

Effect of Antiangiogenic Agents on Experimental Animal Models of Hepatocellular Carcinoma

L X Qin,**MD, PhD*, Z Y Tang,***MD*, X M Li,****MD, PhD*, W Bu,*****MD, PhD*, J L Xia,†*MD*

Abstract

A new therapeutic strategy for treating metastasis in hepatocellular carcinoma (HCC) has entailed the use of antiangiogenic agents such as suramin, BB-94 (Batimastat), TNP-470, and carboxyamido-triazole (CAI, a synthetic inhibitor of non-excitable calcium channels that reversibly inhibits angiogenesis). These agents have been used to treat metastatic model of HCC in nude mouse (LCI-D20 mouse model). The results of these studies are summarized in this paper with emphasis on the inhibitory effects of the drugs on tumour growth, angiogenesis, invasion and metastasis in LCI-D20 mouse models. The results suggest that all of the agents used can significantly inhibit tumour growth, angiogenesis, invasion and metastasis of human HCC in nude mouse models, and may be candidates for the control of recurrence and metastasis after HCC resection.

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Key words: Angiogenesis, Antiangiogenesis, Hepatocellular carcinoma (HCC), Invasion, Metastasis

Introduction

Metastatic spread is the principal reason of treatment failure in solid tumours. Surgery, radiation therapy and chemotherapy have not manifestly altered the prognosis for metastatic tumours. Hence, the research for new avenues for potential novel therapies is necessary.' The process of metastasis involves a cascade of linked, sequential steps involving multiple host-tumour interactions. A detailed understanding of the genetic and molecular events underlying cancer invasion and dissemination offers hope for measures to control metastatic disease. Intervention at any process of metastasis can theoretically result in prevention of metastasis.²

Angiogenesis, a complex process that includes the activation, proliferation, and migration of endothelial cells, disruption of vascular basement membranes, formation of vascular tubes and networks, and linkage to the pre-existing vascular networks, is a prerequisite both for expansion of the primary tumour mass and the metastatic colony. The new blood vessels embedded in the tumour provide a gateway for tumour cell entering into the circulation and metastasizing to distant sites. Studies of the relationship between angiogenesis and metastasis provide potentials for the development of

important diagnostic, prognostic, and therapeutic clinical procedures.¹⁻⁵

With the knowledge that a solid tumour cannot progress without the generation of new blood vessels, a major effort has been focused on understanding the biology of angiogenesis, identification of mediators that normally dampen angiogenesis, and the evaluation of strategies that employ antiangiogenic agents to inhibit the growth of tumours.⁵ There are a variety of approaches that are under evaluation for their ability to suppress tumour neovascularization. These include neutralization or sequestration of angiogenic mediators (such as suramin, anti-VEGF antibody and anti-angiogenin antibody), interference with normal function of receptors for angiogenic mediators (such as chimeric soluble flt-1 IgG heavy-chain protein, and specific inhibitors of Flt-1 receptor), inhibition of functions of basement membrane and extracellular matrix (BB-94, Marimastat, and angiostatic steroids), direct endothelial cell injury (vascular targeting), and unknown mechanisms of action on endothelial cells (such as TNP-470, interferon alpha, angiostatin, endostatin, interleukin 12, retinoids, thalidomide and tamoxifen). Some agents have already produced encouraging results on animal tumours, and a

* Associate Professor

** Professor and Chairman

† Physician

*** Surgeon

**** Surgeon

Liver Cancer Institute, Zhongshan Hospital, Shanghai Medical University, PR China

Address for Reprints: Dr Zhao-You Tang, Liver Cancer Institute, Zhongshan Hospital, Shanghai Medical University, Shanghai 200032, P R China

E-mail: zytang@srcap.stc.sh.cn

dozen or more candidate drugs have been getting into clinical trials.”

Hepatocellular carcinoma (HCC) is ranked the second cancer killer in China since the 1990s. In recent decades, some progresses have been made in the clinical study of HCC, which include small HCC resection, cytoreduction and sequential resection of unresectable HCC, and resection of subclinical recurrence after curative resection. However, spread from local HCC invasion remains a major obstacle to further prolonging survival after resection of HCC. Much have been done to explore the mechanisms of recurrence and metastasis in HCC, and effective ways to prevent and treat them.¹²⁻¹⁸

It is well known that HCC is one of the tumours with rich blood supply and special vasculature. The spread of tumour cells via the blood vessel (including thrombosis in the portal vein) is the commonest pathway of HCC metastasis. It has been previously shown that intratumour microvascular density (MVD), overexpression of vascular endothelial cell growth factor (VEGF) and metalloproteinases (MMPs) are closely related to the progression and metastasis of HCC, suggesting that angiogenesis plays an important role in the invasion and metastasis of HCC.¹⁸ Thus, antiangiogenesis might be one of the possible ways to control HCC metastasis.

To explore new therapeutic modalities for metastasis of HCC, some antiangiogenic agents including suramin, BB-94 (Batimastat), 0-(chloroacetyl-carbamoyl) fumgillin (TNP-470, a potent angiogenesis inhibitor), and carboxyamido-triazole (CAI, a synthetic inhibitor of non-excitabile calcium channels that reversibly inhibits angiogenesis) have been used to treat metastatic models of human HCC in nude mouse (LCI-D20 mouse model) in the authors' institute.¹⁸⁻²⁰ The results of these studies are presented with emphasis on the inhibitory effects on *in vivo* tumour growth, angiogenesis, invasion and metastasis in LCI-D20 mouse models.

Methods

1. **Animal model:** The metastatic nude mouse model of human HCC (LCI-D20) was established in BALB/CA-nu/nu mice by orthotopic implantation of human HCC tissue, which has been maintained for over 50 passages. The tumour maintains its original features after these passages.¹⁵
2. **Tumour growth and metastasis assays:** In the LCI-D20 nude mouse models, mice were sacrificed at the scheduled time of the experiments. The size and weight of the liver tumour xenografts from the animals were calculated. The liver, tumour, and lung of the mice were fixed in 10% buffered formaldehyde, and embedded in paraffin. Sections of the lung and tumour were stained with haematoxylin and eosin. The numbers of lung metastases were counted in a randomized section of the lung. The survival time of

mice was also investigated.

3. **Cell proliferation assay:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to test the sensitivity of agents to cells (including HCC cells and human umbilical vein endothelial cells, HUVECs) *in vitro*.²¹
4. Gelatinase zymography was performed to test the effects of agents on the production of MMPs of tissue extracts and culture cells *in vitro*.²²
5. Boyden chamber and matrigel assay was used to determine the effect of agents on the motility and enzymatic dissolution ability of HUVECs *in vitro*.
6. The intratumour microvessel density (MVD) was evaluated by immunohistochemical staining using polyclonal anti-factor VIII (Duke, Denmark) (ABC method).
7. **Statistics:** The results of some parameters such as tumour size, tumour weight, serum AFP levels, length and number of vessels were expressed as mean $\pm$ SD. The significance of differences between parameters such as tumour size, and metastatic rate in treated and control groups were analyzed at the end of the experiments using Chi-square test and Student's *t*-test. $P < 0.05$ was considered statistically significant.

Effects of Suramin on Tumour Growth, Angiogenesis, Invasion and Metastasis in LCI-D20 Models

Suramin is a polysulfonated naphthylurea originally developed as a form of treatment for African trypanosomiasis. It is an inhibitor of DNA polymerase and reverse transcriptase as well as many other enzymatic systems. It has been reported to inhibit *in vitro* cell growth of normal and neoplastic cell lines, possibly by interaction with the binding of a number of growth factors including platelet-derived growth factor, epidermal growth factor, and transforming growth factor-beta to their specific cell surface receptors. Moreover, suramin is able to inhibit receptor binding of other ligands like low-density lipoprotein and transferrin. It is also able to induce cell differentiation in several experimental systems. Recently, encouraging results were published from clinical trials indicating that suramin may be useful as an antineoplastic agent in the treatment of certain advanced human malignancies. Antitumour activity as measured by a 75% decrease in prostate specific antigen was reported in 30 of 69 patients with hormone-refractory metastatic prostate cancer.^{4-7,23-26} Suramin is currently being tested in a large-scale prospectively randomized trial to confirm these observations.

To investigate the effects of suramin on growth and metastasis of human HCC, the metastatic models (LCI-D20) established in nude mice by orthotopic implantation were used. Suramin was administered by injection *i.p.* (60 mg/kg /dose) on days 1, 3, 6, 9, 14, 21, 28 after the tumour implantation. The primary tumour growth and

spontaneous pulmonary metastasis were evaluated. The results showed that growth, invasion and metastasis of the human HCC xenografts in LCI-D20 models were significantly inhibited by suramin. Pulmonary metastasis was found in 62.5% of suramin-treated mice as compared with 100% of controls ($P < 0.05$) (Table I). In the suramin-treated group, intratumour microvessel density (MVD) was markedly decreased (37.4 ± 23.7 vs. 87.5 ± 16.3 , $P < 0.05$), and the vasculature was more regular, with less sinusoid-like vessels compared with the control group. These suggested that suramin had inhibitory effects on the growth, angiogenesis, invasion and metastasis of human HCC xenografts in nude mice, and could be used to prevent the progression and metastasis of HCC.¹⁹

TABLE I: EFFECTS OF SURAMIN ON TUMOUR GROWTH, INVASION AND METASTASIS IN LCI-D20 MOUSE MODELS

	Suramin-treated group (n = 8)	Control group (n = 5)	
Tumour size (cm)	7.54 ± 5.58	10.80 ± 2.98	<0.05
Abdominal wall invasion (%)	25	100	<0.05
Intrahepatic spreading (%)	37.5	100	<0.05
Lung metastasis (%)	62.5	100	<0.05

Effects of BB-94 on *in vivo* Tumour Growth, Invasion and Metastasis, and Survival of LCI-D20 Mouse Models

Metalloproteinases (MMPs) are a family of enzymes that are crucial to the processes of tumour vascular invasion and angiogenesis, which can degrade basement membrane and the extracellular matrix. The enzymatic dissolution of basement membranes and surrounding tissue matrix may assist the penetration of capillary sprouts during tumour neoangiogenesis and also potentiate the invasion of tumour cells through endothelia during the intravasation and extravasation phases of dissemination and metastasis. Elevated MMP expression in tumour tissue compared with adjacent normal tissue has been documented in human tissue samples from patients with breast, lung, colon, and liver cancer and was associated with more invasive disease.¹⁻⁴ Batimastat (BB-94) is a low molecular weight peptide mimetic that reversibly binds to zinc in the active site of MMPs.²⁷ In animal models, BB-94 inhibited tumour growth, invasion and metastasis in well-tolerated doses.^{28,29} Clinical trials suggested a decrease in the rate of ascites formation in women with advanced ovarian cancer, and inhibition of organ invasion of human colon carcinoma.^{4,6}

To investigate the effects of BB-94 on tumour growth, invasion, metastasis and survival of the LCI-D20 mouse models, 30 mg/kg of BB-94 suspended in PBS (pH 7.3)

containing 0.001% Tween-80 with concentration of 3% was injected i.p. into each mouse model (LCI-D20) every day. Gelatin zymograph technique was employed to study the expression of MMP-2 and MMP-9 in the tumour tissues from LCI-D20 mouse models. Intratumour MVD was also evaluated to test the effect of BB-94 on the tumour angiogenesis. The results showed that BB-94 could significantly inhibit tumour growth, invasion and metastasis of HCC in nude mice, and prolong the survival time of the mice. The intratumour MVD after the BB-94 treatment was less than that in the control tumour tissue. The number of the cells passing through the matrigel in BB-94 treated and control groups was 3.6 ± 3.36 and 22.8 ± 9.09 /field of vision, respectively, ($P < 0.01$). The rest of the results are presented in Table II.

TABLE II: EFFECTS OF BB-94 ON TUMOUR GROWTH, INVASION AND METASTASIS AND SURVIVAL IN LCI-D20 MOUSE MODELS

	Treated group (n = 19)	Control group (n = 19)	P
Tumour weight (g)	2.27 ± 1.17	3.13 ± 0.84	<0.05
Abdominal wall invasion (%)	22	80	<0.05
Intrahepatic spreading (%)	33	80	= 0.055
Lung metastasis (%)	44	100	<0.05
Survival rate in 45 days (%)	100	56	<0.05

Effects of TNP-470 on Tumour Growth, Proliferation, Metastasis of LCI-D20 Mouse Models

TNP-470, a synthetic analogue of fumgillin, which is a naturally secreted antibiotic of *Aspergillus fumigatus fresenius*, has been found to exhibit potent inhibitory effects on the growth of endothelial cells and a variety of solid tumours with relatively few side-effects. There have been many reports about the inhibitory effects of TNP-470 on the growth and metastasis of various kinds of solid tumour.³⁰⁻³⁶ The potent antiangiogenesis effect of TNP-470 has been demonstrated both *in vivo* and *in vitro*.³⁷

To investigate the antitumour and antimetastatic effects of TNP-470 on HCC, TNP-470 (30 mg/kg body weight) was given subcutaneously to nude mice bearing HCC in the LCI-D20 metastatic nude mouse models every other day from days 1 to 15 after tumour implantation. A significant inhibitory effect on the tumour growth and pulmonary metastasis was observed after the TNP-470 treatment. No significant side effect was observed in the treatment (Table III and Fig. 1). These indicated that TNP-470 could significantly inhibit both growth and metastasis of HCC in the nude mice.²⁰ TNP-470 appears to be a potent antimetastatic agent for HCC.

TABLEIII: INHIBITORY EFFECTS OF TNP-470 ON TUMOUR GROWTH AND METASTASIS IN LCI-D20 MOUSE MODELS

	TNP-470 treated group (n = 12)	Control group (n = 12)	P
Tumour weight (g)	0.97 ± 0.34	2.04 ± 0.34	<0.001
Serum AFP level (ug/L)	93 ± 59	769 ± 283	<0.01
Pulmonary metastasis rate (%)	8.3 (1112)	50 (6/12)	<0.05

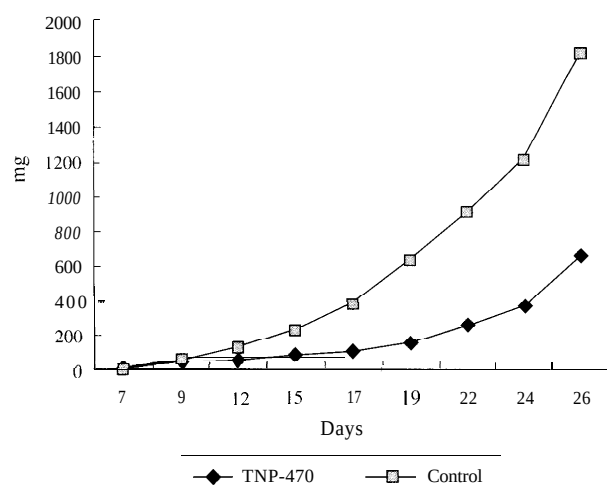


Fig. 1 Inhibitory effects of TNP-470 on tumour growth of LCI-D20 mouse model

Effects of CAI on the Proliferation, Motility, Degrading Extracellular Matrix and Angiogenesis of Tumour Cells

CAI is a novel inhibitor of signal transduction, which inhibits cancer proliferation, invasion and metastasis. It has been demonstrated previously that CAI inhibited receptor-operated calcium influx and release of arachidonic acid and also inhibited proliferation of an array of malignant cell lines. Phase I clinical trial has shown that orally administered CAI can yield plasma concentrations within the effective range without significant toxicity.³⁸⁻⁴⁰ Studies of the biological consequences of CAI revealed that CAI inhibited growth and colony formation in soft agar of a wide array of malignant human and rodent cell lines, and oral administration of CAI to mice bearing human tumour xenografts reduced tumour incidence and arrested tumour growth. The effect of CAI on calcium influx, release of arachidonate, proliferation *in vitro*, and tumour growth and metastasis *in vivo* all occur in the CAI concentration range of 1 to 10 μM .⁴⁰ The effective concentrations for CAI inhibition of calcium influx and arachidonic acid release are in the same range as the concentrations which inhibit tumour growth and metastasis, leading to the hypothesis that the CAI molecule inhibited tumour proliferation and dissemination through modulation of key calcium-mediated path-

ways.⁴⁰

To investigate the inhibitory effects of CAI on angiogenesis induced by HCC, MTT, Boyden chamber and matrigel assays were used to determine the effect of CAI on proliferation, motility and enzymatic dissolution ability of HUVECs *in vitro*. Tumour spheroids from the metastatic tumour line of HCC were implanted into the corneal micropockets of nude mice, and 100 μM CAI was applied as eye drops to the nude mice eyes twice a day to determine the effect of CAI on the angiogenesis induced by HCC cells *in vivo*. The results showed that CAI could significantly suppress the proliferation of HUVECs, reduce motility through the matrigel and inhibit the abilities of degrading extracellular matrix and locomotion (The number of cells passing through the filter membrane of CAI-treated group and controls were 3.8 ± 2.3 and 13.8 ± 4.4 , respectively, $P < 0.05$). Compared with the control group, the angiogenesis induced by HCC on the cornea was markedly inhibited in CAI-treated group, as the number and length of the vessels were less than that in the control group, ($P < 0.05$). These findings suggest that CAI could inhibit neovascularization formed by HUVECs and angiogenesis induced by HCC (Table IV).

TABLEIV: INHIBITORY EFFECTS OF CAI ON ANGIOGENESIS INDUCED BY HCC

	Results (n = 6)	P
Length of vessel (ratio)		
CAI treated	2.7 ± 1.5	<0.05
Control	5.3 ± 0.6	
Number of vessels		
CAI treated	5.7 ± 2.1	<0.05
Control	12.3 ± 2.5	

Conclusions

With a better understanding of the critical and universal dependence of tumour progression on neovascularization, it is rational to hypothesize that suppression of this rate-limiting step could suppress the growth and metastasis of a wide range of tumour types.

The results of these studies suggested that all of the agents used could significantly inhibit tumour growth, angiogenesis, invasion and metastasis of human HCC in nude mouse models, might be used to control the recurrence and metastasis of HCC. Antiangiogenesis as a modality of treatment is likely to be most effective in a low tumour burden state. Thus, the appropriate clinical approach to using antiangiogenesis therapy in HCC is to combine it with conventional therapies (such as surgical resection and transcatheter arterial chemoembolization) to reduce the tumour burden, followed by its use in an adjuvant setting to prevent recurrence, suppress micrometastases, and prolong disease-free survival.

However, the focus of early clinical trials for these drugs has been to define dose-limiting toxicities and to determine a schedule of drug administration for further testing of efficacy. Moreover, antiangiogenic agents may need to be administered continuously at well-tolerated doses. There are still much work to be done before antiangiogenic agents can be used in the clinical settings to control recurrence and metastasis of HCC.

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