Review of Three New Agents That Target Angiogenesis, Matrix Metalloproteinases, and Cyclin-Dependent Kinases

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The search for novel compounds that act through unique pathways has been facilitated by new knowledge in the molecular and biochemical pathways required for neoplastic transformation and metastasis.

Background: Many potential new antineoplastic agents are currently in various stages of clinical development. Three areas of drug development include antiangiogenic compounds, agents that inhibit matrix metalloproteinases, and agents that modulate cyclin-dependent kinases.

Methods: The authors reviewed the available data for endostatin, COL-3, and flavopiridol, each of which is being developed with one of the above-mentioned proposed mechanisms of action. These agents are among the first drugs to reach clinical testing that is focusing on these novel targets.

Results: Endostatin has finished preclinical testing and the first human trials are about to be initiated. COL-3 is in phase I testing in several locations. Phase I studies for flavopiridol have been completed and several phase II studies are underway. It is unknown at this point if any of these agents will provide clinical benefit to patients at doses that do not cause unacceptable toxicity.

Conclusions: These agents are currently at various stages of clinical testing. Albeit promising as potential modulators in molecular and biochemical pathways, continued research is needed into the toxicities and clinical usefulness of these agents.

Introduction

Despite efforts over several decades with standard antineoplastic agents as single agents or in combination, the improvement in the prognosis for patients with advanced neoplasms remains a formidable challenge. This result has prompted interest in defining compounds with unique targets. The search for novel compounds that act through unique pathways has been facilitated by an explosion of new knowledge in the molecular and biochemical pathways required for neoplastic transformation and metastasis.

Much of the current excitement is focused on the inhibition of antiangiogenesis. The excitement regarding the antiangiogenesis drugs even prompted a series of reports in newspapers and periodicals. The attractiveness of this therapy is that it is focused on the endothelial cells, not the tumor. Since endothelial cells are less likely to mutate, targeting new blood vessel formation should result in little drug resistance being developed. Other areas of interest include matrix metalloproteinase inhibitors and cell cycle modulators.

In this paper we review three new agents in various stages of clinical testing — endostatin, COL-3, and flavopiridol — that are examples of antiangiogenesis inhibitors, matrix metalloproteinase inhibitors, and cell-cycle modulators.

Endostatin

Angiogenesis is the recruitment of new blood vessels. It occurs normally during wound healing, menstruation, and the development of an embryo. Vessel growth is normally controlled by a balance of endogenous inhibitors (eg, angiostatin, interferon) and stimulators (eg, platelet-derived growth factor, insulin-like growth factor, interleukin-1, interleukin 6). Elevated levels of stimulators of angiogenesis such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been correlated with the presence of a malignancy.

Tumor angiogenesis is the process of new blood vessel formation within a malignancy. It has been shown that tumor cells stimulate the proliferation of endothelial cells and new capillaries, allowing enlargement of the tumor mass via increased nutritional supply to the cells. Folkman has focused his research career on inhibiting this phenomena as a potential therapeutic modality. He hypothesizes that inhibition of the tumor recruitment of new blood vessels would most likely isolate a tumor to a local primary site and limit its growth to only a few millimeters in diameter.

Epithelial tumors have the ability to grow to a finite size in the absence of vascularization as a result of passive diffusion of nutrients and passive elimination of waste products. Most solid tumors begin as avascularized malignant cells. These lesions are usually thin, flat collections of cells; however, they can extend to as much as 2 mm in size during this period. Some tumors can remain in this avascular state for years in the skin, pharynx, gastrointestinal tract, respiratory tract, genitourinary (bladder) and uterine cervix (cancer in situ). During this time, the tumor is in a nonproliferative state, and metastases are rare. Gibbon et al reported that a tumor implanted near the iris vessel of a rabbit will become vascularized within five days and then will increase in volume 16,000 times over the next two weeks. Numerous other reports have shown similar results with other model systems.

There is increasing experimental and clinical evidence that the microvascular endothelium in a tumor bed exerts tight control over various cancer phenotype, including tumor growth, tumor progression, metastasis, tumor dormancy, and tumor cell apoptosis. The potential clinical importance of angiogenesis has been shown for multiple solid tumors (eg, breast, colon, ovarian). Weidner et al showed a correlation between microvessel count and metastatic prostate cancer. The mean microvessel count within biopsy tissue for the metastatic group was 76.8 vessels per field compared with 39.2 for those without metastasis (P<0.0001). This study supports the theory that the degree of angiogenesis is an important predictor of disease progression.

The inhibition of angiogenesis has been proposed as a potential means for selectively impairing tumor growth. The first compound identified as an angioinhibin was a...
Collagenase inhibitor found in cartilage. Other agents with activity include a corticosteroid plus heparin combination, protamine, platelet factor 4, sulfated polysaccharide-peptidoglycan complex derived from the bacterial wall of Arthrobacter, pentosan, D-penicillamine, TNP-470, gold thiomalate, suramin, thrombospondin, and analogues of vitamin D3.13,14,16 One of the first clinical reports was by White et al17 who observed in a child the regression of pulmonary capillary hemangiomatosis, a disease characterized by active angiogenesis, following the administration of alpha interferon.

Endostatin is a 20 kDa C-terminal proteolytic fragment of collagen XVIII that was first purified from a murine hemangiöendothelioma cell line.18 In the original study by O'Reilly et al,18 recombinant murine endostatin was produced in baculovirus and Escherichia coli expression systems. It demonstrated selective inhibition of endothelial cell proliferation in vitro and antiangiogenic activity in the cell adhesion molecule (CAM) assay, and it suppressed the growth of metastases in a Lewis lung carcinoma model. Furthermore, E. coli-derived endostatin therapy (administered subcutaneously as a nonrefolded suspension in phosphate-buffered saline, 20 mg/kg per day) demonstrated potent antitumor activity in Lewis lung carcinoma, T241 fibrosarcoma, EOMA hemangiöendothelioma, and B16F10 melanoma tumor models.18 Continued endostatin treatment maintained these tumors in a dormant state and microscopic size, and it showed no toxicity or evidence of drug resistance. Discontinuation of endostatin therapy resulted in lethal recurrence of the primary tumors within five to 14 days.

In a subsequent study by Boehm et al,19 recombinant endostatin was administered subcutaneously to mice with Lewis lung carcinomas, T241 fibrosarcomas, and B16F10 melanomas and did not induce drug resistance. Treatment of tumors involving established tumors in the animals, treating with endostatin until regression was achieved, and then discontinuing therapy to allow the tumors to regrow. Interestingly, all tumors became dormant after two to six cycles of treatment and failed to regrow once dormancy began.19 Mice were observed for 103 to 165 days after the onset of the dormant state,19 immunity to the tumors was ruled out by reestablishing the same tumor type in dormant mice at a remote site from the primary site. Furthermore, the study described complete regression and dormancy of Lewis lung carcinomas in mice treated with a combination of endostatin and angiotatin (both at 20 mg/kg per day) for 25 days and later remained disease free for 11 months after therapy was discontinued.19 These data confirmed the potent antitumor activity of endostatin in mouse models. However, there have been conflicting data from other groups on the activity of endostatin.20,22

Hohenester and colleagues23 solved the crystal structure of mouse endostatin at 1.5 Å resolution. The structure spans the 184 C-terminal amino acid residues of mouse alpha1(XVIII) collagen plus the N-terminal sequence APLA.24 Structural analysis of endostatin revealed a relationship to the C-type lectin carbohydrate recognition domain (CRD), despite having lost the calcium-dependent oligosaccharide-binding feature of this protein family.25 The high affinity of endostatin for heparin is explained by the large number of basic residues in its structure, especially arginines. A putative heparin-binding site has been described spanning a cluster of 11 arginine residues.23 The heparin-binding properties of endostatin also suggest a possible antiangiogenic mechanism through signaling interference with heparan sulphate proteoglycans, specifically the bFGF signaling pathway.23

In more recent studies, the crystal structure of endostatin has elucidated zinc-dependent dimerization-binding properties. Ding et al44 reported that zinc is a constituent of both murine and human endostatin in solution. The human endostatin zinc-binding site is localized to the N-terminal loop of the protein, which makes a dimeric contact in endostatin crystals and involves histidines 1, 3, 11, and aspartic acid 76.24 The location of the binding site is adjacent to the precursor cleavage site, suggesting the possibility that zinc may be involved in its activation from its precursor or may be required for antiangiogenic activity.24,25 Indeed, a subsequent study by Boehm et al25 determined that endostatin binds zinc at a 1:1 molar ratio. Furthermore, site-directed mutagenesis of the zinc binding site reduced the biological activity of endostatin.26 The authors concluded that zinc binding of endostatin is essential for its antiangiogenic activity. They also hypothesized that zinc binding may serve two functions: to protect the N-terminus from proteolytic degradation and to stabilize the protein conformation that is important for interaction with a putative endothelial cell receptor.27 More structural analyses will be necessary to clearly elucidate interactions of endostatin with zinc and its role in the antiangiogenic activity of endostatin.

Phase I trials are scheduled to start at M.D. Anderson Cancer Center, the University of Wisconsin, and Harvard University (Dana-Farber Cancer Center and Massachusetts General Hospital). Preclinical toxicology and pharmacokinetic results are still pending. However, the pic-produced human endostatin appears to be an acceptable pharmaceutical preparation for clinical administration.

COL-3

Most cancers have the potential to metastasize. A tumor cell must intravasate, enter the circulation, extravasate, seed, and proliferate at distant sites to develop a distant metastasis. Several processes in both metastasis and angiogenesis require the remodeling of the extracellular matrix. Matrix metalloproteinases (MMPs) comprise a class of enzymes that degrade the extracellular membrane components such as collagenase, gelatinase, and stromelysins.

MMPs are expressed during physiologic processes such as wound repair, reproduction, mammary involution, and tissue growth and remodeling. This class of enzymes is implicated in the following disease processes: atherosclerosis, corneal ulceration, emphysema, osteoarthritis, osteoporosis, rheumatoid arthritis, ulcerative colitis, tumor invasion, and metastasis.29,30 MMPs and TIMPs are secreted by both tumor cells and stromal cells.26,27,31 It is currently believed that an imbalance between active MMPs and TIMPs causes degradation of the basement membrane and allows angiogenesis, tumor growth, and invasion to occur. Therefore, synthetic MMP inhibitors are being developed for their potential ant metastatic and antiangiogenic properties.32

MMP inhibitors have been developed for potential therapeutic use in arthritis, cancer, periodontal disease, and corneal ulceration. Marimastat (BB-2516, British Biotech, Ltd), AG3340 (Agouron Pharmaceuticals, Inc), OGS-2702A (Novartis Pharma AG), BAY 12-9568 (Bayer Corp), D2163 (Chiroscience Group PLC), Ilomastat (GM6001, Glycomed, Inc) and COL-3 (Metastat, CMT-3, CollaGenex Pharmaceuticals, Inc) are MMP inhibitors currently in clinical trials.33 This review focuses on COL-3.

Golub and colleagues34,35 discovered that some tetracyclines could inhibit collagenase. Further manipulation of the tetracycline molecule resulted in the elimination of the antimicrobial properties without destroying the ability to inhibit MMPs.36 The resulting molecule, COL-3 (6-demethyl-6-deoxy-4-dedimethylaminotetracycline), is a highly lipophilic, chemically modified tetracycline.37

COL-3 (5 µg/mL) has been shown to decrease inducible nitric oxide synthase protein expression and nitric oxide production in rat mesangial cells.38 It has been suggested that nitric oxide is involved in angiogenesis. Murohara and colleagues39 have reported that endothelium-derived nitric oxide may mediate angiogenesis by supporting endothelial cell migration by an integrin-dependent mechanism. COL-3 concentrations between 2.5 and 10 µg/mL inhibited nitric oxide production and inducible nitric oxide synthase (iNOS) in murine macrophages in a concentration-dependent manner.40 The decrease in iNOS appears to be due to a decrease in the iNOS protein or an increase in mRNA stability rather than an effect on transcription.40

COL-3 directly inhibits MT1-MMP (membrane-type-1 MMP) expression and activity and pro-MMP-2 expression in osteosarcoma cells.41 The inhibition of MMP-2 activity by COL-3 is competitive in nature. COL-3 has been shown to be cytotoxic to BPH-1 (immortalized nontumorigenic prostatic epithelial cell line), DU-145, PC-3 (human prostate cancer cell lines), and FHS-733 cells(normal human fibroblast cell line).42 Dunning MAT LyLu cells (rat prostatic carcinoma cell line) exposed to COL-3 had an increase in soluble nucleosomes, which suggests apoptosis as a possible mechanism.43 Others have shown that COL-3 induced apoptosis in RAW 264 (mouse macrophage cell line) and U937 cells (human histiocytic lymphoma cell line) but not in human foreskin fibroblasts, ovine articular chondrocytes, MG-63, and murine calvarial cells.44 The MMP-based mechanisms were demonstrated when COL-3 was able to decrease the metastatic lesions in lungs of the MAT LyLu Dunning model and inhibited the invasiveness activity of PC-3 and DU-145 cells across a Matrigel-coated filter at concentrations of less than 3 µg/mL.4

In vivo studies demonstrated that paraplegia in rats resulting from bone metastasis from Dunning MAT LyLu cells was significantly reduced when rats were treated with COL-3 at 40 mg/kg either prior to or at the same time as the tumor injection.45 COL-3 also decreased the number and size of lung metastases in SCID mice inoculated with C8161 cells.42
The absorption of COL-3 after oral administration (5 mg) to rats was found to be delayed ($T_{\text{max}} = 12$ hours) and resulted in a maximum concentration of 4.6 $\mu$g/mL (unpublished data, Y. Liu, et al, 1999). This concentration is within the range found to have produced several of the actions reported in vitro.\textsuperscript{38,40,41} In addition, it had a relatively long half-life of 22 hours compared to the other tetracycline derivatives that ranged from 2 to 38 hours. COL-3 was found to distribute into the lung, brain, spleen, kidney, heart, and liver of the rats 48 hours after a single administration (unpublished data, Y. Liu, et al, 1999).

Since COL-3 inhibited MMP activity and may have other mechanisms of action (inhibition of INOS), COL-3 was entered into phase I clinical trials at the National Cancer Institute (NCI), AIDS Malignancy Consortium, and the University of Texas at San Antonio. Currently, the NCI trial has enrolled 33 patients at four dosage levels (36, 50, 70, and 98 $\text{mg/m}^2$ per day orally). The most significant complications reported to date are photosensitivity and three cases of drug-induced lupus. One patient has stable disease after 17 months of therapy. Preliminary pharmacokinetics suggests that a one-compartment model appropriately describes the data and results in an estimated terminal half-life of greater than 2 days.\textsuperscript{46}

MMP inhibitors are still in the developmental stage and are being studied in combination with cytotoxic agents. Nonetheless, their place in the treatment of cancer has not been clearly defined. Surrogate markers still need to be defined with this class of compounds. Many investigators believe monitoring plasma or serum levels of MMPs will not reflect the compounds’ true activity in the tumor. Since most MMP inhibitors are not cytotoxic, a traditional approach to drug development may prove to be futile and alternative endpoints may need to be explored.

### Flavopiridol

Abnormalities in the cell cycle progression may account for a large number of human tumors. The search for these differences has identified many different proteins that are potential therapeutic targets. One of the key families of proteins controlling cell cycle progression is the cyclin-dependent kinases (CDKs). These enzymes are activated by the formation of complexes with cyclin proteins. There are different cyclin proteins that complex with several different kinases throughout the cell cycle. In turn, these complexes are regulated by several families of small proteins such as p16, p15, p21, and p27.\textsuperscript{47-49}

Flavopiridol can cause cell cycle arrest at $G_1$ or $G_2$ and can inhibit the activation and activity of several CDKs, specifically CDK1, CDK2, and CDK4.\textsuperscript{50-54} Flavopiridol appears to inhibit the activity of CDK2 by blocking the ATP binding site.\textsuperscript{55}

Flavopiridol is the first anticancer agent with potent activity as a CDK inhibitor to enter clinical trials. Flavopiridol can also inhibit other protein kinases, which include protein kinase C as well as protein kinase A and EGFR, but these activities occur only if IC50 concentrations of 10 mmol/L or more.\textsuperscript{47} In addition, certain cell types (e.g., hematopoietic) are notably sensitive to flavopiridol-induced apoptosis.\textsuperscript{56} These cytotoxic effects in vitro models have been confirmed in leukemia/lymphoma xenografts models.\textsuperscript{57} Thus, although not absolutely limited to its effect on CDKs, the spectrum of activities of flavopiridol is potentially very unique in comparison to conventional agents, which heightens interest in examining its clinical activity.

When A549 non-small cell lung carcinoma cells were exposed to flavopiridol in combination with paclitaxel, cytarabine, topotecan, doxorubicin, or cisplatin, enhanced cytotoxicity was noted in vitro.\textsuperscript{58} The enhanced cytotoxicity noted with cisplatin was schedule-independent. For the other agents tested, the cytotoxicity was enhanced (IC50 was reduced by 50% or more compared with single drug controls) when the other agent was added prior to exposure to flavopiridol compared with simultaneous or prior flavopiridol exposure. It was determined that this was the result of flavopiridol-influenced cell cycle arrest in either the $G_1$ or $G_2$ phase. When cells were exposed for 24 hours to flavopiridol and then released, the cells were highly sensitive to the effects of an S-phase active agent (cytarabine or 5-fluorouracil).\textsuperscript{58}

Flavopiridol has also shown to synergistically increase the percentage of mitomycin-C-induced apoptosis in MKN-74 gastric and MDA-MB-468 cells.\textsuperscript{59} Treatment with either mitomycin-C or flavopiridol alone induced apoptosis in less than 18% of either of the cell lines. Sequential treatment with mitomycin-C followed by flavopiridol was significantly better at inducing apoptosis (63% to 76% of cells) than the other order or simultaneous exposure.\textsuperscript{59}

Only one clinical phase I study has been fully reported in the literature to date, while a second study has been reported in abstract form only.\textsuperscript{60} Therefore, the majority of the clinical information described below for this drug was derived from the clinical trial conducted at the NCI.\textsuperscript{60} Flavopiridol was administered as a 72-hour constant infusion in both clinical studies. Dosing began at the rate of 4 mg/m$^2$ per day x 3 and was escalated in successive cohorts of patients. At doses of less than 50 mg/m$^2$ per day x 3, toxicity was quite manageable. At doses of 35 mg/m$^2$ per day x 3 or greater, diarrhea was noted as the major toxicity and resulted in dose-limiting toxicity. The investigators concluded that the diarrhea was consistent with a secretory diarrhea, without leukocytes, mucus, guaiac-positive stools, presence of $C$ difficile toxin, or other laboratory findings that would indicate another cause of the diarrhea was observed. Treatment of the diarrhea consisted of loperamide (2 mg at first onset, then every 2 hours while awake until no diarrhea was present) was successful in managing this toxicity. Another toxicity noted at doses of less than 50 mg/m$^2$ per day x 3 was a flu-like syndrome consisting of anorexia, fatigue, fever, and malaise. Local tumor pain was also noted. The clinical study conducted at the University of Wisconsin determined the maximum tolerated dose (MTD) to be 40 mg/m$^2$ per day x 3.\textsuperscript{61}

Since the therapy with loperamide was successful in managing the diarrhea, an effort was made to establish a higher MTD in the presence of a diarrhea prophylactic regimen. The regimen consisted of high-dose loperamide (every 2 hours while awake until an 8-hour diarrhea-free interval) and cholestyramine (one packet orally three times daily). Doses were escalated up to 122.5 mg/m$^2$ per day until symptomatic hypotension was noted, and the dose was reduced. The dose was further reduced to 78 mg/m$^2$ per day x 3 because of hypotension and uncontrolled diarrhea when patients were treated at 98 mg/m$^2$ per day x 3. The MTD in patients that can tolerate the diarrhea prophylaxis regimen is 78 mg/m$^2$ per day x 2. Three of 18 patients were unable to tolerate such a regimen and developed grade 3 diarrhea after halting the prophylactic therapy. The prophylactic regimen and flavopiridol were tolerated in 14 of 18 patients at 78 mg/m$^2$ per day x 3 days.

It is unclear at this time if the diarrhea is associated only with constant infusion flavopiridol therapy. Further studies using other intravenous dosing strategies (daily bolus infusion x 5) are currently being conducted and should answer this question.

The pharmacokinetic parameter estimates of flavopiridol from the phase I study were as follows: total clearance, 17.23 L/h/m$^2$; terminal half-life, 11.6 hours; and apparent volume of distribution, 12.13 L/m$^2$. The concentration data from many patients (approximately 30%) were found to have a post-infusional increase in concentration at 3 to 24 hours after the end of the infusion. This second peak concentration appeared to occur more often around meal times and was not necessarily associated with any specific time point post infusion. This phenomenon was also reported following intravenous administration of another flavonoid to mice.\textsuperscript{62} This may represent resorption of drug from the gastrointestinal tract.\textsuperscript{63} Flavopiridol has been shown to undergo glucuronidation in hepatic microsomal preparations and in hepatic perfusion studies conducted in rats.\textsuperscript{63,64} In the rat hepatic perfusion studies, two separate glucuronide metabolites were found in the bile of the perfused liver.\textsuperscript{65} Using hepatic microsomal preparations in vitro, only one metabolite was found.\textsuperscript{63,65} The glucuronidation rate was found to vary up to sixfold in microsomal preparations prepared from 48 patients.\textsuperscript{65} It appears that uridine-diphosphate-glucuronosyltransferases (UGTs) from the UGT2 family are responsible for this metabolism. Micosomes from rats (Gunn) and four patients (Crigler-Najjar type-1) that are deficient in UGT1 enzymes were able to metabolize this drug.\textsuperscript{65}

Flavopiridol is being tested in phase II studies in lymphoma (relapsed mantle cell, and intermediate/high-grade non-Hodgkin’s lymphoma), chronic lymphocytic leukemia, and prostate cancer as a 72-hour continuous infusion. A phase I study is currently attempting to determine the MTD of flavopiridol administered as a short daily infusion for five consecutive days every three weeks. Flavopiridol is also undergoing investigation in combination with either cisplatin or paclitaxel as a 24-hour infusion.

It remains to be seen if flavopiridol will ultimately be found to have clinical activity as a 72-hour continuous infusion or with some other route of administration. However, because it represents the first agent that inhibits CDK2 and CDK4, it is an important first step in finding agents that can modulate the cell cycle of cancer cells in vivo.
Conclusions

The three agents that have been reviewed are in various stages of clinical testing. Flavopiridol is in phase II testing. Prolonged administration of flavopiridol has been shown to cause diarrhea. At lower doses, the diarrhea can be controlled, but clinical activity may be decreased. While COL-3 is still in phase I studies, the potential for photosensitivity may prove to be the dose-limiting toxicity and limit its clinical usefulness. The clinical studies for endostatin are only beginning. While the animal studies that have been conducted are promising, it is not known if the same activity will be demonstrated in humans.

Even if some of these agents do not progress into later phases of clinical testing, the information gained from attempting to modulate the indicated pathways is important. Using the information gained from the clinical studies of these agents, newer compounds can be developed that may overcome some of the possible negative attributes associated with these agents.

References
