

# Antitumor activity of irinotecan with ellagic acid in C6 glioma cells

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## SUMMARY

**OBJECTIVE:** Irinotecan-based combination chemotherapies in malignant gliomas need to be examined. The aim of this study was to investigate the synergetic effect of ellagic acid, a natural polyphenolic antioxidant compound, with irinotecan, an inhibitor of topoisomerase I enzyme, on the growth, cadherin switch, and angiogenic processes of a glioma cell line.

**METHODS:** A combination of 100  $\mu$ M ellagic acid and 100  $\mu$ M irinotecan was applied to rat C6 glioma cells for 24th, 48th, and 72nd h. The cell proliferation was evaluated by 5-bromo-2'-deoxyuridine immunocytochemistry. The expression levels of vascular endothelial growth factor, E-cadherin, and N-cadherin were measured using real-time polymerase chain reaction and their immunoreactivities using immunocytochemistry.

**RESULTS:** The treatment of irinotecan with combining ellagic acid enhanced antitumor activity and the synergistic effect of these reduced the cell proliferation of C6 glioma by inhibiting the cadherin switch and promoting the antiangiogenic processes.

**CONCLUSIONS:** Further research is required to prove a negative relationship between C6 glial cell proliferation and irinotecan with ellagic acid application. Our preliminary data suggest that even with the extremely short-term application, irinotecan with ellagic acid may affect glioma cells at the level of gene and protein expression.

**KEYWORDS:** Glioblastoma. Ellagic acid. Irinotecan. Cadherin. VEGF.

## INTRODUCTION

Malignant glioma is one of the common primary brain tumors detected in the adults. These lesions, highly malignant, easily and diffusely infiltrate the tissues, so that the optimal therapy against these tumors is the combination of surgical resection, radiation therapy, and chemotherapy<sup>1,2</sup>.

The successful treatment options are limited for the recurrent gliomas, and progression-free survival is measured as approximately 10 weeks and overall survival as 30 weeks<sup>3</sup>. Therefore, new therapeutic strategies using the combinations of effective compounds with essential chemotherapeutic are essentially required to improve the success of treatments by preventing recurrence and to promote the quality of life of glioma patients<sup>4</sup>.

Irinotecan (Ir), an inhibitor of the topoisomerase I enzyme, has a high anticancer effect on the solid tumors in the gastrointestinal tract. This drug, which easily cross the blood-brain barrier, has been proven cytotoxic and antitumor activity of the brain tumors, such as glial neoplasms with multidrug resistance, in preclinical studies<sup>5,6</sup>. Although research has proved the monotherapy of the Ir as efficient, its activity does not have combined effect with other agents<sup>7</sup>. Its combination

with other chemotherapeutic agents in the malignant gliomas needs further study<sup>1,7</sup>.

Ellagic acid (EA) is a natural polyphenolic compound derived from ellagitannins found in foods with reported antioxidant, anti-inflammatory, and antifibrotic properties<sup>8,9</sup>. However, the potential synergistic effect of Ir with EA, i.e., a common chemotherapeutic agent, is poorly understood for the treatment of gliomas.

The epithelial-to-mesenchymal transition (EMT) provides an aggressive behavior of the tumor cells by reducing the expression or loss of epithelial markers such as adherent junction proteins  $\alpha$ -catenin,  $\beta$ -catenin, E-cadherin and by increasing the expression of mesenchymal markers such as vimentin and N-cadherin<sup>10,11</sup>.

Glial neoplasms are a highly vascular cancer and also rich in the expression of vascular endothelial growth factor (VEGF) that promotes the process of angiogenesis. Antiangiogenic agents may prevent this process and promote regression of existing vessels<sup>12</sup>.

We aimed to demonstrate the antitumor effects of the treatment of Ir with EA in C6 glioma cell line.

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## METHODS

### Cell culture

C6 glioma cells were purchased from the American Type Culture Collection (Manassas, VA, USA), and all procedures were in accordance with steps described in previous studies<sup>13,14</sup>.

### Immunocytochemistry

To determine the immunoreactivities of E-cadherin, N-cadherin, and VEGF, all steps of immunocytochemistry (ICC) and H-SCORE analysis described in previous studies were applied in this study<sup>13,14</sup>.

### Expression analysis

The expression levels of E-cadherin, N-cadherin, and VEGF were determined following all steps of real-time quantitative polymerase chain reaction (qPCR) described in our previous studies<sup>13,14</sup>.

### 5-Bromo-2'-deoxyuridine cell proliferation assay

5-Bromo-2'-deoxyuridine (Br-dU) ICC was used to examine the cell proliferation, and all procedures and scoring were in consistence with steps described in our previous studies<sup>13,14</sup>.

### Statistical analysis

Semi-quantitative and quantitative data from all groups were statistically analyzed by using GraphPad InStat version 3.06 (GraphPad Software, San Diego, CA, USA). All data were represented as mean±SD. The means of continuous variables were calculated using a one-way analysis of variance, and variations between the groups were compared using a post-hoc Tukey's multiple comparison test. A p-value <0.05 was accepted as statistically significant.

## RESULTS

### Combined Ellagic acid and Irinotecan suppresses the cell proliferation

To define the efficacy of Ir with or without EA on the cell proliferation of C6 glioma, the Br-dU proliferation assay was performed, and the scores were semi-quantitatively analyzed. Irinotecan treatment alone significantly inhibits the cell proliferation at the 24th (control: 84.87±2.25; Ir: 47.22±1.91, p<0.001), 48th (control: 88.48±2.37; Ir: 47.25±2.63, p<0.001), and 72nd (control: 86.10±1.65; Ir: 35.98±2.24, p<0.001) hours. In contrast, the combination with EA inhibited the proliferation more distinctly compared to the control group

at 24th (control: 84.87±2.25, Ir+EA: 5.01±0.52, p<0.001), 48th (control: 88.48±2.37; Ir+EA: 8.45±0.99, p<0.001), and 72nd (control: 86.10±1.65; Ir+EA: 1.52±0.63, p<0.001) hours of incubations.

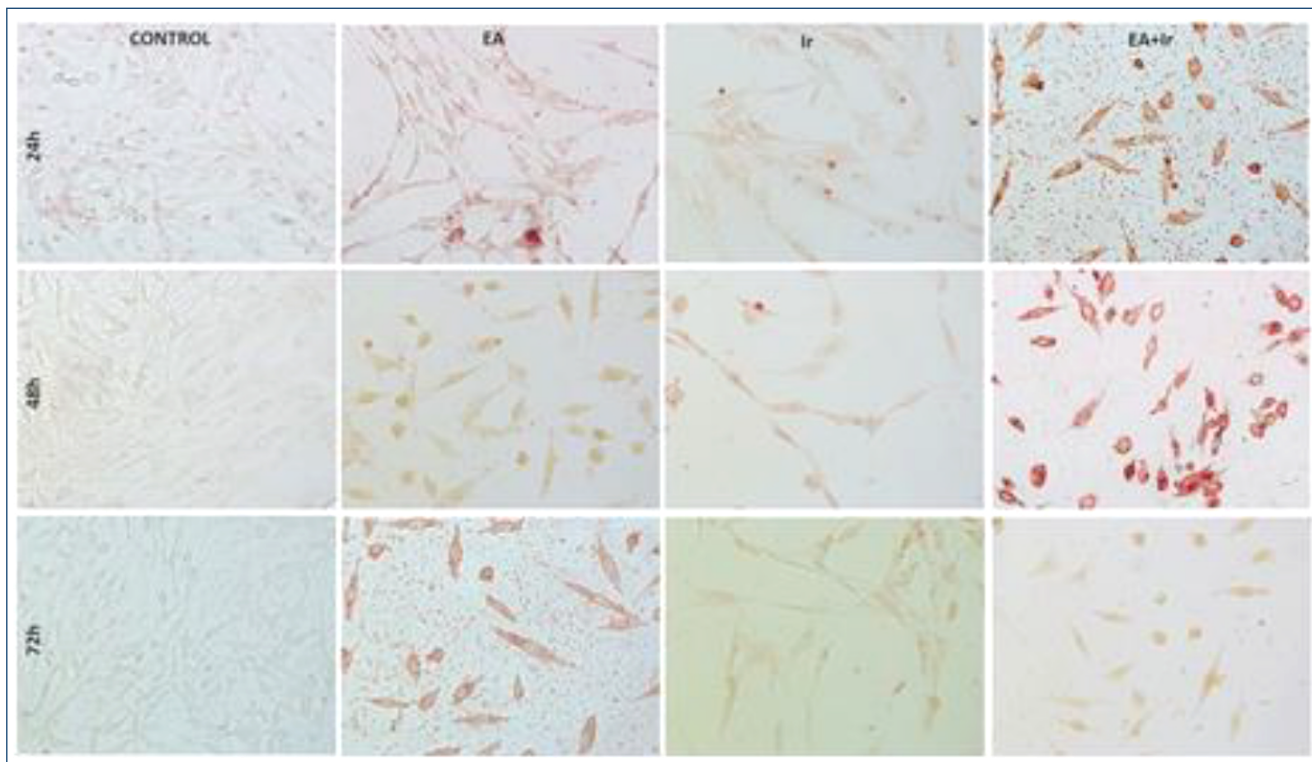
### Combined Ellagic acid and Irinotecan mediates the cadherin switch at the gene and protein levels

The expressions of E-cadherin and N-cadherin were quantified by qPCR. Their protein levels were studied by ICC, as shown in Figures 1 and 2. Treatment with only Ir considerably upregulated the protein levels of E-cadherin expression at all incubation hours in cells compared to the control group at 24th (control: 10; Ir: 45), 48th (control: 8; Ir: 30), and 72nd (control: 5; Ir: 25) hours of incubation (p<0.01) (Figure 1). However, the gene level of E-cadherin was only significantly higher than the control group at 24th incubation time (control: 1.0; Ir: 1.6, p<0.05). In contrast, Ir treatment with EA dramatically increased E-cadherin expression at 24th (control: 1.0; Ir+EA: 3.3, p<0.001), 48th (control: 1.0; Ir+EA: 2.0, p<0.01), and 72nd (control: 1.0; Ir+EA: 1.8, p<0.05) hours of incubation, and protein levels significantly increased at 24th (control: 10; Ir+EA: 80), 48th (control: 8; Ir+EA: 55), and 72nd (control: 5; Ir+EA: 45) (p<0.001) hours of incubation (Figure 1).

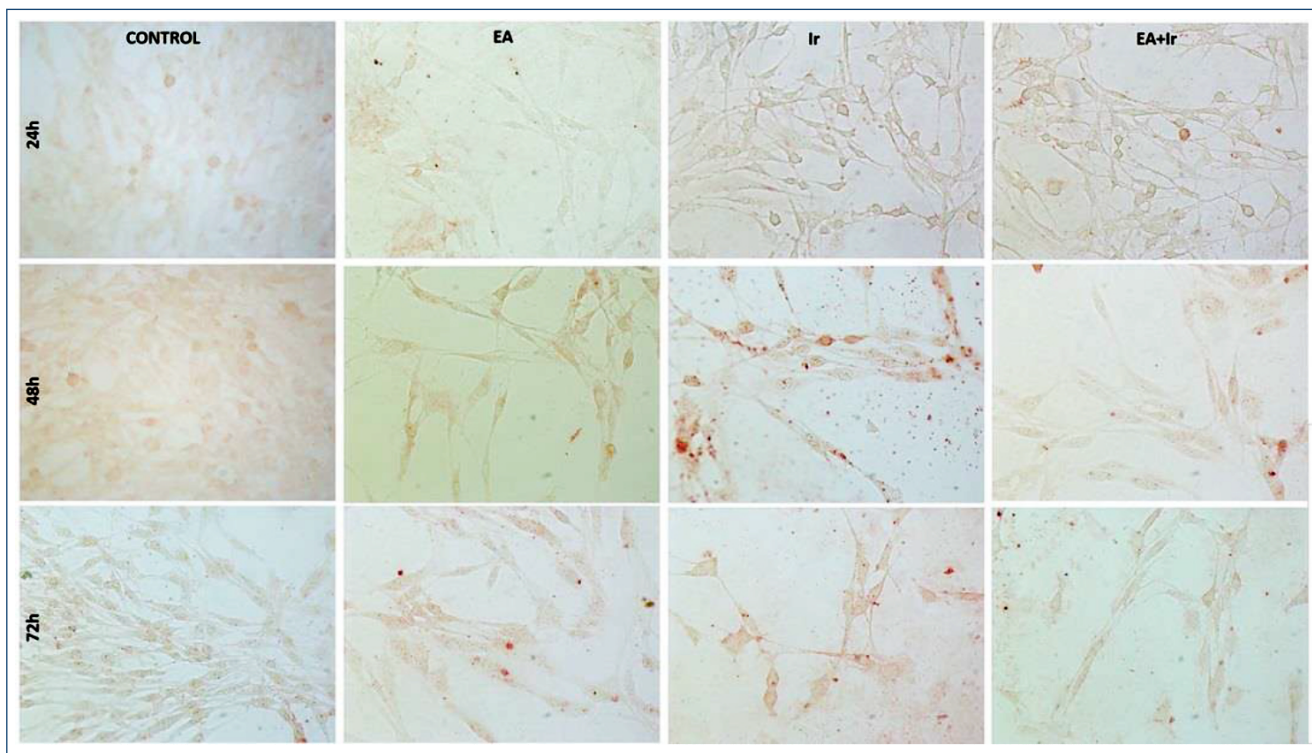
The treatment of Ir without EA reduced N-cadherin gene levels significantly at 24th hour (control: 7.6; Ir: 4.8, p<0.01), but failed to reduce gene levels at 48th (control: 5.1; Ir: 4.0, p>0.05) and 72nd (control: 5.0; Ir: 3.2, p>0.05) hours of incubation, compared to the control group. Irinotecan without EA significantly reduced the N-cadherin protein level at 24th hour (control: 120; Ir: 55) (p<0.01), but failed to reduce at 48th and 72nd hours of incubation (p>0.05) (Figure 2), as well as Ir with EA significantly decreased the gene levels all the time at 24th (control: 7.6; Ir+EA: 3.0, p<0.001), 48th (control: 5.1; Ir+EA: 2.8, p<0.05), and 72nd (control: 5.0, Ir+EA: 1.9, p<0.01). Irinotecan with EA reduced the protein levels of N-cadherin at 24th (control: 120; Ir+EA: 30, p<0.001) and 72nd (control: 62; Ir+EA: 20, p<0.01) hours of incubation (Figure 2).

### Combined Ellagic acid and Irinotecan downregulates the expression of VEGF at the gene and protein levels

The treatment of Ir without EA significantly downregulated the gene levels of VEGF expression at 24th (control: 2.0; Ir: 1.3, p<0.01) hour of incubation compared with the control group, and Ir treatment with EA dramatically downregulated the gene levels of VEGF expression at 24th (control: 2.0; Ir+EA: 1.0, p<0.001), 48th (control: 2.5; Ir+EA: 0.9, p<0.001), and 72nd (control: 1.5; Ir+EA: 0.5, p<0.001) hours. In contrast, Ir



**Figure 1.** Immunocytoreactivity of E-cadherin in the control (C). EA: ellagic acid; Ir: irinotecan. Combination (ellagic acid+irinotecan) groups, compared with the time of exposure. Magnification:  $\times 400$ .



**Figure 2.** Immunocytoreactivity of N-cadherin in the control (C). EA: ellagic acid; Ir: irinotecan. Combination (ellagic acid+irinotecan) groups, compared with the time of exposure. Magnification:  $\times 400$ .

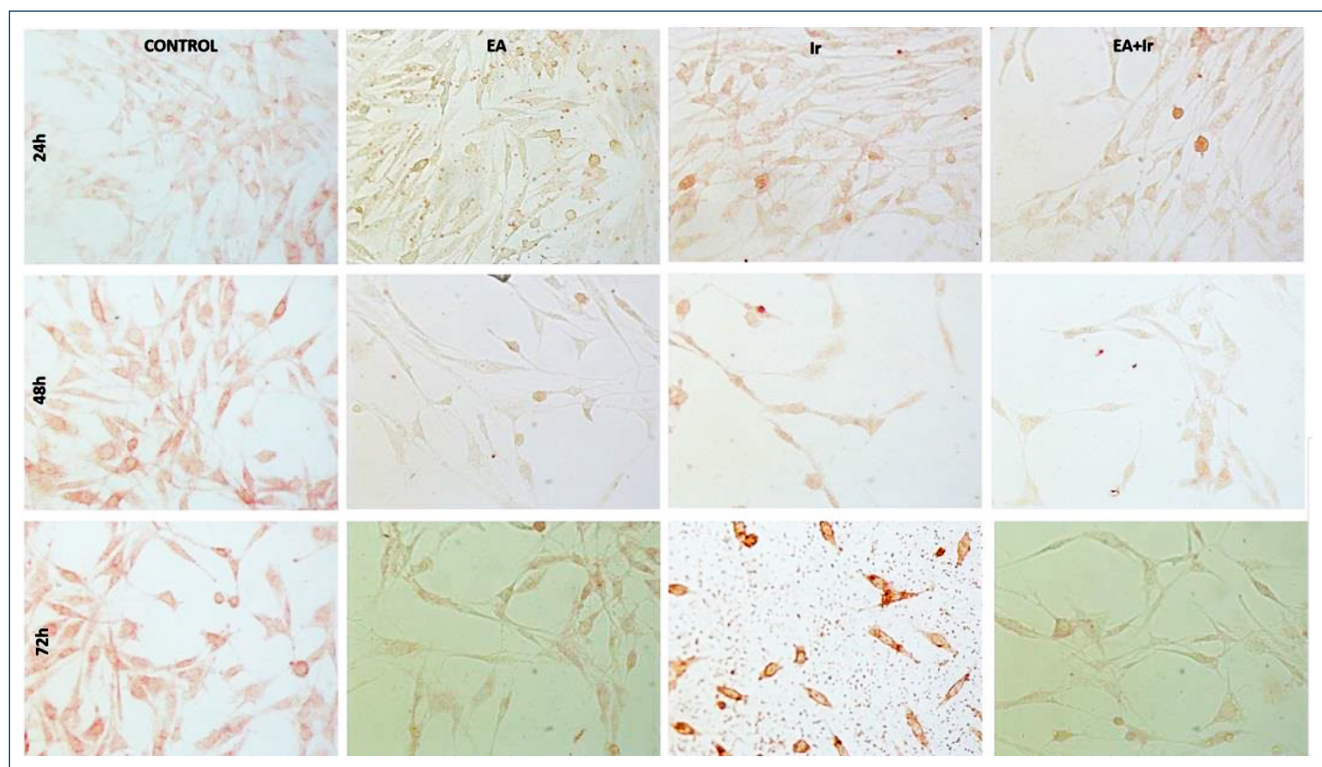
without EA significantly decreased the protein levels of VEGF at 48th (control: 42; Ir: 25,  $p < 0.01$ ) and 72nd (control: 22; Ir: 12,  $p < 0.05$ ) hours of incubation, and Ir with EA decreased significantly at 24th (control: 34; Ir+EA: 6), 48th (control: 42; Ir+EA: 5), and 72nd (control: 22; Ir+EA: 4) hours at all incubation times ( $p < 0.001$ ) (Figure 3).

## DISCUSSION

There are several modern therapies against glioma cells; however, it is still a fatal malignant disease with extremely poor prognosis<sup>1,2</sup>. Irinotecan, a topoisomerase I inhibitor, has been a new option<sup>6</sup>. The active metabolite of Ir is 7-ethyl-10-hydroxycamptothecin (SN-38), produced by the breakdown of the Ir catalyzed by carboxylesterase enzyme<sup>15</sup>. Irinotecan can be directly converted to SN-38 in glioma cells, resulting in an increase in SN-38 level, a decrease in proliferation, increase in the apoptosis, and induction of morphological changes<sup>16,17</sup>. Coggins et al.<sup>7</sup> demonstrated that Ir was effective in animal models of a variety of CNS tumor xenografts. Nakatsu et al.<sup>16</sup> revealed the antitumor activity of Ir, i.e., multidrug resistance, in human GBM cells. In combination

with Ir, the progressive nature and poor prognosis of disease in patients with malignant primary brain tumors compelled the scientists to investigate an alternative agent with novel potent action. Irinotecan, applied as either a monotherapy or a combined therapy with other agents, has been largely studied to treat these malignant and fatal gliomas<sup>15,18</sup>.

These combination therapies with Ir targeting the cadherin switch during EMT have been more beneficial than the conventional mono-chemotherapy regimens used against malignant, persistent, or resistant gliomas<sup>16,17</sup>. EMT represents the process in which cells undergo phenotypic changes by losing the cell polarity and cell-cell junctions. EMT results in a transformation from the unipolar and immobile cells into the mobile mesenchymal cells. This transformation of cells plays a fundamental role in the invasion and metastasis of a variety of cancers<sup>10,11</sup>. E-cadherin and N-cadherin are essential players of EMT process in the mechanisms of invasion and metastasis of tumors, resulting in the therapeutic resistance of gliomas. This study showed that the synergistic effects of EA treatment with Ir via altering the expression of E-cadherin and N-cadherin, as well as the expression of VEGF in a model of C6 glioma cells. The combination treatment of the EA with Ir selectively elevated



**Figure 3.** Immunocytoreactivity of vascular endothelial growth factor (VEGF) in the control (C). EA: ellagic acid; Ir: irinotecan. Combination (ellagic acid+irinotecan) groups, compared with the time of exposure. Magnification:  $\times 400$ .

E-cadherin expression while decreasing N-cadherin expression in a time-independent manner, suggesting a modulatory effect on EMT pathways in GBM cells. Moreover, the treatment EA with Ir decreased the expression of VEGF, regardless of incubation time, suggesting an antiangiogenic effect in glioma cells.

Noronha et al.<sup>10</sup> reported the E-cadherin and N-cadherin in gliomas and suggested that EMT process is compromised by increased N-cadherin expression, causing a poor prognosis, and resistance to the cancer therapies in the patients with glioma. In this study, the EA application with or without Ir considerably reversed the cadherin switch by upregulating E-cadherin expression and downregulating N-cadherin, offering an antitumor activity of EA via interfering with the EMT process in C6 glioma cells.

Angiogenesis plays an essential function in the tumor progression; however, its function is provoked by the altered levels of several proangiogenic factors including VEGF and by the abnormal hypoxic microenvironment of gliomas<sup>19,20</sup>. Therapeutic agents have been developed in combination therapies in order to inhibit this angiogenesis process mostly by targeting the members of the VEGF family, resulting in a decrease in the incidence of gliomas and resultant mortality<sup>14</sup>. Some studies have proposed that EA could inhibit angiogenesis in cancer by decreasing the number of blood vessels<sup>13</sup>. Hosny et al.<sup>21</sup> showed that EA has a significant antiproliferative effect on the in vivo behavior in cancer animal models.

Kamiyama et al.<sup>22</sup> reported that under normoxic and hypoxic conditions, Ir considerably downregulated the expression of VEGF in glioma cells in a time- and dose-dependent manner. Irinotecan has been suggested to inhibit both the endothelial

proliferation and vessel formation and the angiogenic pathways in glioma cells. Additionally, the EMT process is predominantly induced by a hypoxia in the microenvironment and the microvascular proliferation via the expression of VEGF in glioma cells<sup>10,22</sup>. This study also supported these in vitro effects of EA on VEGF expression when combined with Ir, indicating the downregulation of its expression and reduction of its immunoreactivity. Therefore, findings showed that the antitumor activity of Ir with EA enhanced the promoting antiangiogenic processes in glioma cells.

## CONCLUSION

An in vitro antitumor activity of Ir was exerted by combining with EA in C6 glioma cells. Moreover, in vitro and clinical studies are needed to clarify whether a combined strategy leads to a higher efficacy in the treatment of aggressive cancers than do a chemotherapeutic monotherapy alone, and whether these combinations could reduce the dose of agents and minimize the side effects of cytotoxic therapies.

## AUTHORS' CONTRIBUTIONS

**AC:** Conceptualization, data curation, formal analysis, writing – original draft, Writing – review & editing. **BB:** Conceptualization, data curation, formal analysis, writing – original draft, Writing – review & editing. **HO:** Conceptualization, data curation, formal analysis, writing – original draft, writing – review & editing.

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