Effect of glutamine analogue—acivicin on tumor induced angiogenesis in Ehrlich ascites carcinoma

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The inhibition of tumor growth and tumor induced angiogenesis by the glutamine antimetabolite acivicin was evaluated in 6-7 weeks old male Swiss albino mice bearing Ehrlich ascites carcinoma (EAC) transplanted by intraperitoneal (ip) injections of EAC cells. Treatment involving ip injections with two different doses of acivicin (0.05 and 0.4 μg/g body weight/day) in saline revealed decrease in tumor volumes and reduced number of blood vessels on peritoneal wall after 10 and 15 days of treatment when compared to control (i.e. injected with saline only). Vascular hyperpermeability was found to be lesser in the treated groups of mice than the control as indicated by the FITC-D and colloidal carbon assay. Serum VEGF level was found to decrease in the drug treated groups both after 10 and 15 days of treatment. The results thus suggest that acivicin may suppress tumoral angiogenesis through regulation of VEGF level.

Keywords: Acivicin, Angiogenesis, Ehrlich ascites tumor

The modified amino acid acivicin [(α,S,5S)-α-amino-3-chloro-4, 5-dihydro-5-isoxazoleacetic acid; AT-125; NSC-163501] is a fermentation product of Streptomyces svicei. In nature it may play the ecological role of defending the home turf of the producing species and thus encourage other microbes to move towards less hostile environments. Being a glutamine analogue, it was later introduced into the novel niche of cancer therapy.

Glutamine is avidly consumed by the actively growing tumor cells as it (i) transports nitrogen and carbon, (ii) is essential for protein synthesis, (iii) is required for the synthesis of DNA and RNA, and (iv) acts as a major respiratory fuel. Moreover, glutamine is essential for the synthesis of the angiogenic stimulator, fibrin. The extravasation and clotting of plasma fibrinogen in the extravascular space to form fibrin gel is an important prerequisite for the ingrowth of new blood vessels from existing ones. Developing tumors require new vasculature as they grow in order to ensure a constant supply of required nutrients and oxygen while paving way for the elimination of metabolic wastes. Acivicin, a glutamine antimetabolite is currently under Phase II clinical trials and is suggested to disturb glutamine metabolism by hindering its transport, formation or usage in processes such as transamination and translation. It is also believed to interact with glutamine amidotransferases. Keeping the above points in view, and no evidence of the study of the antiangiogenic potentials of acivicin in the past, an attempt has been made in the present study to investigate the tumoral angiogenesis modulatory role of acivicin in the murine ascites tumor model EAC to add a better understanding of its mode of action as an anticancer agent.

Materials and Methods

Animals—Male Swiss Albino mice of 6-7 weeks age were used. The animals were obtained from the animal house of CNCI, Kolkata. They were provided with standard pellet diet and tap water ad libitum, under hygienic conditions.

Chemical—Acivicin was purchased from Sigma Chemical Co. Catalogue No.A-2295. It was dissolved in normal saline and was stable for at least one week at 5°C. Two doses of acivicin (0.05 and 0.4 μg/g body weight/day) were selected with reference to early reports. A stock solution of 50 μg/ml was prepared. It was appropriately diluted with saline to prepare the other dose.
Tumor—The transplanted type of tumor used in the experiment was Ehrlich Ascites Carcinoma (EAC) that was propagated in our laboratories by ip inoculation in male Swiss albino mice.

Experimental design—Animals were injected each with 0.2ml saline solution containing 1×10⁶ EAC cells intraperitoneally. This day was taken as day 0. These EAC bearing mice were randomly sorted into the following groups with 5 mice per group: Group I—mice received saline injections for 10 consecutive days; Group II—mice received injections with acivicin 0.05μg/g body weight for 10 days; Group III—mice were injected with acivicin 0.4μg acivicin/g body weight for 10 days; Group IV—mice received saline for 15 consecutive days; Group V—mice received ip injections with acivicin 0.05μg/g body weight for 15 consecutive days; and Group VI—mice received acivicin 0.4μg/g body weight for 15 days. All the above-mentioned injections were given intraperitoneally starting from day 1 after tumor inoculation. Mice were killed by chloroform anesthesia. Portions of the parietal peritoneum were dissected out and after washing with PBS they were fixed overnight in 10% formalin at room temperature, rinsed in PBS (two times for 30 min each), dehydrated and embedded in paraffin. Giemsa staining was performed on 5-7μm paraffin sections, cleared in xylene, mounted in DPX and observed under light microscope.

FITC-dextran (FITC-D) leakage assay—FITC-dextran is a macromolecular tracer that diffuses out through hyperpermeable blood vessels. Tumor-induced angiogenesis is associated with vascular hyperpermeability. To determine the extent of vascular permeability, the leakage of FITC-dextran from the plasma to the tissues was studied in control and treated groups. FITC-D of average molecular weight 70,000 was a gift from Dr. S. Mondol, Harvard Medical School, Boston, USA. Both control and acivicin treated mice (treated for 15 consecutive days) were injected iv with 1.42mM FITC-D in PBS. Animals were sacrificed 30 min following tracer injection. To visualize FITC-D microscopically, diaphragms were rapidly excised and immersed in 70:30 mixture of ethanol:10% formalin. After 4 hr fixation at room temperature tissues were dehydrated in ascending grades of ethanol (70-100%) over 24 hr, cleared in xylene, and mounted under cover slip on glass slides and viewed under a fluorescence microscope.

Measurement of VEGF by Enzyme linked immunosorbant assay (ELISA)—Serum VEGF level was measured by ELISA. Wells in an ELISA plate were incubated with serum samples in 1:10 dilution. VEGF in serial dilutions of known concentrations were used to draw the standard curve. After incubating overnight at 4°C wells were washed with PBS-Tween wash buffer thrice. They were then incubated with goat polyclonal Ig-G, (SantaCruz Biotechnology Cat # sc-1881) for overnight at 4°C. After washing with PBS-Tween thrice, wells were incubated at room temperature for 2 hr with anti-goat Ig-G (whole molecule) obtained from Sigma (Cat. No. A-4174). Finally, after washing thrice with PBS-Tween wash buffer, the substrate solution containing ortho-phenyl-diamine and hydrogen peroxide was added to each well. The plate was kept in dark for about 30 min and O.D. was measured at 490nm in ELISA reader (Tecan Spectra, Pacific Biotech).

Colloidal carbon assay—Colloidal carbon is a tracer that is leaked out of the vessels due to hyperpermeability and can be visualized in tissues by both macroscopy and by high-resolution light microscopy. Both drug treated and control mice (after 15 days of drug treatment) were injected, iv, with 0.1ml of a 1:5 dilution (in PBS) of colloidal carbon. One hour later, animals were sacrificed by chloroform anesthesia. Portions of the peritoneal membrane were dissected out and after washing with PBS they were fixed overnight in 10% formalin at room temperature, rinsed in PBS (two times for 30 min each), dehydrated and embedded in paraffin. Giemsa staining was performed on 5-7μm paraffin sections, cleared in xylene, mounted in DPX and observed under light microscope.

Results

Effects of acivicin on tumor volume—EAC ascites tumor was palpable only after 5-6 days and accumulation of ascites fluid was observed only after day 7 of the inoculation of tumor cells. The volume of ascites fluid was decreased markedly in drug treated series in comparison to that of control (P<0.001 in case of
acivicin treatment with 0.4μg/g body weight for 15 days, Fig. 1). It was also revealed that the body weight of EAC tumor-bearing mice was decreased by 33.2% in the high dose drug treated group in comparison to that of the control group (unpublished data).

**Effect of acivicin on tumor induced angiogenesis**—Extensive angiogenic response was observed from 5 days after the inoculation of 1x10⁶ EAC cells ip in male Swiss albino mice. The number of new blood vessels on the peritoneal lining after inoculation of tumor cells was much more in the control than in the drug treated (low and high dose) groups after 15 consecutive days of drug administration. Acivicin treatment markedly reduced the number of newly growing blood vessels in the peritoneal lining membrane.

Kinetics of the newly formed blood vessels in the peritoneal membrane of EAC ascites tumor model in control and treated series is presented in Fig. 2. More than 50% decrease in blood vessel count per square millimeter was found in the group treated with 0.4μg acivicin/g body weight for 15 consecutive days (P<0.01).

**Effects of acivicin on regulation of VEGF secretion by tumor cells**—A direct correlation between the increasing level of VEGF and the extent of induced angiogenic response was observed (Fig. 3). Acivicin was found to regulate VEGF secretion by tumor cells. A maximum of 82.8% inhibition in serum VEGF level was found when mice were treated with the high dose of acivicin (0.4μg/g body weight) for 10 consecutive days (P<0.01).

**Effects of acivicin on vessel hyperpermeability as indicated by colloidal carbon assay**—Microscopic study of 5-7μm sections of the peritoneal membranes of normal, tumor-bearing control and acivicin treated groups of mice showed that almost no carbon leakage was observed in normal mice (Fig. 4A). In contrast,
Fig. 4—Giemsa stained sections of the peritoneal membranes of mice groups injected iv with colloidal carbon; 4A: the regular apposition of muscle bundles in normal mouse (non tumor bearing); 4B and 4C: section belonging to tumor bearing control (inoculated with $1 \times 10^6$ cells/mouse) mouse indicates that microvessels underwent a dramatic increase in cross-sectional area, becoming large and thin walled with persistent hyperpermeability to colloidal carbon. These vessels were congested with erythrocytes and were hyperpermeable to colloidal carbon. Tumor cells (T) were commonly adhered to the peritoneal surface, individually and in clumps; 4C: enlarged vessel lumen in the group treated with low drug dose (treated for 15 days). Tumor cells (T) showed adherence to peritoneal membrane. In high dose acivicin treated group, very less colloidal carbon leakage was observed; 4D and 4E: section from high dose drug treated mouse (treated for 15 days) showing very few tumor cells and colloidal carbon leakage was not much detectable.
extensive carbon labeling was detected in tumor bearing control mice (Fig. 4B and C). Tumor cells were also found attached to peritoneal surface as cell clumps (T). However, the leakage of colloidal carbon was found to be highly reduced in the acivicin treated groups (Fig. 4D and E).

Effects of acivicin on vessel hyperpermeability as indicated by FITC-D leakage—After 30 min of intravenous FITC-D injection, the diaphragms of mice were observed under fluorescence microscope. Extensive leakage of FITC-D was found in EAC tumor bearing mice receiving only saline. The extravasation of the tracer was, however, reduced in the drug treated series (Fig. 5A-D).

**Discussion**

Antiangiogenic therapy provides a promising avenue in cancer treatment. Thus, there is need for exploring sources that act as antiangiogenic agents. Acivicin, already under Phase II clinical trials in humans is known to possess antineoplastic effects against L1210 tumors, P388 mouse leukemias, M5076 mouse ovarian tumor and xenografts of a human mammary tumor and an oat cell carcinoma in nude mice. It is known to exert its antineoplastic effects by inhibiting glutamine amidotransferases including GMP synthetase, CTP synthetase, glutamyl transpeptidase, formylglycineamidine ribonucleotide synthetase, and

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**Fig. 5—Fluorescence visualization of FITC-D from the diaphragm of different groups of mice; 5A: fluorescence from the diaphragm of normal mouse; 5B: high fluorescence in tumor bearing control mouse; 5C: that diaphragm of low dose acivicin treated mouse showing somewhat reduced FITC-D leakage when compared to the control group; 5D: scanty fluorescence in high dose acivicin treated groups.**
carbamoyl phosphate synthase\(^{18}\), in mammalian and protozoan systems.

The results obtained in the present experiment indicate that tumor induced angiogenesis was inhibited by acivicin. The degree of tumoral angiogenesis was assessed by counting the peritoneal blood vessels, measuring the VEGF level and the hyperpermeability of blood vessels to macromolecules. Moreover, the rate of tumor angiogenesis was correlated with tumor volumetric growth rate.

Induction and maintenance of angiogenesis requires interaction of many growth factors with their respective receptors, which then activate endothelial cells. Vascular endothelial growth factor (VEGF), a multifunctional cytokine, is abundantly expressed and secreted by most human and animal tumors examined\(^{19,20}\) and is known to cause hyperpermeability of tumor blood vessels, thus inducing tumoral angiogenesis. In the present study, results indicate that acivicin decreased serum VEGF levels in treated groups of mice and this correlated with the reduced angiogenesis.

Fibrin is a major component of the initial stroma in several tumor types\(^{21,22}\). Fibrin acts as scaffold for both invasive cancer and endothelial cells, thereby contributing to tumor growth and neovascularization\(^{21,22}\). Structure of fibrin is an important determinant of angiogenesis. Modifications of the structure of fibrin network alter its sensitivity towards proteolytic degradation\(^{23,24}\), which affect tube formation. It is already known that both glutamine and histidine are structural components of fibrin. Therefore, depletion of any of these amino acids will hamper angiogenesis. Evidences suggest that the in vivo levels of histidyl t-RNA and glutaminyl t-RNA may be lowered by acivicin treatment\(^{7}\). Acivicin is also known to inactivate HisHF enzyme to block histidine biosynthesis in E. coli\(^{7}\). Therefore, whether this specific interaction occurs in mammals is still to be found. All these collectively suggest that acivicin may disturb fibrin structure and thus affect new blood vessel formation. The present results suggested that acivicin acts as an antiangiogenic agent. However, further studies are needed to understand its mechanism of action.

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References


