RESEARCH COMMUNICATION

Modulation of Metastatic Potential of B16F10 Melanoma Cells by Acivicin: Synergistic Action of Glutaminase and Potentiation of Cisplatin Cytotoxicity

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Abstract

Treatment for metastatic melanoma has mostly been unsatisfactory despite advances in ongoing medical research. Here we investigated the role of acivicin, a glutamine analogue, singly and in combination with either E. coli glutaminase or cisplatin, on the growth, angiogenic activity and invasiveness of B16F10 cells in vitro and after allografting in C57BL/6 mice. B16F10 melanoma colonization in the lungs of mice was measured by monitoring colony counts. Host toxicity was assessed with reference to tumor bearing host's weight and survivability. Acivicin promoted melanoma dormancy and reduced melanoma associated angiogenic factors like VEGF level and vessel diameter. Acivicin in combination with glutaminase significantly suppressed tumor growth by 66.7% and increased life-span by 43.5% without host toxicity. Tumor VEGF content was significantly lowered by combination therapy as assessed by ELISA. Accelerated cytotoxicity, reduced invasion and enhanced apoptosis of melanoma cells were exhibited in vitro by combined than by single agent treatment. Moreover, invasion of melanoma cells through matrigel chambers was reduced in the presence of acivicin and glutaminase in combination. These findings support future studies of acivicin in combination with other anticancer agents for prevention of melanoma metastasis.

Key Words: Acivicin - melanoma - metastasis - glutaminase - cisplatin
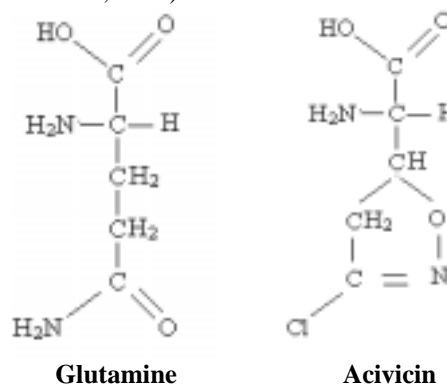
Introduction

New means for treating and controlling melanoma development are of extreme importance because it is an aggressive tumor, with a propensity to metastasize, and is resistant to most current therapeutic regimens (Satyamoorthy et al., 2001). Early primary melanoma is highly curable, but once it disseminates, it becomes almost certainly fatal. Despite advances in ongoing basic science research and innovative clinical approaches such as biotherapy, vaccines and regional perfusion therapy, treatment for metastatic melanoma has been largely unsatisfactory. However, several new treatment strategies do show promise.

Acivicin [(alpha S, 5S)- alpha-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; AT-125; NSC- 163501], a fermentation product of Streptomyces svicei (Poster et al., 1981), shows antineoplastic activity against L1210 tumors (Martin et al., 1974; Rosenfeld et al., 1981), P388 mouse leukemias (Rosenfeld et al. 1981; Ardalan et al., 1982) and human breast and lung tumor xenografts in athymic mice (Williams et al., 1990). It has also shown benefits in a phase I trial of solid tumor treatment (Baruchel et al., 1995). Acivicin is a glutamine antagonist (Hanka et al, 1973). It is believed to interact with a class of glutamine requiring enzymes called glutamine amidotransferases (Poster et al.,1981; Prajda, 1985; Smith et al., 1995; Weber et al., 1984). They are the rate-limiting enzymes of *de novo* purine and pyrimidine biosynthesis (Ahluwalia et al., 1990; Medina et al., 1992).

Acivicin is also a well-known competitive nonreversible inhibitor of gamma-glutamyl transpeptidase (GGT) (Allen et al.,1980; Reed et al., 1980) that catalyzes the hydrolysis of glutathione. B16F10 melanoma is a highly metastatic murine cell line, which has the capacity to successfully form pulmonary tumor colonies in their syngeneic host, the C57BL/6 mouse. It is previously reported that B16 melanoma cells with high glutathione (GSH) content show high metastatic activity *in vivo* (Obrador et al., 2002).

Glutamine   **Acivicin**

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Reports by other workers suggest that cotreatment of cancer cells with acivicin and bacterial glutaminase enzyme has a synergistic antiproliferative effect (Holcenberg, 1979), which might be due to action on the common target - glutamine. On the other hand, combination of acivicin with cisplatin inhibits the capacities of the pyrimidine pathways, resulting in an efficient reduction of DNA synthesis (Futami et al., 1989). The connection between angiogenesis and melanoma progression has become an attractive target for new melanoma therapies. Drugs that interfere with angiogenesis and that can be feasibly combined with conventional anticancer agent would be extremely desirable. Therefore, the present work was designed to test the hypothesis that acivicin in combination with cisplatin might block melanoma associated angiogenesis. We also examined the effects of acivicin and glutaminase or cisplatin combination stress on in vitro melanoma cells.

Materials and Methods

Cell line

B16F10 murine melanoma cells were obtained from the National Centre for Cell Science, Pune (India) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100μg/ml streptomycin in a humidified incubator at 37°C and 5% CO_2.

Drugs and enzyme

Acivicin [(alpha S, 5S)- alpha-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid] was purchased from SIGMA Chemical Co. (Cat No. A-2295). For in vitro assays, it was dissolved in DMEM medium and passed through bacterial filter to prepare a 5μM solution. Glutaminase from *E.coli* was purchased from SIGMA (Cat No. G8880). Cisplatin (Sigma, Cat. No. p-4394) was dissolved in PBS to make a 1μg/μl solution. All the reagents were passed through bacterial filter prior to use. In case of animal studies, acivicin was dissolved in normal saline and this was stable for one week at 5°C (Holcenberg, 1979). A dose of 0.4μg acivicin/g body weight/day and 1IU glutaminase/mouse/day was selected for mice treatment with reference to previous reports (Ghosh et al., 2004; Roy and Maity, 2005).

Animals

Five to six week old male C57BL/6 mice were purchased from the National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad. They were maintained in the animal care facilities of the animal house of CNCI. They were housed in polypropylene cages and were kept in a room maintained at 25±2°C with a 12:12 hr light/dark cycle. They were given free access to standard laboratory feed and water ad libitum, under hygienic conditions.

Cytotoxicity assay

The effect of treatment agents on B16F10 cell viability and proliferation was determined using a Cell Proliferation Kit I, Boehringer Mannheim, Germany (MTT, 1465007) according to the manufacturer's instructions. Cells were plated on 96 well dishes at $1x10^4$ cells/well and exposed to 5 and 10μM acivicin singly or in combination with 0.1 IU bacterial glutaminase or 5μg cisplatin. Readings were taken in an ELISA plate reader after 24 hours of incubation.

Apoptosis assay

Annexin V selectively binds to phosphatidylserine, relocalised to external side of cell membrane during early stages of apoptosis (Vermes et al., 1995), and this has enabled the use of fluorescein-labeled annexin V for identification of cells undergoing apoptosis. Cells were also stained with propidium iodide to distinguish early apoptotic cells from necrotic cells. B16F10 cells were cultured in vitro and treated with 50μM acivicin. Cells were then stained with Annexin V and propidium iodide and apoptosis of cells was studied by flow cytometry and fluorescence microscopy. FITC-labeled annexin V apoptosis detection kit (BD Biosciences) was used. Ten thousand events were collected with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The percentages of live, dead and apoptotic cells were determined.

Morphological analysis

B16F10 melanoma cells were seeded on sterile glass coverslips and cultured *in vitro* for 24 hours. They were then treated with different concentrations of acivicin for another 24 hours and their morphological appearances were compared with untreated controls after staining with hematoxylin and eosin.

Matrigel invasion assay

The invasion and metastasis of tumor cells require cell migration (Entschladen and Zanker, 2000; Mareel and Leroy, 2003). Invasive behavior was examined *in vitro* using Biocoat Matrigel Invasion Chambers (Becton Dickinson Labware, Bedford, MA) as per the manufacturer's instructions. Briefly, $1x10^5$ cultured B16F10 melanoma cells were trypsinized, washed and resuspended in serum-free medium and placed in the top portion of the invasion chamber. The lower portion of the chamber contained 10% FBS as a chemoattractant. Cells were exposed to 5μM acivicin alone or in combination with either 0.1 IU E. coli glutaminase or 5μg cisplatin. After 4 hours, cells that had invaded through the matrigel and migrated to the bottom chamber were fixed in 100% methanol, stained with Giemsa, photographed and counted. The percentage invasion was determined as follows: (average number of cells invading through the matrigel insert membrane/average number of cells invading through the control insert membrane) x 100.

In vivo experimental pulmonary metastasis assay

For the *in vivo* experimental pulmonary metastasis assay, 0.2 ml of trypsin detached B16F10 cells ($1x10^5$ cells/ml in DMEM) were injected into the tail veins of C57BL/6 mice. The viability of cells was >90% as determined by trypan blue exclusion. Acivicin and glutaminase, singly and in combination, were injected ip from one day after tumor inoculation in the respective treatment groups of

mice. Control mice received saline ip. Each group consisted of six mice. Mice were sacrificed after three weeks. The lungs were removed and visible colonies on the surfaces were counted with the help of dissecting microscope. The lung and body weights were also recorded. The experiment was carried out according to the guidelines of the current laws of CPCSEA (Ethical Committee for the purpose of control and supervision of experiments on animals), India.

Histopathological characterization

For histological studies, small portion of lungs were removed and fixed overnight in 10% formalin. Fixed tissues were rinsed in 1X PBS, subsequently dehydrated in 35, 50 and 70% ethanol and embedded in paraffin. Sections (5μm) were deparaffinized in xylene and rehydrated in 100, 95, 70 and 50% ethanol and 1X PBS. Hematoxylin and eosin (H&E) staining of sections was carried out using standard reagents (Qauligens) and protocols. 5μm cryosections of lung tissues were also stained by routine H&E staining to locate melanoma invading regions.

Immunohistochemical analysis of VEGF expression

Sections (5μm thick) of formalin fixed, paraffin-embedded melanoma colony bearing lung specimens were analyzed by immunohistochemistry with goat polyclonal Ig-G (1:100 dilution; SantaCruz Biotechnology Cat. No. sc-1881) against VEGF (Huang et al., 2000). Anti-goat IgG (1:500, Sigma Chemical Pvt Ltd, Cat. No. A-4174) served as secondary antibody and sections were visualized using aminoethyl carbazole substrate chromogen. The slides were examined under a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Negative controls were conducted using nonspecific IgG.

Effects on host life-span

A parallel set of experiments was carried out with 5 mice per group receiving the same treatments. The numbers of surviving mice were recorded daily. Survivability was measured as: Percentage increase in life span= % ILS= [mean survival time (treated)/mean survival time (control)]-1 x 100.

Statistical analysis

All in vitro experiments were repeated in triplicate and similar results were obtained. The data between control and treated series were analyzed by Student's t-test. A p-value less than 0.05 was considered to be significant.

Results

Cytotoxicity assay

Colorimetric assays using MTT labeling reagent revealed that when compared with untreated controls, cell viability was reduced by 19.5, 43.8 and 45.8% by acivicin, glutaminase and cisplatin, respectively (see Figure 1a). About 83.1 and 71.7% reduction in cell survival was observed when acivicin was combined with glutaminase and cisplatin respectively.
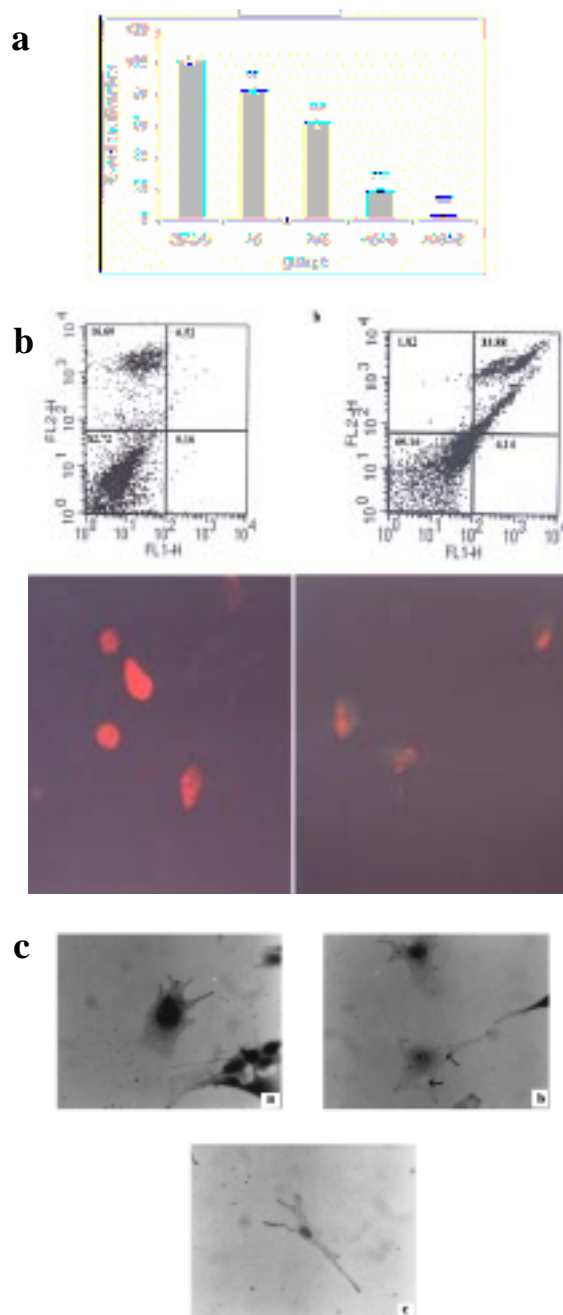

Figure 1. a) Proliferation data; b) Flow cytometric and fluorescence findings, a, control, b, 50μm avicidin; c) Morphological features of B16F10 cells cultured on coverslips, a, control, b, 50μm, c, 200μm (note membrane changes at arrows)

Induction of apoptosis by acivicin

About 4.14 and 33.9% cells were found in early and late stages of apoptosis respectively after 50 μM acivicin treatment (see Figure 1b). These figures were only 0.16 and 0.52% in untreated control group suggesting a role of acivicin in inducing tumor cell apoptosis.

Altered cell morphology

Microscopical analysis revealed morphological aberrations in cells after 24 hour treatment with acivicin (see Figure 1 c). Treatment with 50μM acivicin solution resulted in membrane irregularity and cytoplasmic disruption giving a broken appearance. Pronounced

membrane irregularity, cell shrinkage, cytoplasmic blebbing and relatively flattened appearance were observed in cells after treatment with 200µM acivicin. These indicate cytotoxic effects of acivicin on B16F10 melanoma cells *in vitro*.

Matrigel invasion assay

Because migration and invasion are important in malignancy, we examined the effect of acivicin (alone and in combination with glutaminase and cisplatin) on these properties of B16F10 cells. The in vitro invasion assay showed that acivicin reduced cellular invasion through the artificial basement membrane by 25%. Invasion was reduced by 93.9 and 87.8% when acivicin was applied in combination with glutaminase or cisplatin, respectively. The values were 37% with glutaminase and 48% with cisplatin alone.

In vivo lung colonization

Treatment with 0.4µg acivicin/g body weight/day resulted in a decrease in the pulmonary colony count. Lung colony count was reduced by about 30% and 66.7% with acivicin and its combination with glutaminase, respectively. The number of colonies correlated with the increase in relative lung weight (%). This weight ratio was reduced from 1.43 in the controls to 1.31, 1.23 and 1.01% by acivicin and glutaminase, alone and in combination, respectively, concordant with the lesser number of colonies in these groups of mice.

Histological analysis

Clumps of black colored melanoma cells were found in the lung sections of the melanoma-bearing control group. Reduced numbers of invading melanoma cells were observed in the lung sections of mice after treatment with acivicin. These numbers were found to be further reduced in the drug plus enzyme group. Hematoxylin and eosin staining of paraffin-embedded sections revealed some large blood vessels in the lungs. Closer examination showed melanoma cells in the lumina of these vessels. The density of such vasculature was lower in the drug treated group. The diameter of the blood vessels was much smaller in the acivicin treated group than that in the control group.

VEGF expression

To determine whether acivicin treatment could alter VEGF expression in B16 melanoma colony bearing lungs, we performed immunohistochemical analysis on sections of fixed, embedded lungs using antibody against VEGF. The immunohistochemical analysis shows that VEGF accumulates in lungs of control tumors at high levels . Lungs treated with acivicin exhibited a much reduced VEGF level, indicating that acivicin actively inhibits VEGF expression in B16 colony bearing lungs.

Survivability

The mean percentage increase in host life span was 43.46% in the mouse group receiving combination treatment while it was only 21.61 and 20.6% in the drug and enzyme treated groups, respectively.

Discussion

Melanomas present a great challenge because of their aggressive nature after dissemination (Sunand and Schuchter, 2001). Dacarbazine is the only single agent approved for the treatment of melanoma. However, responses following dacarbazine therapy are usually short-lived, sometimes with complete remission where patients do not experience any significant increase in survival (Serrone et al., 2000). Therefore, search for better therapeutic options is of urgent concern. Tumor cell membranes have multiple components that participate in the process of metastasis. So, here we focused on the effects of acivicin, a membrane γ-glutamyl transpeptidase (GGT) ectoenzyme inhibitor on viability and invasive properties of the highly metastatic B16F10 melanoma cell line which is known to express high levels of GGT activity (Obrador et al., 2002).

Our results show that acivicin exhibits cytotoxic effects and reduces matrigel invasion by B16F10 cells *in vitro*. Acivicin is an antagonist of glutamine, an amino acid known to be used avidly for energy, protein and purine synthesis by tumor cells. Glutamine uptake and usage in processes such as transamidation and translation is disturbed by this drug (Smulski et al., 2001). This might explain why acivicin inhibits the proliferation of B16 cells. The data obtained suggest that in vitro invasion of B16F10 melanoma cells is reduced when treatment with acivicin was combined with bacterial glutaminase or cisplatin. Although synergistic antineoplastic effects of bacterial glutaminase with acivicin is preestablished (Roberts and Rosenfeld, 1980), our report for the first time suggests synergism in anti-invasive activity of this combination regimen in B16F10 cells *in vitro*.

Results indicate that acivicin increased the cytotoxic property of cisplatin in an MTT assay and cellular invasion in an in vitro matrigel invasion assay. It is suggested that GGT accelerates tumor growth and increases tumor resistance to cisplatin (Marie et al., 1999; Pompella et al., 2006).

Morphological aberrations like cell shrinkage and membrane blebbing were induced by acivicin treatment supporting the previously observed cytotoxic effects of the drug on cultured B16F10 cells. In vitro acivicin was able to cause apoptotic cell death, which was assayed by double staining with Annexin V-FITC and propidium iodide. An earlier report suggested that apoptosis is induced in V79 cells by acivicin independent of GGT activity (Aberkane et al., 2001).

Acivicin is a potent inhibitor of CTP and GMP synthetases (Lyons et al., 1990; Nakamura et al., 1995) and thus it disrupts nucleotide synthesis. Clearly a p-53 dependent arrest of the cell cycle in G0/G1, leading to apoptosis, could be caused by nucleotide depletion alone and this might explain the observed apoptotic effects of acivicin.

Results showed that in mice, experimental metastatic lung colony formation was reduced by acivicin treatment. This was confirmed by macroscopic and microscopic examination of lungs. In addition, acivicin prolonged the life span of such mice. Membrane bound GGT enzyme

activity expressed by a number of malignancies including melanoma (Paolicchi et al., 2002) can act as a basal source of superoxide, H_2O_2 and other prooxidants. Prooxidant condition generally activates NF-κB (Meyer et al., 1994). In melanomas, high constitutive levels of NF-kB activation are usually observed and this is implicated in the expression of genes involved in cell proliferation and metastasis. Interference of GGT activity by acivicin might impair NF-κB transcriptional activity, thus affecting tumor cell dissemination and metastasis.

Angiogenesis, the process of neovascularisation from preexisting blood vessels, is a prerequisite for outgrowth and metastasis of tumors (Folkman, 1995; Griffioen and Molema, 2000). Histological analysis revealed that blood vessel diameter and density decreased in B16F10 colony bearing lung tissues after acivicin treatment. In human tumors, angiogenesis could be related to metastases and patient survival in case of cutaneous melanomas (Srivastava et al., 1988). The expression of VEGF, a potent angiogenic cytokine (Ferrara, 1997), is a prerequisite for melanoma tumor growth and metastasis (Claffey et al., 1996; Oku, et al., 1998). Acivicin treatment reduced VEGF expression in colony bearing lung tissues as assessed by the intensity of immunostaining. VEGF expression in melanoma is predictive of both metastasis and relapse (Erhard, et al., 1997; Marcoval, et al., 1997; Salven, et al., 1997; Neitzel, et al., 1999; Vlaykova, et al., 1999; Mowlavi and Malafa, 2000; Ugurel, et al., 2001). Acivicin in normal mice showed increase in hemoglobin level and RBC count.

The results thus permit us to conclude that acivicin could be a good chemotherapeutic agent as it inhibits cell survival, metastasis, promotes apoptosis of B16F10 melanoma cells as well as inhibits VEGF mediated angiogenesis. The antimetastatic activity can be enhanced by a therapy involving combination of acivicin with glutaminase or cisplatin. Thus future studies are warranted to establish that acivicin may have therapeutic benefit in melanoma patients when applied in combination with a suitable agent.

Acknowledgements

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