Effects of aloe emodin on U87MG glioblastoma cell growth: in vitro and in vivo study

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Abstract
Glioblastoma, the most aggressive and malignant form of glioma, appears to be resistant to various chemotherapeutic agents. Hence other approaches have been investigated to target more pathways involved in glioblastoma development and progression. Here we investigate the anti-cancer effect of Aloe-Emodin (AE), an anthraquinone compound presents in the leaves of Aloe arborescens, on human glioblastoma cell line U87MG. U87MG were treated with various concentrations of AE (20 and 40 μM) for different times (24, 48, and 72 hr). Cell growth was monitored by daily cell count after treatments. Growth analysis showed that AE significantly decrease proliferation of U87MG in a time and dose dependent manner. FACS analysis demonstrates a block of cell cycle in S and G2/M phase. AE probably induced also apoptosis by releasing of apoptosis-inducing factor: PARP and Lamin activation leading to nuclear shrinkage. In addition, exposure of U87MG to AE reduced pAKT phosphorylation. AE inhibition of U87MG growth is a result of more mechanism together. Here we report that AE has a specific growth inhibition on U87MG also in in vivo. The growth of U87MG, subcutaneously injected in nude mice with severe combined immunodeficiency, is inhibited without any appreciable toxic effects on the animals after AE treatment. AE might represent a conceptually new lead antitumor adjuvant drug.

Keywords
adjuvant chemotherapy, aloe emodin, anthraquinone, apoptosis, brain cancer, cell cycle, glioblastoma, natural drug, temozolomide

1 | INTRODUCTION

Glioblastoma multiforme is the most severe and common brain tumor that affects adults. It is a malignant and infiltrating tumor, characterized by expansive and rapid growth. These aspects, together with a high angiogenesis, cellular heterogeneity, and the presence of tumor stem cells, capable of proliferating and generating glial neoplastic cells1,2 contribute to an unfavorable prognosis: the average survival for this type of cancer is 14 months3 both for patients treated surgically and for those treated with radiotherapy or chemotheraphy with a rate 2-year survival rate of 2%.4 There are numerous histopathological variants of GBM: in any case, the common characteristics of all types of GBM are cellular and nuclear pleomorphisms microvascular proliferation and necrosis. In addition, GBM cells possess a high capacity to activate numerous resistance mechanisms (cellular defense factors, DNA repair) in response to chemotherapy and radiotherapy, complicating the effective therapy for this tumor. The current clinical practice is based on a standard therapeutic program that involves the surgical resection of the solid tumor volume and the radiotherapy treatment combined with the temozolomide chemotherapy, known as the Stupp protocol.5 In most cases, the therapy does not reveal the ability to completely control the tumor, after a period of time that varies from patient to patient, neoplasia occurs. Although there has been great progress in identifying the molecular targets involved in
the growth and development of GBM, the genetic processes underlying tumor progression have not yet been fully understood.\textsuperscript{6} The ineffectiveness of traditional therapeutic methods is pushing most of the research to discover new molecules to be used as adjuvant therapy for GBM treatment.\textsuperscript{7,8} In the last decade, bioactive phytochemicals have been regarded as low or completely non-toxic compounds for the treatment of many forms of cancers, including brain tumors.\textsuperscript{9} A substance that seems very promising is aloe. The fresh aloe leaves contain various groups of chemical compounds such as glycoproteins, polysaccharides, anthraquinone derivatives, vitamins, minerals, amino acids, and many others, showing multidirectional therapeutic action. Emodin (1,3,8-trihydroxy-6-methylantraquinone) (Figure 1) is a naturally occurring anthraquinone derivative and an active ingredient of Chinese herbs, including \textit{Rheum palmatum},\textsuperscript{10} \textit{Polygonum cuspidatum}, \textit{Polygonum multiflorum},\textsuperscript{11} \textit{Aloe vera},\textsuperscript{12} and \textit{Cassia obtusifolia}.\textsuperscript{13} These herbs have been wildly used as traditional medicines in many countries, especially in eastern Asia. In the last 3 years, there have been many reports on the anti-tumor and anti-inflammatory effects of emodin. Furthermore, emodin has been demonstrated to possess a wide spectrum of pharmacological effects, such as antiviral, antibacterial, anti-allergic, antiosteoporotic, anti-diabetic, immunosuppressive, neuroprotective, and hepatoprotective activities. These pharmacological properties suggest that emodin might be a valuable therapeutic option for the prophylaxis and treatment of various diseases, including asthma, atopic dermatitis, osteoarthritis, diabetes and diabetic complications, atherosclerosis, Alzheimer’s disease (AD), hepatic disease and several types of cancers, such as pancreatic cancer, breast cancer, hepatocellular carcinoma, and lung carcinoma.\textsuperscript{14} There is no available information, so far, on mechanisms of emodin inhibition cell growth in human glioblastoma cell line U87MG in vitro and in vivo. In the present study, we investigated the effects of AE on U87MG human glioblastoma cells in vitro and in vivo.

2 | MATERIALS AND METHODS

2.1 | Materials

We used a human GBL continuous cell line (U87MG) (American Type Culture Collection). For treatments in vitro and in vivo we used Aloe emodin (AE) (Sigma). For proteins analysis we used the following antibodies: mouse monoclonal antibody anti-cyclin D2, mouse monoclonal antibody anti-p21, mouse monoclonal antibody anti-p53 and rabbit polyclonal antibody anti-pAKT, and rabbit polyclonal antibody anti-PARP (Poly (ADP-ribose) polymerase) and mouse monoclonal antibody anti-Lamin A (Cell Signaling).

2.2 | Cell cultures

U87MG human glioblastoma continuous cell lines were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum (Invitrogen), in culture dishes (Becton Dickinson, Lincoln Park, NJ), at 37°C, 5% CO\textsubscript{2}, and 95% of humidity. The medium was changed every 3 days. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

2.3 | Aloe emodin treatment of human GBM cell lines

To evaluate, in vitro, the response to treatment with AE, human glioblastoma cells (U87MG) were plated in 24-well plates, 2.5 × 10\textsuperscript{3} cells per well, in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and incubated at 37°C in an atmosphere containing 5% CO\textsubscript{2}. The day after the cells were treated with AE at following concentrations 20 and 40 μM. At 24, 48, and 72 hr after treatment we made a cell count by Burker chamber, in the presence of trypan blue dye (Sigma) to discriminate live cells from the dead ones.

2.4 | Analysis of cell cycle proteins after treatment with AE

Proteins were extracted from U87MG treated with AE (20 and 40 μM) in Triton X-100 lysis buffer (Tris-HCL 10 mM, EDTA 1 mM, NaCl 150 mM, Triton X-100 1%, Naf 1 mM, 1 mM Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}, 1 mM Na\textsubscript{3}VO\textsubscript{4}, protease inhibitors 1x). Proteins (40 μg) were separated by SDS-PAGE on a polyacrylamide gel to 12.5% and transferred to nitrocellulose membranes (Amersham) by electroblotting. The membranes were incubated for 2 hr at room temperature in 5% milk diluted in T-TBS 1X, and then incubated over night at 4°C with primary antibodies specific and 1 hr with secondary antibody. Each membrane was then incubated with mouse monoclonal anti-β-actin (1:10 000;
Santa Cruz Biotechnology). Proteins were detected by chemiluminescence using ECL Western blotting (Amersham). The signals were detected by a digital scanner and quantified by densitometric analysis (Scion Image software).

For cyclin D2, P21, p53, PARP [Poly (ADP-ribose) polymerase], and Lamin A analysis, cells were plated at a density of 500 000 cells for plate, in DMEM without FCS for 48 hr. After re-added 10% FCS, induction was performed with AE (20 and 40 μM) for 24–48 and 72 hr. It used anti-cyclin D2, P21, P53, PARP, and Lamin A antibody (1:1000; Cell signaling). For pAKT detection, treatments with AE (20 and 40 μM) were performed at 15 min, 30 min, 1 hr, 2 hr, and 4 hr. The membranes were incubated over night with anti-pAKT antibody (Cell Signaling, 1:1000) in 2.5% BSA.

2.5 | FACS analysis by flow cytometry

The U87MG human GBM cells were plated (25 × 104) in DMEM with 10% FBS and treated with AE (20 and 40 μM) for 24, 48, and 72 hr. After treatment, cells were trypsinized, washed in sample buffer (glucose 0.1% in HBSS), fixed in 70% ethanol, and stored at 4 °C overnight until the day of analysis. Before analysis, propidium iodide (50 μg/mL) was added for 30 min at room temperature. Flow cytometry (FC) analysis of the cell cycle was performed with Gallios instrument (Beckman Coulter).

2.6 | Immunofluorescence

U87MG cells were plated in culture slides (BD falcon), 400 000 per well and treated with AE 40 μM for 48 hr. At the end of the treatment the cells are fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton. Incubation was performed with 10% goat serum for 1 hr and incubated with primary antibody anti-p21 (1:100 cell signaling) over night at 4 °C. Secondary anti rabbit antibody Cy3 was added (1:200 Vector) for 1 hr and DAPI coloring (Vector). The cells were visualized at a fluorescence microscope at 20 × magnification.

2.7 | U87MG cell implantation

Male nude mice CD1 (Charles River, Calco, CO, Italy; 20–22 g body weight) were housed in a controlled conditions (temperature 22°C; humidity, 40%) on a 12-hr light/dark cycle and observed daily. The experiments were carried out according to the regulations for the care and use of animals promulgated by National Institutes of Health; ethics committee approval for animal studies N 159/2016-PR (Risp prot. 20234.1), that was approved December 1, 2015 before start the study. After anesthesia with ketamine 100 mg/kg and xilazine 10 mg/kg i.p., mice were subcutaneously implanted with 1 × 10^6 U87MG cells/0.5 mL. At 7 days after inoculation treatments are carried out with AE (50 mg/kg), intraperitoneal, 5 days for week for 3 weeks. In another set of experiment, nude mice with U87MG, after 1 week from inoculation, are treated with AE 100 mg/kg, administrated 5 days a week by gavage for 4 weeks. Tumor size was measured by caliper.

Subsequently, the mice were sacrificed by cervical dislocation after anesthesia, and tumors removed, were fixed in formalin, dehydrated in ethanol at increasing concentrations and embedded in paraffin. From each tumors were cut sections of 4 μm thickness. The sections were stained with Mayer’s hemalum (Diapath) and Eosin (Diapath) and subjected to microscopic evaluation.

2.8 | Immunohistochemistry

We analyzed cell cycle and apoptosis proteins by immunohistochemistry on subcutaneous tumors taken from CD1 nude mice and included in paraffin. Immunohistochemistry was performed on 4 μm thickness sections using BenchMark XT automatic immunostainer (Roche). We used mouse monoclonal antibodies anti-Ki67 and anti P53 (prediluted; Roche Diagnostic), rabbit polyclonal antibody anti-Caspase 3 and mouse monoclonal antibody anti-Caspase 8 (1:1000, Cell signaling).

3 | RESULTS

3.1 | Aloe emodin reduces U87MG human glioma cells growth

The effect of AE on the proliferation of human glioblastoma U87MG cells was analyzed by setting up growth curves. Different concentrations of AE (20 and 40 μM) were tested at different treatment times (24, 48, and 72 hr). As shown in Figure 1A, the effect of AE on cell proliferation is significant (P < .001) at all drug concentrations and at different treatment times. Furthermore the effect of AE on the inhibition of cell growth is strictly dependent on the progressive increase of the dose and the treatment time. The same experiment also analyzed the effect of Aloe arborescens (total extract from leaves), used with a concentration of 8%; also with this natural compound we have a significant reduction (P < .001) of cell proliferation in the three treatment times, with a greater effect at 72 hr (Figure 1B).

3.2 | Cell cycle analysis

To evaluate how AE inhibits the proliferation of U87MG cells, a cell cycle analysis was performed using FACS in control cells and cells treated with AE 20 and 40 μM at 24, 48, and 72 hr. The results of the FACS analysis show that AE determines a blockage of the cell cycle in S and G2/M phase. As shown in the Figure 2, in S phase and in G2/M phase we found an increase in the percentage of the number of cells by both concentrations of AE used (20 and 40 μM). A greater effect of the substance can be found in the sample treated with AE 40 μM at 72 hr; in S phase at 72 hr 40 μM AE causes an increase of the number cells by 25% compared with the control, while in the G2/M phase the percentage of cells increases is 18%. In the first phase of the cell cycle (G0/G1), however, we have a decrease in the percentage of cells due to the effect of AE at both concentrations (20 and 40 μM). These data show how the AE acts on the cell cycle, resulting in a block in the S phase or G2/M phase, both in pre-mitotic stages (Figure 2).

3.3 | Evaluation of the expression of cell cycle proteins in U87MG treated with aloe emodin

Western Blot analysis was used to evaluate the effect of AE on the expression of some proteins involved in cell cycle regulation and
blocking mechanisms. Protein samples were extracted from U87MG, treated for 24, 48, and 72 hr with AE at 20 and 40 μM. To evaluate the effect of the substance on cell cycle arrest, the expression of CDK2 protein (33 kD) was analyzed. In the samples treated with AE (Figure 3A), the expression of CDK2 decreases significantly only at 48 hr of treatment with both concentration. The decrease in cyclin CDK2 consequently leads to an increase in tumor suppressor: in fact, as shown in (Figure 3B), the expression of the p53 protein (53 kD) undergoes a significant increase (P < .001) compared with the control already starting from 24 hr of treatment and for both concentrations. Finally, to investigate the effect of Aloe on cell cycle progression, the protein p21 (21 kD) was analyzed; its expression increases in samples at 20 and 40 μM AE at 24 and 48 hr (Figure 3C). P21 increase was detectable as in immunofluorescence assay (Figure 3D). These results suggest that AE determines a block of the cell cycle in which is involved precisely the treatment with AE 20 and 40 μM, was evaluated the phosphorylation of pAKT ser. There is a reduction in phosphorylation of AKT only at 1, 2, and 4 hr from the treatment. (Figure 5A, B).

3.4 | Aloe emodin leads activation of start apoptosis

To further analyze the AE-induced start of apoptotic event, we performed a large time course experiment from 24 to 72 hr with 20 and 40 μM of AE. Immunoblot analysis using antibodies specific to them reveals a decrease of PARP [poly(ADP-ribose) polymerase] normal form (116 kD) expression at 48 and 72 hr, and activation of Lamin A (74 kD) with evident increase of cleaved band (50 kD) (Figure 4B,C).

The pro-apoptotic AE effect is already visible at first observation of cells under a microscope. Figure 4A shows U87MG control cells that have a typical fusiform, while U87MG cells treated with 40 μM AE for 72 hr exhibits a round shape and a reduced volume, that are typical characteristics of apoptotic cell.

3.5 | In vivo experiment

In first experiment we implanted U87MG cells subcutaneously onto right side of nude mice (N8), after 1 week from implantation, mice were divided randomly into two groups: control and AE treated one. Mice were treated 5 days for a week with AE 50 mg/kg by intraperitoneal injection for 3 weeks. In other experiment mice (N8) were treated daily with AE 100 mg/kg by gavage 5 days for a week, and the treatment was prolonged for 4 weeks. At the end of the treatment animals were sacrificed and tumors taken from subcutaneous were weighed and were subjected to histological analysis. Tumor size was evaluated weekly by using a caliper. The evaluation of tumor size, by caliper measurement analysis confirmed that, in mice treated with AE, tumor size significantly reduced to about 40% (Figure 6).

Cytologically, tumors were composed of large pleomorphic cells with abundant eosinophilic cytoplasm (Figure 7). Hematoxylin-eosin staining showed that tumors were composed of dysmorphic cells, typical of glioblastoma, with high cell density; the tumors treated with AE showed the same cell morphology but a marked reduction in cell density was also confirmed by the decrease of Ki 67 in the immunohistochemical staining. Finally, to show that even in the tissues there is an evidence of pro-apoptotic effect of AE also in vivo we have used the
FIGURE 3 Downregulation of cyclin D2 and induction of p21 and p53 in U87MG cell line after treatment with AE. Western blotting and densitometric analysis of (A) cyclin D2, (B) p53, (C) p21 in continuous GBM cell line U87MG after treatment with AE (20 and 40 μM) applied to the cultures at 24, 48, and 72 hr. Thirty micrograms of protein were loaded for each lane. The blot was repeated three times with similar results. Values are the means ± SEM of three individual determinations. One-way ANOVA and Dunnett test; *P < .05, **P < .001, ***P < .0001. On the right (3D) there is a representative picture of immunofluorescence with antibody anti p21 of U87MG cells control and U87MG treated with AE 40 μM for 48 hr [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 AE inhibits GBM cell growth by inducing apoptosis. (A) Cell morphology of U87MG after AE treatments, cell morphology changed, cell became rounded without the cell prolongations similar apoptotic cells. (B) AE induces decrease of normal PARP. (C) AE activates Lamin a by cleavage; figure shows normal Lamin A MW (74 kD) and cleavage form (50 kD). Values are the means ± SEM of three individual determinations. One-way ANOVA and Dunnett test; *P < .05, **P < .001, ***P < .0001 (Lamin A normal form); ΔP < .05, ΔΔP < .001, ΔΔΔP < .0001 (Lamin A cleaved form) [Color figure can be viewed at wileyonlinelibrary.com]
P53, caspase 8, and caspase 3 antibody. Immunohistochemistry for P53, caspase 8, and caspase 3 showed evidently stronger staining in the tumor slides of the mice treated with AE.

4 | DISCUSSION

Despite standard treatments, recurrence and drug resistance remains the main cause of treatment failure in glioblastoma. The difficulty in treating this malignant disease lies both in its inherent complexity and numerous mechanisms of drug resistance.15

The reasons for drug resistance in glioblastoma include drug efflux, hypoxic areas of tumor cells, cancer stem cells, DNA damage repair, and miRNAs. Many potential therapies target these mechanisms, including a series of investigated alternative and plant-derived agents, and new pharmaceutical formulation such as nanoparticles and viral vectors, together with new strategy entailing the use of monoclonal antibodies, vaccines, and immunotherapy agents, such as check-point inhibitor will be considered to help combat drug resistance.16 The emerging new data on the potential of plant-derived therapeutics should also be closely considered and further investigated.17
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For better results of cancer therapy, the research is dealing with the discovery of new natural substances that can be used as adjuvant drugs in the treatment of glioblastoma. One of these substances is aloe emodin, which has shown an anti-proliferative effect in several tumor cells and, moreover, has shown to be able to decrease the vitality of human colon cancer cells. In this study we analyzed the effects of AE on the proliferation of human glioblastoma U87MG cells, and the results obtained indicate that AE could be a new strategy for the treatment of brain cancer. We performed an in vitro study on U87MG to evaluate the proliferation in different concentrations of AE (20 and 40 μM) at different times (24, 48, and 72 hr). Both concentrations used inhibit cell growth already at 24 hr of treatment, but the maximum inhibition is found at 48 and 72 hr, where about 50% of the cell growth is stopped. Several studies have shown that AE blocks the G2/M cell phase in HELA cervical carcinoma cells. In fact, the substance we tested, in addition to inhibiting the proliferation of glioblastoma cells is also able to induce a cell cycle blockage in U87MG cells. This was demonstrated by FACS analysis; the results showed that long-term treatment (24, 48, and 72 hr) with AE (20 and 40 μM) resulted in an increase in the percentage of cells in S and G2/M, causing, therefore, a cell cycle arrest in these two phases. To further analyze the cell-cycle arrest mechanism induced by AE, the expression of p53, p21, and cyclin CDK2 proteins was analyzed by Western Blot analysis. The results of the analysis showed that AE consistently increases the level of expression of the proteins (p53, p21) involved in the control mechanism of the cell cycle progression. The expression of CDK2, active protein in the S phase of the cell cycle undergoes a decrease only at 48 hr of treatment with AE 20 and 40 μM. Our results revealed, therefore, that the arrest of the cell cycle induced by AE is associated with the increase of p53, p21 and the reduction of cyclin CDK2. We can conclude that in vitro, in U87MG, AE determines: the reduction of cell proliferation and the cell cycle blockage in S and G2/M phases. The MAPKs play a critical role in the regulation of cell growth, differentiation, and in the control of cellular responses to cytokines and stressor. It also was investigated that phosphorylation of Akt serine in U87MG glioblastoma cells treated at short times treatment (2 and 4 hr) with AE (20 and 40 μM) there is a decrease of phosphorylation that could be responsible of blocking of proliferative signals. We investigate the pro-apoptotic AE effect that is already visible at first microscopic observation of the cells. The image represented U87MG control cells with a typical fusiform morphology and U87MG cells treated with AE 40 μM for 72 hr with a round shape and a reduced volume, typical features of apoptotic cell. To confirm the pro-apoptotic effect of AE expression of signaling apoptotic was analyzed by western blot; there is a reduction of expression of the normal form of PARP1 (116 kD) in cells treated with AE compared with the control, after treatment and activation of Lamin A.

In our first in vivo studies, in nude CD1 mice treated with AE 50 mg/kg/day (with i.p. administration for 15 days), we find a 40% reduction in tumor mass compared with that in control mice. We show some pathological modifications induced by AE, including direct evidence in vivo of its anti-proliferative effect as it is evident by decrease of Ki67 positive cells in AE treated slides and pro-apoptotic effect also in vivo: increase P53, and caspase 6 in brain of AE treated mouse. The findings that the systemic infusion of AE, a drug that can cross the blood–brain barrier, limits the growth of the tumor inside the mouse brain, is particularly encouraging from a point of therapeutic view and suggests that the inhibition of cell proliferation outweighs any possible detrimental effect of the drug on tumor growth. An important feature of AE, which emerged from our studies, is the inefficiency of producing toxic, acute, or chronic effects, both in the human glioblastoma cell line (U87MG) and in animal models (nude CD1 mice). The selective in vitro and in vivo killing of human glioblastoma U87MG cells by AE, indicates aloe as novel anticancer adjuvant agent.

**FIGURE 7** AE reduces the growth of U87MG cells and induces apoptosis also in vivo. UM87Mg cells were subcutaneously implanted into right of nude mice in order to produce an orthotopic xenograft model of glioblastoma. Mice were treated with AE (50 mg/kg, i.p.) daily, starting 7 days after tumor implantation. Both vehicle (N8) and AE (N8) administered mice were sacrificed at 3 weeks and tissue sections were stained with hematoxylin/eosin. A. Shows representative pictures of subcutaneous tumor. In control mice treated with vehicle (left pictures) a clear cut eosinophilic high density tumoral cell were apparent; while this density cell was markedly reduced in AE treated mice (on the right). When tumor sections were stained for Ki67 (a protein which associates with cell proliferation) we observed a clear reduction in AE treated mice slides. In picture are visible the increase of expression of apoptotic protein P53, caspase 3 in AE treated mice slides compared with control one [Color figure can be viewed at wileyonlinelibrary.com]
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