# Anti-Tumor Effect of CDA-II on a Human Glioma Cell

**Hongyan Wang**
**Xueyun Zhong**
**Frank C Liu**
**Yanfang Qin**

Department of Pathology, Medical College of Jinan University, Guangzhou 510032, China.

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**OBJECTIVE** To examine the effect of uroacitide (CDA-Ⅱ), an extraction product from normal human urine, on proliferation and differentiation of human glioma SWO-38 cells.

**METHODS** The effects of CDA-Ⅱ on cellular survival and colony formation were determined by MTT and colony-formation assays. The in vivo anti-tumor effect of CDA-Ⅱ was examined on transplanted SWO-38 cells in nude mice. In addition, the aterations in cell morphology induced by CDA-Ⅱ were observed by H&E staining.

**RESULTS** Treatment of SWO-38 cells with 1~5 mg/ml of CDA-Ⅱ for 3 days, resulted in 39.49%± 5.27%~65.05%± 5.89% growth inhibition with an $IC_{50}$ of 2.52 mg/ml. Based on the colony-formation assay, 10 days of CDA-Ⅱ treatment at a level of 0.3~2.1 mg/ml caused 23.45%± 0.62%~96.22%±1.01% inhibition with an $IC_{50}$ of 1.03 mg/ml. Furthermore, the inhibitory response was dose-dependent. CDA-Ⅱ at dosage of 500 mg/kg or 2,000 mg/kg per day for 4 weeks significantly suppressed the growth of human glioma SWO-38 cells in nude mice, with inhibition being 47.77% and 79.94%, respectively ($P < 0.05$, n=10). H&E staining and light microscopy revealed that CDA-Ⅱ induced differentiation of the SWO-38 cells.

**CONCLUSION** CDA-Ⅱ has a significant anti-tumor effect on the human glioma SWO-38 cells, and is a potential and natural anti-glioma therapeutic reagent.

**KEYWORDS: CDA-Ⅱ, glioma, anti-tumor effect, proliferation, induced differentiation.**

Glioma is the most common primary malignancy of the human central nervous system and accounts for 35.26%~60.96% of primary intracranial neoplasms. Surgery followed by radiation therapy with or without chemotherapy is the common mode of treatment, but a majority of gliomas respond poorly to treatment. The mean survival expectancy of patients varies from 6 months to less than 2 years after multimodal therapy.[1] It is of vital importance for glioma therapy to find effective anti-tumor drugs with little toxicity. Recently, more and more researchers have focused on finding safe and nontoxic natural anti-tumor reagents for neoplasic therapy. CDA-Ⅱ is a biological mixture containing natural active components which has been isolated and purified from normal human urine. It has been shown that CDA-Ⅱ [2,13] can significantly inhibit proliferation and induce differentiation of various cancers. But its effect on glioma is not completely clear. The aim of this study was to investigate a CDA-Ⅱ

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anti-glioma effect, and provide experimental support for its potential clinical application.

## MATERIALS AND METHODS

### Materials

The human glioma cell line SWO-38 was established in our own laboratory.[3] CDA- II was kindly provided by Everlife Pharmaceutical Co. (Hefei, Anhui). MTT was purchased from the Sigma Co. BALB/c nude mice (SPF level) of both genders, weighing 17～20 g, were obtained from the Experimental Animal Center of Medical College, Jinan University, and used at the age of 4~6 weeks.

### Method

#### *Cell culture*

The SWO-38 cells were cultured as a monolayers in RPMI 1640 with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5 % $CO_2$ at 37℃ . To maintain the cell vitality, passage was conducted after 3 to 5 days. The cells were digested with 0.25 % trypsin during passage.

#### *MTT assay*

For the MTT assay, cells were harvested during logarithmic growth and digested with 0.25% trypsin. The density of the cells was adjusted to $1.5 \times 10^4$ cells/ml with RPMI 1640 culture medium and seeded into 96-well plates (100 μl/ well). After a 24 h incubation period, culture medium containing CDA- II of various concentrations from 1 to 5 mg/ml were added to the plate (100 μl/ well , 4 wells /each concentration). Culture medium (100 μl ) without CDA- II served as control. After 72 h, 20 μl of 5% MTT was added to each well followed by incubation for 4 h. The supernatants were removed and 150 μl of DMSO added followed by shaking for 15 min until the crystals dissolved completely. The optical density of each well was read on a Minireader 450 at 570 nm. The wells with no cells were used as the zero point of absorbance. Inhibition of cell growth was calculated using the following equation: (1 − average A value of experimental group/average A value of control group) × 100%. Each experiment was repeated at least three times.

#### *Colony formation assay*

The cells were digested by 0.25% trypsin, re-suspended in culture medium and aliquots added into 6-well plates at 100 cells per-well. Following cell adherence (24 h), the media were replaced with various dilutions of CDA- II (0.3 mg/ml to 3.0 mg/ml) or RPMI 1640 (as control) and allowed to incubate for 10 days. After the incubation period, the cells were washed three times with PBS and fixed in 4 ml of methanol for 15 min, then stained with Giemsa for 30 min. Finally the cells were washed with tap-water and dried at room temperature. The number of clones were counted under 4× by light microscopy examining nine visual fields at random. The inhibition of colony formation was calculated with the following equation: (1 − average clone number of experimental group /average clone number of control group) × 100% . Each experiment was repeated at least three times.

#### *In vivo anti-tumor assay*

BALB/c nude mice were injected subcutaneously in the neck with glioma SWO-38 cells at $1 \times 10^6$ cells/mouse. After spontaneous growth for 28 days, the nude mice which had transplant volumes of 0.5~1 $cm^3$ were selected and randomly divided into 3 groups: high-dosage group, low-dosage group and control group. Each group had ten nude mice. CDA- II was diluted into the following concentrations: 40 mg/ml and 10 mg/ml. The experimental groups were injected daily ip at 0.2 ml/10 g for 28 days, respectively. The control group was injected with normal saline at the same volume. The life signs, body weight and tumor volumes were monitored twice weekly. The nude mice were sacrificed 28 days after the first treatment and the tumors removd intact and weighed. The toxicity of the CDA- II was based on comparing the final body weight of the mice with that at the start of the injections. If the body weight ratio was larger than 0.8, we considered the drug to be safe, otherwise, it was

considered to be toxic.[4,5] The effect of CDA- II on the glioma was assessed by measuring tumor volume and weight. Tumor volumes were calculated with the following equation where a and b are the length and width of the tumor respectively: Tumor volumes= a × b × b/2. The anti-tumor ratio= (1 −average tumor weight of experimental group/average tumor weight of control group)□100%.

### *Morphological observation*

The same quantity of cells was plated on bacteria-free cover slides in 6-well plates and allowed to completely adhere for 24 h. Then the slides were treated with media containing 0 or 3 mg/ml CDA- II and after three days the slides were washed with PBS 3 times following fixing for 10 min in acetone, the cells were stained with hematoxylin and eosin (H&E) and viewed under a light microscope.

### Statistical analysis

Data were reported as $\bar{x} \pm s$ and were compared by using the *t*-test, a *P*-value < 0.05 was considered as statistically significant. Professional statistical computer software SPSS 10.0 was employed to analyze all data.

## RESULTS

### Inhibitary effects of CDA- II on the proliferation of SWO-38 cells

As shown in Table 1, CDA- II inhibited the growth of the glioma SWO-38 cells after exposure to 1.0 to 5.0 mg/mL CDA- II for 72 h. At a concentration of 1.0 mg/ml, the degree of inhibition was 39.49% . Compared to the control cells, the amount of inhibition at all levels of CDA- II was statistically significant ($P < 0.01$). Furthermore, the inhibitory effect of CDA- II displayed a dose-dependent pattern with the greater inhibition at the highest CDA- II concentration. The $IC_{50}$ of CDA- II was 2.52 mg/ml.

### Effects of CDA - II on colony formation of the glioma SWO-38 cell line

CDA- II significantly inhibited colony formation of glioma SWO-38 cells (Table 2). At a level of 0.3 mg/ml, clonogenicity was markedly suppressed, and as the concentration of CDA- II was increased, the inhibition of colony formation increased. At concentration of 3 mg/ml, CDA- II completely suppressed cellular growth. The result showed that CDA- II effectively inhibited proliferation of the glioma cells in a dose-dependent manner. The $IC_{50}$ was 1.03 mg/ml.

**Table 1. Inhibition of proliferation of glioma SWO-38 cells treated with CDA- II for 72 h (%)**

| Concentration of CDA- II (mg/ml) | Proliferation inhibition($\bar{x}\pm s$) |
|---|---|
| 0 | 0 |
| 1 | 39.49 ± 5.27* |
| 2 | 46.05 ± 5.09* |
| 3 | 54.04 ± 3.37* |
| 4 | 61.35 ± 2.97* |
| 5 | 65.05 ± 5.89* |

Compared to controls, *$P<0.01$.

**Table 2. The inhibition of colony formation of SWO-38 cells treated with CDA- II for 10 days (%)**

| Concentration of CDA- II (mg/ml) | Inhibition of colony formation($\bar{x}\pm s$) |
|---|---|
| 0 | 0 |
| 0.3 | 23.45 ± 0.62* |
| 0.6 | 31.69 ± 2.09* |
| 0.9 | 44.50 ± 2.57* |
| 1.2 | 56.64 ± 1.50* |
| 1.5 | 67.54 ± 1.20* |
| 1.8 | 81.55 ± 1.73* |
| 2.1 | 96.22 ± 1.01 |
| 3 | 100 |

Compared to 0 mg/mL, *$P < 0.01$.

### Inhibitary effects of CDA- II on transplant growth in vivo

Food intake and life signs of all nude mice were normal during the observation period except that one of the control mice died from an unknown reason during the fifth week. The ratio of every nude mouse end-body weight and beginning-body weight was larger than 0.8. This result indicated CDA- II had no side effects on the nude mice. CDA- II significantly

suppressed the glioma cell growth as the tumor volumes of the treated groups were markedly less than that of the control group ($P < 0.05$). The inhibitary effect of CDA- II at a dosage of 2,000 mg/kg/d was obviously greater than that of 500 mg/kg/d ($P < 0.05$) (Fig.1). The inhibition of growth was 79.94 % and 42.77 % respectively ($P < 0.05$, n=10)(Table 3). The tumor weights of the CDA- II groups were 0.68 ± 0.68 g and 1.94± 1.10 g, while the average tumor weight of the control group was 3.39± 1.09 g ($P < 0.05$). The inhibition of both treated groups was larger than 30 %. CDA- II suppressed the growth of these tumor cells in a dose-dependent manner.

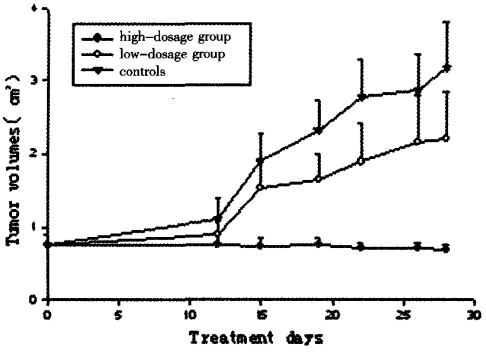


**Fig.1.** The effect of CDA- II on SWO-38 xenograft volume.

### Effects of CDA- II on morphology of glioma cells

The morphological changes induced by CDA- II in the SWO-38 cells are illustrated in Fig.2. In the absence of CDA- II, SWO-38 cells were anchorage-dependent cells, the majority were polygonal and cytoplasmic processes were relatively short. When cell growth was productive, they grew in layers and lost contact inhibition. After exposure to 3 mg/ml of CDA- II for 3 days, the cells became flat, the cytoplasm increased, the nuclei became small, so the nuclear/cytoplasmic ratio fell. Cytoplasmic processes also increased and became narrow. Cell bodies were larger, and developed a stellate configuration. These properties suggested that the SWO-38 cells had differentiated toward astrocytes.

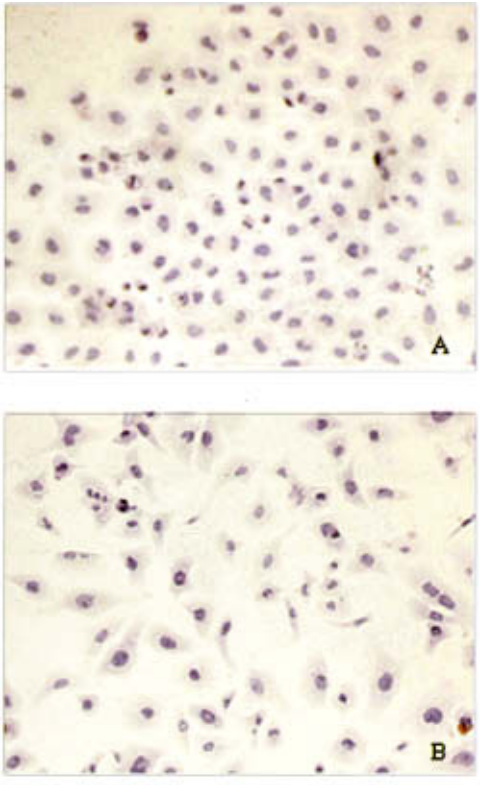


**Fig.2.** The morphological changes of golima cells after treatment with CDA- II (H&E× 200) (A) Controls: SWO-38 cells without CDA- II treatment. (B) 72-h treatment with CDA- II induces maturational changes characterized by a more stellate cell shape and longer processes.

**Table 3. The inhibitary effect of CDA- II on SWO38 cell growth in nude mice**

| Groups | n | Weight (beginning/end, g) | Tumor weight (g) | Inhibition (%) |
|---|---|---|---|---|
| Controls | 9 | 17.70 ± 1.52 / 23.77 ± 2.00 | 3.39 ± 1.09 | - |
| Low-dosage | 10 | 18.61 ± 2.69 / 23.78 ± 2.89 | 1.94 ± 1.10* | 42.77 |
| High-dosage | 10 | 16.68 ± 2.85 / 23.64 ± 2.33 | 0.68 ± 0.68** | 79.94 |

Compared to controls*: $P < 0.05$; **: $P < 0.01$.

## DISCUSSION

Malignant glioma is one of the most lethal tumors arising in the central nervous system. Due to its biological characteristics such as local invasive growth, common recurrence, resistance to traditional chemotherapeutic drugs, multi-drug resistance, and presence of the blood brain barrier, up to this time there is no therapy for this fatal malignancy. Now there is a focus of the pharmaceutic industry to screen for effective anti-tumor drugs with low-toxicity from natural resources. CDA- II is comprised of a group of natural metabolic products, which have been isolated and purified from normal, fresh human urine. As a new anti-tumor reagent, CDA- II has been utilized for clinical therapy of multi-solid tumors in Japan, USA and China.[6,7] Chinese researchers reported that CDA- II [8] had a considerable effect on many solid tumors of the alimentary canal, lung, breast cancer as well as glioma, etc, and evidently alleviated the patient's symptoms. CDA- II has few side effects and could markedly improve the quality of life of terminal cancer patients. [8,9] Considering the considerable effect of CDA- II treatment of glioma patients, we studied the anti-tumor effect of CDA- II on the human glioma SWO-38 cell line and found that CDA- II significantly inhibited its proliferation in vivo and in vitro. Cell morphological changes showed that CDA- II could induce glioma cell differentiation.

A basic technique in screening for anti-tumor drugs is to determine whether or not the drug can inhibit cell proliferation. In our study, we utilized the MTT and in vitro colony formation assays and found that CDA- II significantly inhibited proliferation of the glioma cells in a dose-dependent manner. However, the $IC_{50}$ for CDA- II based on the two methods was different, a finding that may be related to the two following reasons: first, the implanted cellular density and treatment time of CDA- II differed; second, the indexes of observation were different. The former aims at cell survival and growth, and the later quantitatively analyzes the proliferative ability of a single cell.

In our experiments, we established an animal model by transplanting the glioma in nude mice and observed the effect of CDA- II on the tumor growth. The results indicated that CDA- II significantly suppressed the growth of the glioma. The growth inhibition of a high-dosage group was greater than that of a low-dosage group. We also found that CDA- II had no side effect on the change in body weight of any of the mice, and food intake and life signs were normal during experimental period. These data indicate that CDA- II has a potential anti-glioma effect.

In our study, the SWO-38 cell morphology showed the following characteristic changes after CDA- II treatment: cells became flat, the cytoplasm increased, nuclei were reduced, and the ratio of nucleus to cytoplasm decreased. Cytoplasmic processes also increased and became narrow. Normal astrocytes have an obviously different morphological character when compared to tumor cells. These changes in cellular morphology are thought to be advantageous and a sensitive sign of differentiation, suggesting that CDA- II induced the SWO-38 cells to differentiate toward astrocytes. Our previous studies [10-12] have shown that CDA- II induced apoptosis and potentiated $As_2O_3$-induced apoptosis in hepatoma cells, producing a significant synergic effect. In our present study, we found apoptosis was not apparent, but the malignant phenotype of the SWO-38 cells was reversed and the cells were induced toward differentiation. However, the mechanism is not clear.

Lin et al. [13] reported that CDA- II was an effective scavenger of free radicals and played a major role in protecting the brain of rats against excessive amounts of reactive oxygen species. It is well known that free radicals [14] can affect many targets involved in cellular proliferation and differentiation such as signal transduction, $Ca^{2+}$ homeostasis, protein phosphorylation, and activators of transcription. It seems possible that one of the mechanisms by which CDA- II induces differentiation is to influence signal transduction and gene expression by acting as a scavenger for free radicals.

In conclusion, our results demonstrated that CDA- II has a potential anti-glioma effect and is a promising differentiation inducer. To expand its application, it is necessary to explore further the molecular mechanism

of CDA- Ⅱ action at the gene level.

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