Cytotoxic effect of diferuloylmethane, a derivative of turmeric on different human glioblastoma cell lines

Ismael Bilal¹, Sijia Xie², Muna S. Elburki³, Zahra Aziziaram⁴, Sangar Muhammad Ahmed⁵, Salah Tofik Jalal Balaky⁶*

ABSTRACT

Glioblastoma is a fatal brain tumor, and the standard treatment for this cancer is the surgical removal of the tumor followed by chemotherapy with temozolomide and radiotherapy. Because chemotherapy has many side effects, the use of compounds extracted from natural herbs, due to fewer side effects, can be a good alternative or supplement to chemical drugs in cancer treatment. In this study, curcumin (diferuloylmethane), known as the main active ingredient of turmeric, was used to evaluate its cytotoxicity on four human glioblastoma cell lines (U373, U251, D54, and T98G). Among these cell lines, U373 was temozolomide resistance, and T98G was photodynamic treatment resistance. These cell lines were treated with increasing concentrations of diferuloylmethane. Survival percentage was assessed by MTT assay and the trypan blue staining method was used to evaluate the rate of cell death and confirm the results of the MTT assay. The results showed that diferuloylmethane has a cytotoxic effect on U251, D54, and T98G cell lines. This effect was higher in high concentrations of diferuloylmethane on U251 and D54 than on U373. Therefore, according to the results of the current study and further studies, curcumin (diferuloylmethane) can be considered an effective complementary treatment in the treatment of glioblastoma.

Keywords: Brain Tumor, Cancer, Curcuma longa, Medicinal Herbs, Supplement Medicine

1. Introduction

Glioblastoma is the most common primary brain tumor in the adult population, with a prevalence of 4-5 cases per 100,000 cases [1; 2]. Glioblastoma multiforme cells invade the brain parenchyma dramatically and are naturally resistant to apoptosis, drug and radiotherapies [3].

Common treatments for cancer have side effects and limitations such as anemia, appetite loss, bleeding and bruising (thrombocytopenia), constipation, delirium, diarrhea, edema (swelling), fatigue, fertility issues, flu-like symptoms, hair loss (alopecia), infection and neutropenia, lymphedema, memory or concentration problems, mouth and throat problems, nausea and vomiting, nerve problems (peripheral neuropathy), immunotherapy and organ-related inflammation, pain, sexual health issues, skin and nail changes, sleep problems, urinary and bladder problems [4].

For instance, temozolomide, as a specific type of brain cancer medicine, interferes with the growth of cancer cells, which are then destroyed by the body. Since the growth of normal body cells may also be affected by temozolomide, other side effects may occur [5]. For this reason, the need for using natural compounds that have few side effects and are cheap has been felt to treat cancer [6].

¹ Department of nursing, College of nursing, Hawler medical University, Erbil, Iraq
² Department of Periodontics, Inner Mongolia Normal University, Hohhot, Inner Mongolia, China
³ Department of Periodontics, Faculty of Dentistry, University of Benghazi, Libya
⁴ Research and Development Department, Giga Biotics, San Diego, California, USA
⁵ Department of Health Sciences, College of Health Sciences, Hawler Medical University, Erbil, Iraq
⁶ Medical Microbiology Department, College of Health Sciences, Hawler Medical University, Hawler, Kurdistan Region, Iraq
*Corresponding Author: Salah Tofik Jalal Balaky (Salah.balaky@hmu.edu.krd)
Medicinal herbs could be the best source for various medicines [7; 8]. Recently, due to different therapeutic properties, medicinal herbs have been considered by many researchers around the world [2]. In modern medicine, several studies have been conducted to find the potential effects of various extracts of medicinal herbs, such as growth inhibitors of some tumors [9]. Moreover, different compounds (including colchicine, vincristine, vinblastine, podophyllotoxin, and taxol) have been isolated from various herbs, which are used against different types of tumors [10; 11].

One of the most studied medicinal herbs is turmeric. Turmeric is the dried rhizome powder of Curcuma longa plant, which is composed of a wide range of phytochemicals, including curcumin, demethoxycurcumin, bisdemethoxycurcumin, curcumol, tetrahydrocurcumin, turmerones, turmeric, and turmeronol [12; 13].

The main ingredients in turmeric are curcuminoids, which also produce the yellow color of turmeric [14; 15]. The most significant curcuminoid, to which the most healing properties of turmeric are attributed, is curcumin, which was first extracted from turmeric in 1815, and its molecular formula was discovered, in 1910. Curcumin, with the molecular formula C_{16}H_{12}O_{6} and the chemical name diferuloylmethane, is the most considerable molecule isolated from plants that have been investigated in recent years [12; 16]. This molecule is inherently hydrophobic and does not dissolve in water, but dissolves in substances such as dimethyl sulfoxide, acetone, ethanol and oil [17; 18]. Turmeric contains about 3-8% curcumin (depending on the growing season), although other plant species also contain some curcumin [2; 19].

In various research and studies, the antiseptic, anti-inflammatory and antioxidant properties of turmeric have been proven and have been proposed as a complementary treatment for Alzheimer's, diabetes, asthma, stomach ulcers, etc [20].

The anti-cancer properties of turmeric have been tested in various clinical trials and cell culture media at different stages, and their effective mechanisms in the treatment of this disease are also being investigated [21]. The goal of this study was to test the cytotoxic effect of diferuloylmethane, as a derivative of turmeric (Curcuma longa), against four human glioblastoma cell lines (U373, U251, D54, and T98G).

2. Materials and methods

2.1 Cell culture

Cell lines U373, U251, D54, and T98G were purchased from Sigma-Aldrich, USA. Among these cell lines, U373 was temozolomide resistance and T98G was photodynamic treatment resistance. All four cell lines were cultured in a DMEM medium (Gibco-Invitrogen, USA) containing 10% FBS.

2.2 Growth curve plot

In order to find the appropriate number of cells for culture in each 16-well plate and also to determine the appropriate time interval, growth curves were plotted for each cell line U373, U251, D54, and T98G. To plot the growth curve, after centrifugation of the cell suspension, cell sediment was dissolved in 1ml of culture medium containing 10% FBS. Then a volume of suspension containing the desired number of cells was calculated and cultured in a final volume of 150μl of culture medium containing 10% FBS at different time intervals. U373, U251, and T98G cell lines were cultured with 3.33×10^{5}, 1.33×10^{5}, 0.66×10^{5}, and 0.33×10^{5} cells/ml throughout 0 to 72 hours. The D54 cell line was cultured with 2.4×10^{5}, 1.33×10^{5}, 0.6×10^{5}, and 0.3×10^{5} cells/ml for 0 to 96 hours. After the desired period, the survival rate was measured by MTT assay.

2.3 Treatment of cell lines with different concentrations of diferuloylmethane

1.33×10^{5} cells per ml of U373, U251, D54, and T98G cell lines were cultured with increasing concentrations of diferuloylmethane (Sigma-Aldrich, USA) in each well of 96 well plates. The final volume of each well was 150μl. After the doubling of cell lines, the survival rate was measured by MTT assay.

2.4 Measurement of survival rate by MTT assay
MTT is commonly used to determine the number of living cells in studies of drug toxicity [22]. To prepare MTT solution with a concentration of 5 mg/ml, 50 mg of MTT powder was dissolved in 10 ml of distilled water and after passing through a 0.22μm filter, it was maintained in 1.5 ml microtubes at -20°C. To measure survival, 10μl of MTT solution was added to each well of a 16-well plate, and the cell culture plate was incubated at 37°C. After three hours of incubation time, 100μl of the supernatant was removed to dissolve the resulting crystals and 100μl of DMSO was added, instead. Then, the crystals formed at the bottom of each well were completely dissolved and the optical density (OD) of the cells was read at 492 nm.

2.5 Cell death percentage

To assess the percentage of cell death, cell line D-54 was selected and treated with 96μM diferuloylmethane or 0.4% DMSO (as a control) for 96 hours according to MTT assay. Subsequently, the percentage of cell death was measured by trypan blue staining (Sigma-Aldrich, USA). In this method, after staining with trypan blue and cell count, the survival percentage was calculated by the following formula:

\[
\text{Cell survival rate} = \frac{\text{Number of living cells}}{\text{Total number of cells}} \times 100
\]

2.6 Statistical tests

Statistical analysis of the results was performed by GraphPad Prism 6 software. All experiments were performed three times, and in each experiment, the samples were tested three times. Statistical analyzes were performed by one-way ANOVA or t-test, and \(P\) values less than 0.05 were considered as the criteria for significant differences between the groups. All data are reported as Mean ± SEM.

3. Results

3.1 The appropriate number of cells for treatment and growth curve of cell lines

To determine the appropriate number of cells for treatment, the growth curve of cell lines with a different number of cells was drawn by MTT assay in intervals of 0 to 96 hours. By the growth curve, the doubling time of the cells number can be determined, and also the number of cells that were suitable for treatment with diferuloylmethane can be distinctive. Since the best time interval for cell treatment is when the cells have passed the delayed growth stage, have the opportunity to double, and are in the logarithmic stage of rapid growth or growth, hence 72 hours was selected for cell lines U373, U251, and T98G and 96 hours were selected for the D54 cell line. 1.33×10\(^5\) cells/ml in a final volume of 150µl, which cell growth was neither too slow nor too fast, was selected for all four cell lines (Fig. 1). In Fig. 1, curves 1 to 4 related to cell lines (a) U373, (b) U251, (c) D54, and (d) T98G. About the number of cells, U373, U251, and T98G cell lines were 3.33×10\(^5\), 1.33×10\(^5\), 0.66×10\(^5\), and 0.33×10\(^5\) cells/ml. D54 cell line was 2.4×10\(^5\), 1.33×10\(^5\), 0.6×10\(^5\), and 0.3×10\(^5\) cells/ml.
Fig. 1. Growth curve of human glioblastoma cell lines; curve number (1) is $3.33 \times 10^5$ cells/ml, (2) is $1.33 \times 10^5$ cells/ml, (3) is $0.66 \times 10^5$ cells/ml, and (4) is $0.33 \times 10^5$ cells/ml on U373, U251, and T98G cell lines. About D54 cell line, (1) is $2.4 \times 10^5$ cells/ml, (2) is $1.33 \times 10^5$ cells/ml, (3) is $0.6 \times 10^5$ cells/ml, and (4) is $0.3 \times 10^5$ cells/ml.

3.2 The effect of diferuloylmethane on human glioblastoma cell lines

Cell lines were treated with increasing concentrations of diferuloylmethane at intervals relating to the doubling time of each cell line. After the desired period, cell survival percentage was calculated by MTT assay and GraphPad Prism 6 software. The control sample was treated with 0.4% DMSO (as diferuloylmethane solvent). The survival percentage of the control sample was considered equal to 100 and the survival percentage of other samples was calculated according to the control sample. The results showed that diferuloylmethane decreased the survival percentage in U373, U251, and T98G cell lines in a concentration-dependent form ($P<0.05$). Among them, cell line D54 was the most sensitive cell line with a concentration of 200μM of diferuloylmethane. Each experiment was performed 3 times and in each experiment, the samples were repeated 3 times (Fig. 2).
Fig. 2. Survival chart of human glioblastoma cell lines after treatment with increasing concentrations of diferuloylmethane; the error axis in all columns is reported as mean ± SEM (*** = P<0.001, ** = P<0.01, * = P<0.05)

To confirm the results of the MTT assay, the rate of cell death was assessed by trypan blue staining. In this method, D54 cells were treated with a concentration of 200μM diferuloylmethane, and then the cell death rate was calculated. The basis of the trypan blue staining method is based on the fact that living cells have a healthy cytoplasmic membrane and due to the selective permeability of the cell membrane, trypan blue does not pass through it, but the cytoplasmic membrane of dead cells is lost, therefore, trypan blue easily enters the cell, and as a result, these cells appear blue under a microscope. The results of this test were consistent with the results of the MTT assay (Fig. 3).

Fig. 3. The survival rate of D54 cancer cell lines treated with diferuloylmethane by trypan blue staining; the error axis in all columns are reported as mean ± SEM (* = P<0.05)

3.3 Comparison of diferuloylmethane inhibitory effect on the human glioblastoma cell lines survival

The cytotoxic effect of diferuloylmethane on human glioblastoma cell lines showed that at concentrations of 100 and 200μM diferuloylmethane, the survival of the U251 cell line decreased by 35.7% and 21.72%,
respectively \( (P<0.05)\) (Fig. 4). Since cell line U251 is resistant to the chemotherapeutic drug temozolomide, the cytotoxic effect of diferuloylmethane on this line is very important. In addition, at a concentration of 100μM diferuloylmethane, the survival of the D54 cell line was 25.96%, which was lower than U373. These results indicate that diferuloylmethane at high concentrations has a different inhibitory effect on the survival of human glioblastoma cell lines.

**Discussion**

Glioblastoma multiforme is the most common invasive and the deadliest type of primary brain tumor in adults [3]. They originate from astrocytes, a type of glial cell. This tumor has cells that look very different from normal cells, usually occur in adults and most forms in the brain. The survival period after the initial diagnosis is 12-15 months [23]. It has a relatively low incidence, but 2.5% of cancer deaths belong to this cancer, and it is more common in developed countries and men. One of the most important risk factors after having male gender is the age of 50 to 74 years [24].

One of the most important treatments for glioblastoma is chemotherapy. Despite the efficacy of chemotherapy, normal hematopoietic cells, intestinal epithelial cells, and hair matrix keratinocytes are prone to the toxic effects of these drugs [25]. The use of herbs alone or in combination with these drugs reduces their side effects [26]. On the other hand, herbal medicines can overcome drug resistance and kill resistant cells by targeting multiple mechanisms and molecules [27]. Turmeric is one of the medicinal herbs whose anti-cancer effect has been proven in various types of cancers [28]. Since this plant has a variety of compounds, in this study, for the first time, the role of diferuloylmethane, as one of the most active derivatives of turmeric, was investigated on glioblastoma cancer [28].

In this study, the effect of diferuloylmethane on the survival of human glioblastoma cell lines showed that diferuloylmethane has a concentration-dependent cytotoxic effect on U251, D54, and T98G cell lines (Fig. 2). The results of trypan blue staining in diferuloylmethane-treated cells also confirmed the results of the MTT assay (Fig. 3). Although neither MTT assay nor trypan blue staining identifies the target molecule or component of diferuloylmethane in a cancer cell, it is obvious that it disrupts the mitochondria and cytoplasmic membrane of these cancer cells. In previous studies, the inhibitory and concentration-dependent effect of diferuloylmethane on the survival of clone cancer cell lines and promyelocytic leukemia has been proven [29]. Also, studies have shown that curcumin (diferuloylmethane) can prevent papillomavirus-induced cervical cancer by inhibiting NF-κB transport into the nucleus and reducing the expression of human papillomavirus (HPV) oncogenes and inducing apoptosis in HPV-infected cells [30]. Other mechanisms suggested for the anti-cancer properties of curcumin include suppression of HPV oncoproteins and increased expression of tumor suppressor genes such as P53 [31]. Although curcumin induces apoptosis in a variety of cells, including pancreatic cancer, it does not affect esophageal cancer cell lines, and this may indicate the presence of various mechanisms of curcumin in cancer cells. For example, in most cells treated with curcumin, apoptotic membrane molecules, including BCL2, increase, but not in esophageal carcinoma [32].

In this study, the inhibitory effect of diferuloylmethane on the survival of the U251 cell line was greater than the U373 cell line (Fig. 4). One of the mechanisms of drug resistance in the U251 cell line is the
increase in MDR1 expression. MDR1 is a membrane protein that plays an important role in multiple drugs resistance. Based on a study on the expression of genes involved in drug resistance in glioblastoma, it was found that MDR1 gene expression is increased in MRD+ patients [33]. NF-κB, as a transcription factor for MDR1, increases transcription of this gene. Since diferuloylmethane inhibits NF-κB [34], this plant derivative can also exert its cytotoxic effect on the U251 cell line through this pathway. Of course, more research is needed to support this claim [35].

In this study, it was also shown that the inhibitory effect of diferuloylmethane on the survival of the D54 cell line is greater than U373 (Fig. 4). One of the possible reasons for the difference in the cytotoxic effect of diferuloylmethane on the D54 cell line compared to the U373 cell line could be a mutation in the p53 gene in the U373 cell line, which inhibits the function of diferuloylmethane on the p53 signaling pathway. Therefore, further studies and additional experiments are necessary to prove this claim.

5. Conclusion

Curcumin (diferuloylmethane) is an active ingredient in turmeric, which its healing properties have been considered by many scientists, for several years. In this regard, we tried to study the cytotoxic effect of diferuloylmethane on four human glioblastoma cell lines (U373, U251, D54, and T98G). Our results showed that diferuloylmethane has a cytotoxic effect on U251, D54, and T98G cell lines. The effect was high in higher concentrations of diferuloylmethane on U251 and D54 than in U373. The application of curcumin in cancer therapy continues to emerge, as natural curcumin was found to increase the cytotoxic effects of several anticancer drugs and could be used in combination with anticancer therapy and several recent studies support this novel therapeutic approach.

Conflict of interest

None of the authors have any conflict of interest to declare.

Consent for publications

All authors have read and approved the final manuscript for publication.

Availability of data and material

The authors have embedded all data in the manuscript.

Authors’ contributions

I.B. helped in doing and manuscript writing, S.X. helped in manuscript draft writing, M.S.E. helped in reviewing the manuscript, Z.A. helped in study design and doing, S.M.A. helped in data collection, Salah T.J.B. helped in data analysis, manuscript writing, doing, and article drafting.

Funding

No company, institution, or organization paid for the research.

Ethics approval and consent to participate

The authors did not use human or animals in the research.

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