Acivicin with glutaminase regulates proliferation and invasion of human MCF-7 and OAW-42 cells—An in vitro study

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Tumor cells intensely utilize glutamine as the major source of respiratory fuel. Glutamine-analogue acivicin inhibits tumor growth and tumor-induced angiogenesis in Ehrlich ascites carcinoma. In the present study, antitumor properties of acivicin in combination with glutaminase enzyme is reported. Acivicin along with \textit{E. coli} glutaminase synergistically reduced \textit{in vitro} proliferation and matrigel invasion of human MCF-7 and OAW-42 cells. Effects of single and combined treatments with acivicin and glutaminase on angiogenic factors were also analyzed in these cell lines. Co-administration of the treatment agents inhibits the release of VEGF and MMP-9 by cells in culture supernatant significantly than single agent treatments. The result suggests that combination of acivicin with glutaminase may provide a better therapeutic option than either of them given separately for treating human breast and ovarian cancer. However, further studies are required to be conducted \textit{in vivo} for its confirmation.

Keywords: Acivicin, Glutaminase, Invasion, MCF-7, OAW-42, Proliferation

Glutamine related therapies are effective in managing cancer cells, as glutamine has multiple contributions to tumor growth, which include participation in protein, purine, pyrimidine and energy metabolism\textsuperscript{1}. Low glutamine concentration reduces proliferation and induces phenotypical and functional differentiation of neoplastic cells in culture\textsuperscript{2}. Being an essential component of the angiogenic stimulator fibrin\textsuperscript{3}, glutamine also takes part in neoangiogenesis facilitating tumor growth and metastasis \textit{in vivo}. Given the multifold importance of glutamine for the survival of cancer cells, regulation of its availability, transport and usage provides an interesting approach in cancer treatment.

Acivicin [{alpha S, 5S}-\textit{D}-alpha-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid] is a glutamine analogue obtained as a fermentation product of \textit{Streptomyces sviceus}\textsuperscript{4} and is a modified amino acid. Antineoplastic activity of acivicin against a broad spectrum of murine and human tumor models\textsuperscript{5} is known and it has already entered phase II clinical trials\textsuperscript{6}. Cytotoxic effects of acivicin may be mediated by its interaction with glutamine amidotransferases, thus hindering glutamine transport, formation or usage in transamidation and translation processes\textsuperscript{7}. However, the effect of acivicin on tumor-induced angiogenesis remains unexplored.

Evidences suggest that acivicin and succinylated \textit{Acinetobacter} glutaminase-asparaginase produces synergistic antitumor effects on P388 tumor cells and Ehrlich carcinoma in mice\textsuperscript{8}. Subthreshold doses of glutamine-antagonist would become depleted by glutaminase\textsuperscript{9}. Bacterial glutaminase is reported to be effective in lowering tumor burden with increased life span of the host\textsuperscript{10}.

In the present communication, the \textit{in vitro} proliferation and invasion of human MCF-7 and OAW-42 cells in presence of \textit{E. coli} glutaminase and acivicin singly and in combination have been reported. Moreover, the effect on MMP-9 and VEGF released by these cells in culture was also examined in presence of acivicin and glutaminase given singly and in combination.

Materials and Methods

Cells — Human breast cancer cell line MCF-7 and human ovarian cancer cell line OAW-42 were purchased from National Center for Cell Sciences, Pune, India. MCF-7 and OAW-42 cell lines were cultured in minimum essential medium (MEM) and Dulbecco’s minimum essential medium (DMEM) respectively, with 10% FBS in a fully humidified air atmosphere containing 5% CO\textsubscript{2} at 37°C.
Drug and enzyme — Acivicin (Sigma, Catalogue No. A-2295) was dissolved in normal saline and was stable for one week at 5°C. A stock solution of 15µM (A15) acivicin in cell culture medium was prepared and was diluted to make 5µM (A5) and 10µM (A10) solutions before treatment. Glutaminase from *E. coli* (G) was purchased from Sigma (Catalogue No. G-8880).

MTT assay — Colorimetric assay using MTT labeling reagent was separately performed with MCF-7 and OAW-42 cells to determine cell proliferation in presence of glutaminase and acivicin *in vitro*. Actively growing cells were seeded in three 96-well microtiter plates at a density of 1×10⁴ cells/well. Triplicate wells were treated with the following: (1) culture medium only (control), (2) A5, (3) A10, (4) A15, (5) 0.1 IU G, (6) A5+0.1 IU G, (7) A10+0.1 IU G, (8) A15+0.1 IU G. Incubation times for the three plates was 24, 48 and 72 hr respectively after which cell proliferation assay (MTT assay) was performed using Cell Proliferation Kit I, Boehringer Mannheim, Germany (MTT, 1465007) according to manufacturer’s instructions.

Matrigel invasion assay — The capacity to invade basement membrane determines the invasive potential of tumor cells. About 2.5×10⁵ MCF-7 and OAW-42 cells were separately treated with (1) medium only (control), (2) A5, (3) 0.1 IU G, (4) A5+0.1 IU G. Invasion through matrigel membrane was analyzed using BD BioCoat Matrigel Invasion Chamber (Catalogue No. 354480, Becton Dickinson Labware, USA) according to manufacturer’s guidelines.

VEGF measurement by ELISA — Vascular endothelial growth factor (VEGF) is a potent and specific mitogen for vascular endothelial cells capable of stimulating angiogenesis during tumor formation. For *in vitro* quantification of VEGF, cleared supernatants of MCF-7 and OAW-42 cell culture medium were collected. Briefly, 5 × 10⁴ cells/well in six well plates were seeded in serum–free medium and incubated at 37°C for 12 hr. After washing in serum-free medium, the monolayers were incubated with (1) serum-free medium only (control), (2) A5, (3) 0.1 IU G, (4) A5+0.1 IU G. Incubation for 24, 48 and 72 hr respectively when compared to controls (Fig. 1a). On combining with treatment with (1) serum-free medium only (control), (2) A5, (3) 0.1 IU G, (4) A5+0.1 IU G, in 1ml serum-free medium at 37°C. MMP-9 in cell culture supernatants was measured by ELISA using MMP-9 ELISA kit (Oncogene Research Products, Cat. No. Q1A56) following manufacturer’s instructions.

Statistical analysis — The results (mean±SE) of cell proliferation, invasion and ELISA assays were subjected to statistical analysis by Student’s *t*-test. The level of significance was set at *P*<0.05. All experiments were repeated twice using triplicates of each sample.

Results

Cell proliferation using MTT assay — Acivicin reduced MCF-7 cell proliferation in a dose dependent manner except that with 15µM acivicin after 48 hr incubation. Bacterial glutaminase led to 46.5, 47.5 and 50.3% proliferation of MCF-7 cells after incubating for 24, 48 and 72 hr respectively when compared to controls (Fig. 1a). On combining with

![Fig. 1](image-url) — Effect of acivicin and glutaminase on percentage of proliferation of MCF-7 cells (a) and that of OAW-42 cells (b). Results represent mean±SE value of triplicates. A5, A10 and A15 are 5, 10 and 15 µM acivicin solutions respectively. G, Glutaminase from *E. coli*. P values: *<0.05 when compared to acivicin; **<0.01 when compared to acivicin; ***<0.001 when compared to acivicin; ^<0.05 when compared to glutaminase.
acivicin, this proliferation was significantly lowered after 24 hr with 5 and 10 µM acivicin ($P<0.05$) and after 48 hr with 10 µM acivicin only ($P<0.05$). Proliferation of the breast cancer cells was significantly inhibited by acivicin-glutaminase combination compared to acivicin treatment alone ($P<0.05–0.001$) after 24 and 48 hr of culture, indicating an enhanced antiproliferative effect of this combination regimen.

Both dose and time-dependent effect was exerted by application of acivicin on the multiplication of OAW-42 cells. Percentage of proliferation dropped to 46.8, 57.3 and 63% that of the control after glutaminase treatment alone (Fig. 1b). Results show that the inhibition of cell proliferation by glutaminase gradually decreased from 24 to 72 hr suggesting a loss of enzyme activity with time. In OAW-42 cells, synergistic inhibition was found with combination of glutaminase and 5 µM acivicin after 24 hr incubation. Proliferation was significantly lower in the group treated with the agents in combination than acivicin treated group ($P<0.05–0.001$).

Matrigel invasion assay — To measure in vitro invasiveness of the metastatic MCF-7 and OAW-42 cells in presence of acivicin and glutaminase, matrigel invasion assay was performed. After 24 hr treatment with 5 µM acivicin and 0.1 IU glutaminase given separately, MCF-7 cell invasion was about 77.1 and 63.3% respectively (Fig. 2). When these cells were treated with both the agents simultaneously, only about 12.3% cell migration through the artificial basement membrane was obtained ($P<0.001$ when compared to treatment with either agent alone). In OAW-42 cells, single agent treatments with acivicin and glutaminase resulted in 70.3 and 48.6% invasiveness in this cell line (Fig. 2). About 19.9% OAW-42 cells migrated through the matrigel membrane after cotreatment with both agents. Similar to MCF-7 cells, OAW-42 cells showed significantly low invasiveness with acivicin-glutaminase combination treatment ($P<0.001$ compared to acivicin and $P<0.05$ compared to glutaminase single agent treatment).

Release of VEGF in culture supernatant — As quantified by ELISA, the concentration of VEGF in culture supernatants of acivicin treated MCF-7 cells was 18.6, 29.6 and 19.8% lower than the respective control groups after 24, 48 and 72 hr respectively. With glutaminase alone, a maximum reduction in VEGF release from cells was about 30.81% after 48 hr ($P<0.001$). Co-treatment of MCF-7 cells with both the agents decreased VEGF release by 36.5, 48.4 and 37.9% as compared to controls after 24, 48 and 72 hr respectively (Fig. 3a). Thus, synergistic effect was achieved by combination treatment after 72 hr of incubation achieving a statistical significance ($P<0.001$). In OAW-42 cells, VEGF level was reduced in culture supernatants after acivicin and glutaminase treatments but they were not statistically significant. However, the combination of both these agents significantly decreased ($P<0.05$) VEGF content of these cells (Fig. 3b).
Quantification of MMP-9 — Acivicin treatment reduced MMP-9 release by MCF-7 cells which was significant after 48 and 72 hr \( (P<0.05) \). The respective reductions after glutaminase treatment were 29, 40.5 and 17% in these cells (Fig 4a). MMP-9 release was lowered significantly after combination treatment with acivicin and glutaminase \( (P<0.01-P<0.001) \). In OAW-42 cells, treatment with acivicin and glutaminase resulted in significant decrease of MMP-9 content of culture supernatant \( (P<0.001) \) compared to control. Maximum reduction in MMP-9 level was obtained after 24 hr of combined application of acivicin and glutaminase (Fig. 4b).

**Discussion**

Acivicin is well known for its antineoplastic effects in a variety of tumor models. However, it has still not been studied for its antiangiogenic properties. The antiangiogenic properties of this drug in EAC tumor model have been reported\(^{13}\). The present communication is an extension to study whether acivicin can affect the release of some angiogenic factors such as VEGF and MMP-9 by human tumor cells in vitro. In addition, the effect of acivicin on the proliferation and invasion of these cells were also evaluated. Importantly, it was also tried to find out if combination of glutaminase to acivicin treatment could enhance their anticancer properties in vitro.

In the present study, *E. coli* glutaminase and the glutamine analogue acivicin reduced MCF-7 and OAW-42 tumor cell proliferation singly and in combination in vitro. Early reports suggested that the breast cancer cells MCF-7 possess low glutamine synthetase activity and so proliferated at slow rates in glutamine-deficient medium even when supplemented with purine nucleosides\(^{11}\). The results further support the importance of glutamine utilization for the proliferation of both MCF-7 and OAW-42 cells.

Bobrovnikova-Marjon et al.\(^{12}\) suggested that no appreciable cell loss was obtained in TSEb cells cultured in glutamine free medium for 48 hr. However, in MCF-7 and OAW-42 cells, treatment with acivicin and glutaminase might block utilization of endogenous glutamine in addition to that of medium-supplemented glutamine, thus leading to rapid death of tumor cells in vitro. Thus glutamine deprivation of tumor cells might inhibit their proliferation. With acivicin or glutaminase therapy, there was a gradual decrease in viable cell count with the passage of time.

Previous *in vivo* studies in EAC model\(^{13}\) indicated a reduction in serum VEGF level in tumor bearing hosts treated with acivicin when compared to untreated hosts. Data obtained in the present work suggest an overall reduction in VEGF release in culture supernatants of MCF-7 and OAW-42 cells after acivicin or glutaminase treatment with more effective reduction after combination treatment with both the agents. Early reports showed that the mRNA levels and positive rates of VEGF protein are significantly higher in epithelial ovarian cancer than in normal ovarian tissue\(^{14}\). VEGF is expressed at high levels in breast cancer specimens compared with normal breast tissue, and suppression of VEGF function inhibits breast tumor formation\(^{15}\). In the present study, lower VEGF concentration was obtained in culture media of acivicin and glutaminase treated cells compared to untreated controls, thus indicating enhanced antiangiogenic property of these agents in combination than that of either agent alone.

MMP-9 or type IV collagenase is a matrix metalloproteinase, which has important functions in tumor invasion, angiogenesis and metastasis. MMP-9 is implicated in regulating tumor angiogenesis via a mechanism that involves VEGF release from ECM stores\(^{16}\). MCF-7 human breast carcinoma cell line expresses very low MMP-9 levels\(^{17}\). The amount of combination
MMP-9 released by MCF-7 and OAW-42 cells in culture very well correlated with their matrigel invading property in vitro. Result obtained in the present study suggests that both acivicin and glutaminase have the potential of reducing MMP-9 release by tumor cells in culture. Similar to that of VEGF, this reduction was enhanced by the combination regimen. Thus the antiangiogenic effects of acivicin and glutaminase may be mediated, at least in part, through regulation of tumor-cell released VEGF and MMP-9.

The glutamine analogue-acivicin and the glutaminolytic enzyme-glutaminase affect a common event: glutamine metabolism by tumor cells. Glutamine is known to reverse the cytotoxic effects of acivicin in cell culture as well as overcome the analogue provoked inhibition of discrete glutamine-dependent amidotransferases. Depletion of glutamine by glutaminase might potentiate the antineoplastic activities of acivicin. It was observed in the present study that acivicin-glutaminase combination is effective in reducing proliferation and invasive property of human MCF-7 and OAW-42 cells. Moreover, this combination is highly effective in decreasing secretion of VEGF and MMP-9 by these cells in culture supernatant. The present in vitro results showing effects of combination treatments with E. coli glutaminase and acivicin thus suggest the potentiality of this combination as a future therapeutic modality for gynecological cancer.

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References

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