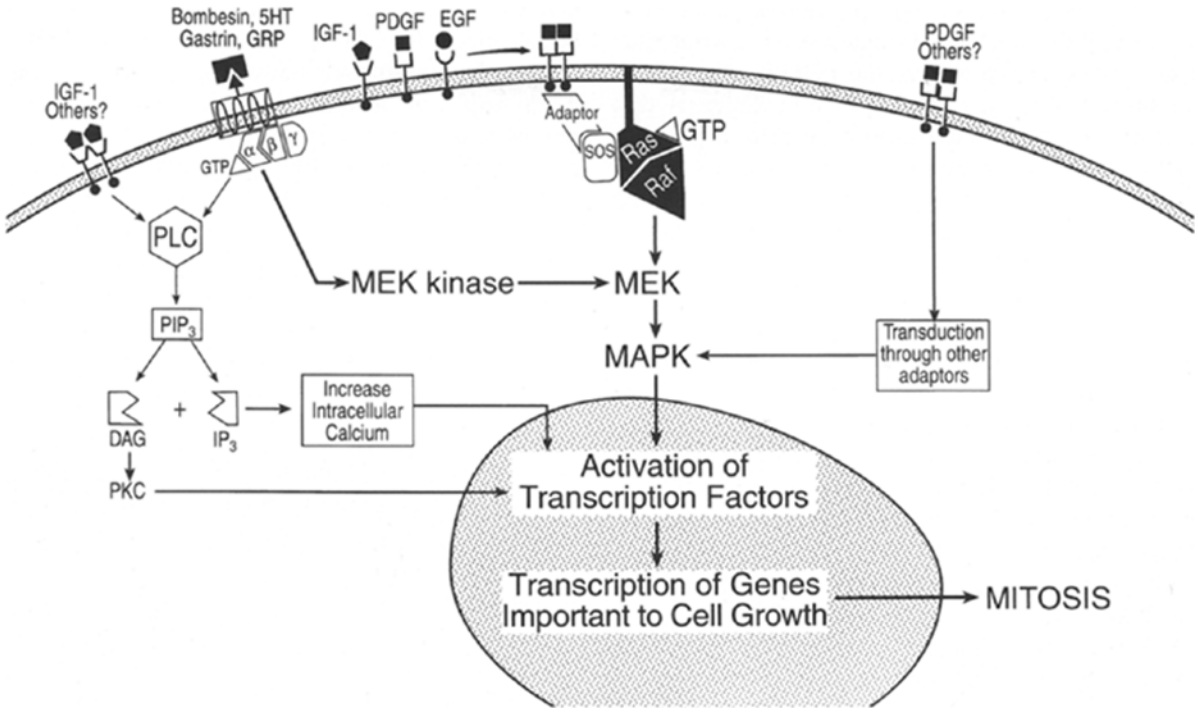

Fig. 3 Redundancy and degeneracy in signal transduction pathways. Several examples of growth facors and mitogenic hormones that signal via tyrosine kinase receptors or seven transmembrane serpentine receptors are shown. The existence of multiple parallel pathways of signal transduction that converge on several intermediates, such as Ras/Raf, the MAP kinase cascade, and phospholipase C (PLC), is emphasized. Ras is illustrated attached to the plasma membrane by a farnesyl anchor. Some receptors, such as the IGF-1 and PDGF receptors, are known to act via multiple pathways. In this diagram, cytoplasmic PLC is depicted interacting with IGF-1, but the subcellular location of PLC when it interacts with IGF-1 is not known. *5HT*, serotonin; *GRP*, gastrin-releasing peptide; *PIP3*, phophotidylinositol triphosphate; *DAG*, diacylglycerol; *IP3*, inositol triphosphate; *PKC*, protein kinase C

tion intermediates (*ras* family, *raf*, etc), and mutations in one or more of these genes have been found in a wide variety of human solid tumors [71, 122]. The family of *ras* genes, which includes Ha-ras, Ki-ras, and *N-ras*, has been of particular interest because *ras* is one of the most commonly mutated gene families found in human malignancies, including >50% of colorectal cancers, >90% of pancreatic cancers, and a substantial minority of non-small cell lung cancers [13, 133, 149]. Furthermore, as described above, Ras protein plays a pivotal role in the relay of signals from many growth factor receptors to the nucleus. Transfection with oncogenic forms of *ras* in combination with one of a number of other oncogenes is sufficient to transform primary cell cultures in vitro [51, 179].

Ras protein is associated with the inner aspect of the plasma membrane and translocation from cytoplasm to membrane requires posttranslational modifications which begin with farnesylation of the cysteine residue (the fourth amino acid residue from the C terminus of the protein). Attachment of the farnesyl group is followed by proteolytic removal of the three C-terminal amino acid residues. The attachment of the farnesyl group (a 15-carbon lipid tail) allows insertion of the molecule into the plasma membrane and is required for Ras activity [56, 73, 175]. Inhibitors of farnesyl protein transferase (FPTase) have attracted interest as Ras inhibitors and antineoplastic drugs.

Specific farnesylation inhibitors should only affect the limited number of intracellular proteins known to undergo farnesylation. Furthermore, FPTase inhibitors may be designed with relative specificity for the C-terminal peptide sequence in Ras that is recognized by the transferase. Based on the structure of tetrapeptides that are competitive inhibitors of FPTase, several drugs have been designed that potently inhibit Ras farnesylation in cells and reverse transformation by mutant *ras* genes [48, 74, 83, 140, 142]. Both Gibbs et al. and Sebti's group have demonstrated that their drugs inhibit the proliferation of cell lines that carry activating *ras* mutations, and both groups have demonstrated inhibition of the growth of tumor xenografts in nude mice. In tumor xenograft models, the FPTase inhibitor L-739,749 had activity against tumors bearing *Ha-ras*, *Ki-ras*, and *N-ras*, but not against tumors with mutant *raf* and wild-type *ras*. FTI-276 was active against a xenograft that harbored both oncogenic *Ki-ras* and a *p53* deletion, but not against a lung cancer xenograft that lacked these mutations [155]. L-739,749 was also active against a pancreatic cancer cell line, PSN-1, which carries mutations in *Ki-ras*, *p53*, and *c-myc*. These observations are promising because they suggest that even malignant cells with multiple genetic abnormalities may be sensitive to Ras inhibition if they carry oncogenic *ras* mutations. Since FPTase inhibitors also inhibit normal Ras function, they will probably be active against tumor cells transformed by upstream mutations in the Ras-Raf-Mitogen-activated protein (MAP) kinase pathway (Fig. 3).

Sebti's group also demonstrated that their synthetic FPTase inhibitors did not block platelet-derived growth factor (PDGF) stimulation of MAP kinase in NIH 3T3

cells, although they did inhibit processing and function of endogenous Ras [97]. Since MAP kinase is an intermediate that lies downstream from Ras in the PDGF signal transduction pathway (Fig. 3), this observation suggests that PDGF activation of MAP kinase can occur by a parallel, redundant pathway. Redundancy of fundamental signal transduction pathways is not surprising and offers the possibility that FPTase inhibitors may not be intolerably toxic, even if they suppress Ras signaling pathways in normal cells. Redundant pathways also seem to provide a mechanism for FPTase inhibitor resistance. Cell lines that are resistant to FPTase inhibitors are found to have active MAP kinase despite effective Ras inhibition [142].

Despite these redundancies that can bypass Ras to activate the MAP kinase pathway, FPTase inhibitors inhibit the growth of many tumor cells. Not only do FPTase inhibitors abrogate Ras activity, but they have additional downstream effects. In the absence of farnesylation, oncogenic Ha-Ras protein acts as a dominant negative inhibitor of Ras activity [47]. Unfarnesylated, oncogenic Ras forms a stable complex with Raf and prevents the translocation of Raf from the cytoplasm to the membrane. However, a nononcogenic Ras modified to prevent farnesylation does not interact with Raf [102]. This finding suggests a model whereby cells with an oncogenic *ras* gene would be much more sensitive to the effects of an FPTase inhibitor than would normal cells. Even partial inhibition of farnesylation might provide a pool of oncogenic Ras to inhibit Raf activity in tumor cells containing *ras* mutations, whereas in normal cells with wild-type *ras*, Raf activity would not be affected. Since mutations of *Ki-ras* are associated with malignancies more commonly than mutations of *Ha-ras*, it will be interesting to learn whether unfarnesylated, oncogenic *Ki-Ras* behaves similarly.

A limitation of FPTase inhibitors may be lack of specificity for Ras. A growing number of proteins are known to be farnesylated, including nuclear lamins A and B, skeletal muscle phosphorylase kinase, and several retinal proteins, as well as a number of unidentified polypeptides [49, 75], and geranylgeranylation is 5- to 10-fold more common than farnesylation [48]. Therefore the tumor specificity of FPTase inhibitors will depend upon their ability to inhibit FPTase without inhibiting other protein-prenyl transferases. The FPTase inhibitors in development are 10- to 1000-fold more potent against farnesyl transferase than against geranylgeranyl-protein transferase types I and II; therefore it seems likely that drugs can be dosed such that they inhibit farnesyl transferase almost exclusively.

The peptidomimetic FPTase inhibitors also display marked differences in potency in inhibiting farnesylation of Ha-Ras versus Ki-Ras. This suggests that FPTase inhibitors can be designed that are relatively specific for Ras compared to other farnesylated proteins. However, as with the biologically directed drugs discussed above, phase I dose-finding studies must determine the biologically effective dose required to block the intended target. In the case of FPTase inhibitors, surrogate endpoints could include assays of Ras farnesylation, Ras activity, MAP kinase activity, and immunohistochemical staining for membrane-bound Ras. If dominant negative inhibition of Raf activity by unfarnesylated, oncogenic Ras is confirmed to be an important mechanism of action of FPTase inhibitors, the most sensitive assay for inhibitors of Ras farnesylation may be downstream inhibition of Raf kinase activity in tumor biopsy samples. As Ras inhibition by FPTase inhibitors is cytostatic, but not cytotoxic, these drugs will also require prolonged administration, and it will be important to develop orally active formulations.

Drug combinations

A comprehensive evaluation of new drugs must take into account their potential in combination with one another or in combination with existing cytotoxic agents. Numerous experiments provide evidence for such synergy. As described above, angiogenesis in the rabbit cornea model is closely associated with hydrolysis of the extracellular matrix and tumor invasion is dependent on both angiogenesis and MMP activity. This finding raises the possibility that angiogenesis inhibitors and MMPIs will be useful in combination. Ionizing radiation and most cytotoxic drugs cause DNA damage that induces apoptosis, and tumors containing mutations that inhibit the apoptosis pathway, such as overexpression of bcl2 or loss of *p53*, are resistant to these treatment modalities [103, 146]. Antisense compounds that inhibit *bcl2* expression and angiogenesis inhibitors such as endostatin and angiostatin which increase the propensity of tumor cells to apoptosis may increase tumor sensitivity to radiation and cytotoxic drugs. In vitro, *c-raf* inhibition sensitizes cells to paclitaxel. Ras inhibitors, which are expected to affect a more proximal step in the same signal transduction pathway, might show a similar effect with paclitaxel. On theoretical grounds, inhibitors of angiogenesis may also be more active in combination with inhibitors of Ras farnesylation because the angiogenic factors FGF and VEGF bind tyrosine kinase receptors, the signals of which are transduced through the Ras pathway.

The potential for effective combinations among different types of drugs that act via different mechanisms holds out hope for more effective therapies, but it adds complexity to the evaluation of novel agents. It is impractical to test all of the theoretically interesting combinations, but the emerging biologically targeted compounds that demonstrate target-specific activity in single-agent trials will need to be evaluated in combination regimens, even in the absence of single-agent anticancer activity.

Conclusions

A host of new anticancer drugs directed at specific biological targets is currently in development, and a few of the more promising examples of these are described above. While some of these drugs may induce apoptosis and regression of existing tumor masses, they have been select-

Table 5 Types of clinical trials of biologically targeted drugs

Trial	Drug dose	No. of patients	Disease specific?	Controlled?	Major endpoints
Phase I	Dose escalation	Small (10–30)	No	No	Drug pharmacology Biologically effective dose MTD Major toxicities
Phase II	Fixed dose or pharmacologic endpoint (AUC or time over threshold concentration)	Intermediate (20–50)	Yes	Not usually	Effect on biological target Pharmacokinetics Disease response Toxicities
Phase III	Fixed dose	Large (>50)	Yes	Yes	Overall survival Time to progression Symptomatic improvement

ed for their ability to modulate one or more molecular processes that are considered important in neoplastic transformation, invasion, or metastasis specifically, and not for their cytotoxic properties. Therefore they will require a strategy for clinical evaluation and development different to that commonly used for cytotoxic, antineoplastic agents.

Most of these compounds are directed at targets that are functional in at least some normal tissues: active angiogenesis is required for endometrial regeneration and wound healing, and, presumably at a low basal rate, for maintenance of the integrity of the normal vasculature; telomerase activity is required by stem cell populations, such as those in the bone marrow, liver, skin, and at mucosal surfaces; and signal transduction through the Ras-Raf-MAP kinase pathway is used by a wide array of growth factors and cytokines. Therefore biologically targeted drugs are likely to have systemic toxicities if used at high doses and for prolonged periods of time. Their potential for specificity lies in the expectation that some malignant cells or malignant processes, such as invasion and metastasis, are more sensitive to interference with the chosen target than are normal tissues.

Since biologically targeted drugs are not expected to be absolutely specific for their targets, thorough pharmacokinetic analysis will be necessary to determine optimal dosing to achieve drug concentrations that are expected to inhibit the intended target with minimal cross-reactivity with related processes. In the case of cytotoxic drugs, biological effects in animal systems (mouse, dog, and monkey) correlate well with the biological effects in humans when the drugs are dosed to achieve similar pharmacokinetic endpoints such as area under the (concentration) × (time) curve (AUC) or time of exposure above a threshold concentration [23]. It will be important to make similar correlations between pharmacokinetic measures and biological effects among animal species for the biologically targeted drugs now in development. In the case of antisense PS-ODNs targeted against *c-raf* and *pkcα*, preliminary data from mouse xenograft tumor models indicate that the time of exposure above a threshold plasma concentration is important.

Pharmacokinetic studies are relatively simple compared to assays for effect on a biological target. Therefore in initial clinical trials it may be prudent to confirm that pharmacokinetic endpoints that are known to be biologically effective in animals are achieved before assays that are expensive and require potentially dangerous biopsy procedures are undertaken in humans.

Compounds that inhibit angiogenesis or MMPs are expected to be most effective at inhibiting growth and invasiveness of micrometastases; therefore they may be effective in prolonging survival even if they do not cause primary tumor shrinkage and may require chronic administration. Similarly, small molecules, antisense compounds, and FPTase inhibitors that are directed against telomerase, Ras- or Raf-mediated growth factor signal transduction, or inhibitors of apoptosis such as Bcl2 or mutant P53 may be more effective in inhibiting further tumor growth than shrinking established, bulky disease. If any of these agents alters the balance of cell proliferation and apoptosis in favor of apoptosis, mass lesions may diminish, but bulk tumor response may not be necessary for improved survival.

Assessment of long-term endpoints such as time to progression, time to disease recurrence, or ultimately survival will require large, randomized studies. To justify such resources it will be necessary to determine that the drug has biological activity and to demonstrate that an optimal dose and schedule have been defined in phase III trials. Since many of these compounds are directed against specific molecular properties of tumor cells, initial clinical trials with biologically targeted agents should be limited to populations of patients who are known to have or are expected to have the relevant target and in whom the presence of the target can be confirmed prior to treatment.

Particularly attractive diseases for the assessment of biologically targeted drugs are those in which neoadjuvant therapy followed by resection is an acceptable treatment approach. These include stage IIIA non-small cell lung cancer, locally advanced esophageal cancer, osteosarcoma, and locally advanced breast cancer. In these diseases, studies can be designed such that the tumor is evaluated for the presence of a specific target at initial biopsy, and the effect of the experimental drug on the target is reevaluated following tumor resection. If responses are observed, they can be correlated with the presence or absence of the intended target; if clinical responses are not seen, relation-

ships between drug dose and molecular response can still be established.

Dose escalation, phase I trials in small numbers of patients should be directed at determining the dose and schedule required both to achieve the AUC or time over a threshold that was biologically effective in animals and to achieve the desired effect on the intended biological target in humans. In addition, at the biologically effective dose phase I and phase II trials will need to assess systemic toxicity.

Phase II trials should be disease specific and evaluate larger numbers of patients at the biologically effective dose and schedule. Ideally, this dose will be less than the MTD because many biologically targeted drugs will require prolonged administration and have the potential to react with targets other than the primary antitumor entity. Extensive evaluation of pharmacokinetic and biological endpoints will be required in phase II trials to determine that these endpoints are achieved by the dose and schedule being analyzed. If these endpoints are not realized, the more expensive phase III trial cannot be justified (Table 5).

This approach raises the concern that the presumed biological target may not be the important target for the drug's antitumor activity, eg, ambiguity of the target for farnesylation inhibitors, or that the chosen assay of the surrogate endpoint may be more sensitive to the drug than the tumor in vivo. Ultimately, large, randomized phase III trials that evaluate survival will be necessary to demonstrate the efficacy of these novel drugs.

The molecular insights into the mechanisms of cancer that have accumulated over the past two decades are now being translated into novel approaches to biologically targeted therapies. Compounds are becoming available that modulate some of the cellular processes thought to be fundamental to the neoplastic phenotype. The next essential step is to devise thoughtful clinical trials that will effectively evaluate these novel agents with consideration of the biological processes that led to their development. Without this consideration there is a high risk that promising agents will be overlooked and ineffectual agents will be pursued inappropriately.

Acknowledgements Anthony L. Boral was supported by National Institutes of Health training grant 5T32CA09172-23.

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