Biotech Studies 33(1), 67-73
http://doi.org/10.38042/biotechstudies.1472022

PROOF
RESEARCH PAPER

BIOTECH STUDIES

Rhein inhibits cell proliferation of glioblastoma multiforme cells by regulating the tgfß and apoptotic signaling pathways

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How to cite:
Çetinkaya, S. (20234). Rhein inhibits cell proliferation of glioblastoma multiforme cells by regulating the tgfß and apoptotic signaling pathways. *Biotech Studies*, *33*(1),67-73. http://doi.org/10.38042/biotechstudies.1472022.

Article History
Received 10 September 2023
Accepted 26 March 2024
First Online 22 April 2024

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Keywords
Apoptosis
Cell viability
Colony formation
Rhein
TGF-ß

Abstract

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) is a plant metabolite found in rhubarbs. It inhibits cell proliferation and stimulates apoptosis in *in vivo* and *in vitro*. However, research into the molecular mechanisms of action is insufficient for recommending it as a therapeutic agent. Therefore, this study aims to investigate the antiproliferative, apoptotic, and antimetastatic effects of rhein by targeting the TGF-β signaling pathway, and apoptotic pathway in glioblastoma cells (U87 GBM). In this study, the XTT assay was utilized to determine cell viability, the colony formation assay to measure cell survival and proliferation, RT-qPCR for the analysis of gene expressions, and ELISA for the detection of proteins. U87 GBM cells were treated with varying concentrations of rhein (5-100 µM) in a time-dependent manner (24, 48 h), after which the percentage of cell viability was calculated. The colony formation assay was performed by treating cells with the IC_{50} dose of rhein. According to the XTT assay, the IC_{50} dose of rhein was determined as 10 µM at 24 h. The ability to form colonies was significantly decreased in the cells of the treatment group. According to the gene expression analysis, rhein increased the mRNA levels of *CASP3*, *-8*, *-9*, *BAX,* and *TGF-ß1* genes, while a notable decrease was observed in the *BCL-2*, *SMAD2*, *SMAD3,* and *TIMP1* genes. In conclusion, it was determined that rhein induces apoptosis via the non-canonical TGF-β pathway.

Introduction

Rhein, scientifically known as 4,5-dihydroxyanthraquinone-2-carboxylic acid, represents a lipophilic anthraquinone compound identified as a metabolite in various plants, including the *Rheum* species (*R. tanguticum, R. officinale, R. palmatum* L.) (Polygonaceae), *Cassia tora* L. (Fabaceae), *Polygonum multiflorum* Thunb., *Aloe barbadensis* Miller (Asphodelaceae) and *P. cuspidatum* (Polygonaceae) (Figure 1) (Zhou et al., 2015). From an ethnobotanical perspective, these plants are traditionally used for treating inflammation, diabetes, bacterial, and helminthic infections (Henamayee et al., 2020). Pharmacologically, several studies have demonstrated its hepatoprotective (Bu et al., 2018), nephroprotective (Meng et al., 2015), anti-inflammatory (Wang et al., 2020), antioxidant (Xu et al., 2017), anticancer (Henamayee et al., 2020), and antimicrobial (Nguyen & Kim, 2020) activities. Because of these bioactivities, its use in the treatment and prevention of various diseases, such as osteoarthritis, hepatic disorders, and cancer, has been extensively researched.

Despite recent significant advances in cancer treatment, the search for therapeutic agents from plant-derived sources continues to be popular due to drug resistance and lower side effects (Atanasov et al., 2015). In this regard, chemotherapeutic drugs such as paclitaxel (taxol), vincristine, vinblastine, and docetaxel are effective drugs that are still used clinically (Habtemariam & Lentini, 2018). Rhein has been found to suppress the growth and proliferation of diverse

cancers, such as breast cancer (Chang et al., 2012), pancreatic cancer (Yang et al., 2019), hepatocellular carcinoma (Wang et al., 2020), colon cancer (Zhang et al., 2021), and lung cancer (Yang et al., 2019). These studies have determined that rhein can modulate different signaling steps in its molecular action mechanisms, thereby stimulating cell apoptosis and suppressing invasion and metastasis. Considering the current information, studies on the potential of rhein to be a therapeutic agent against cancer are intriguing. However, studies that elucidate its mechanism of action at the cellular and molecular levels are insufficient to recommend it as an effective therapeutic agent.

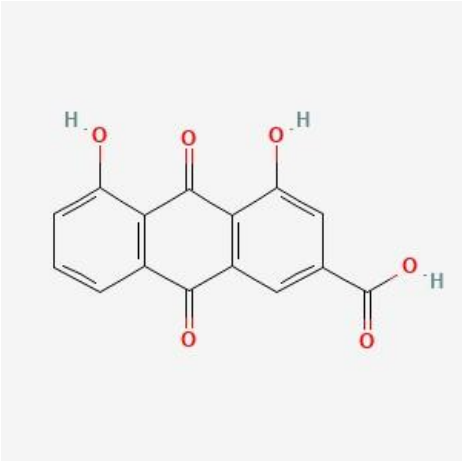

Figure 1. Chemical structure of the rhein (http://www.chemspider.com/Chemical-Structure.9762.html).

In recent times, within the context of discovering potential therapeutic agents, the transforming growth factor-β (TGF-β) signaling pathway has emerged as a major focus due to its key roles in diseases such as pancreatic cancer and its dual functionality (Tewari et al., 2022). This pathway is intricately linked to apoptotic processes, serving as a critical mediator in both promoting and inhibiting cell death, depending on the cellular context (Ramesh et al., 2009). The TGF-β pathway, through its complex interactions with downstream molecules, can trigger apoptosis by influencing the expression of genes directly involved in the cell death mechanism (Sánchez-Capelo, 2005). In light of this, the current study seeks to delve into the antiproliferative, apoptotic, and antimetastatic effects of rhein on glioblastoma cells (U87 GBM) by specifically targeting the TGF-β signaling and apoptotic pathways. Toward this goal, an extensive analysis has been conducted on the effects of rhein against U87 GBM cells, focusing on cell survival, proliferation, apoptotic, and metastatic effects regulated through the TGF-β pathway. For the gene expression changes, the expressions of *TGF-β1, SMAD1, SMAD2,* and *TIMP1*, which are associated with the TGF-β pathways, as well as apoptotic-related genes *CASP3, CASP8, CASP9, BAX,* and *BCL-2*, have been examined. This provides an understanding of how TGF-β signaling can direct cellular fate towards apoptosis. The ability of cancer cells to form colonies is considered a significant indicator of their proliferation and metastatic potential, reflecting the progression of the disease and the capacity to develop resistance to treatment. Inhibiting colony formation can prevent the spread of cancer cells and the growth of tumors, thereby enhancing the effectiveness of therapeutic strategies and aiding in the control of the disease. In this context, the colony-forming capacity of U87 GBM cells and the effect of rhein on this capacity have been investigated.

Materials and Methods

Cell culture and treatment

The U87 GBM cell line was obtained from the American Type Culture Collection (ATCC), located in Virginia, USA. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM-F12) from Sigma-Aldrich, USA, which was enriched with 10% fetal bovine serum (FBS) from Capricorn, Germany, and 100 U/ml of penicillin-streptomycin (10 mg/mL) from Capricorn Scientific, Ebsdorfergrund, Germany, all maintained at a temperature of 37°C and a CO_2 level of 5%. Rhein was acquired from Sigma-Aldrich (R7269 Merck; Germany) and dissolved with 0.1% DMSO at room temperature to make a stock solution. The solution was then stored at -20°C until it was needed for use.

Cell viability assay

The XTT cell proliferation assay was employed to determine the cytotoxic effect of rhein on U87 cells, according to the manufacturer's instructions (Biological Industries, 20-300-1000). U87 cells were seeded ($2x10^3$ cells/well) into 96-well plates. U87 cells were distributed into 96-well plates at a density of $2x10^3$ cells per well. After a 24 h incubation period, the cells were exposed to various concentrations of rhein, ranging from 5-10-15-20-30-40-50-75-100 µM, and incubated for additional periods of 24 and 48 h. The XTT solution was added to each of the wells and the plates were incubated for 4 h. Following incubation, the absorbance of the samples was measured using an ELISA microplate reader (BioTek, Epoch) at a wavelength of 450 nm, with 630 nm serving as the reference absorbance. To evaluate the cytotoxic efficacy of the rhein, IC_{50} values of the samples were calculated.

Colony formation assay

The method commonly known as "colony formation" is widely employed for examining the survival and proliferation capabilities of cancerous cells. In this study, the colony formation assay was carried out to evaluate the colony forming capacity of rhein on U87 GBM cells. Cells were seeded in 6-well plates at a density of $2x10^3$ cells per well and then incubated for 24 h. After the incubation period, the cells were treated with rhein and then subcultured every two days. The media were washed with PBS at the end of day 10 and fixed with 100% methanol at -20 °C. Then, the colony numbers of the control and dose groups were determined by

staining with 1.0% crystal violet for 10 min and photographed with an inverted microscope. Colony forming capacity was calculated according to colony forming numbers in each group (Güçlü et al., 2022).

Total RNA extraction, cDNA synthesis, and RT-qPCR

The expression changes of the apoptosis and TGF-β signal-related genes were evaluated using real time quantitative polymerase chain reaction (RT-qPCR) analysis. U87 GBM cells were seeded in 6-well plates at a density of $2.5x10^4$ cells per well and then incubated for 24 h at 37°C in an atmosphere containing 5% CO^2. Subsequently, cells were treated with the IC_{50} dose of rhein, and then total RNA isolation was performed with RiboEx reagent (GeneAll, 301–001). Each of the RNA sample concentrations and quality were measured using a nanodrop spectrophotometer (Thermo Scientific, USA). The DNase I enzyme (Thermo Scientific, USA) was used to avoid possible DNA contamination. Then, the purified RNAs were reversed into cDNA via the iScriptTM cDNA Synthesis Kit (Bio-Rad, 170–8891). To quantitatively assess mRNA expression levels, the BrightGreen 2x qPCR MasterMix (abm, Canada) was utilized as per the instructions provided by the manufacturer. The expression levels of *CASP3*, *CASP8*, *CASP9*, *BAX,* and *BCL-2* genes in the apoptosis pathway and *TGF-β1*, *SMAD2*, *SMAD3,* and *TIMP1* genes in the TGF-β pathway were assessed using SYBR in RT-qPCR analysis, conducted on an Applied Biosystems (Foster City, California, USA) instrument. The primer sequences for the studied genes were sourced from IDT PrimerQuest (https://eu.idtdna.com/Primerquest/Home/Index). The oligonucleotide sequences utilized in the RT-qPCR reactions are listed in Table 1. The conditions for the RT-qPCR were set at 95°C for 4 min, followed by 40 amplification cycles, each consisting of 95°C for 10 s, 60°C for 60 s, and 72°C for 4 min.

Table 1. Primer sequences of the selected genes used in RT-qPCR.

GENE NAME	PRİMER SEQUENCES
GAPDH	F: 5-GTCAACGGATTTGGTCGTATTG-3
	R: 5-TGTAGTTGAGGTCAATGAAGGG-3
CASP3	F:5-GAGCCATGGTGAAGAAGGAATA-3
	R:5-TCAATGCCACAGTCCAGTTC-3
CASP8	F:5-GCCCAAACTTCACAGCATTAG-3
	R:5-GTGGTCCATGAGTTGGTAGATT-3
CASP9	F:5-CGACCTGACTGCCAAGAAA-3
	R:5-CATCCATCTGTGCCGTAGAC-3
BAX	F:5-GGAGCTGCAGAGGATGATTG-3
	R:5-GGCCTTGAGCACCAGTTT-3
BCL-2	F:5-GTGGATGACTGAGTACCTGAAC-3
	R:5-GAGACAGCCAGGAGAAATCAA-3
TIMP1	F:5-GTCAACCAGACCACCTTATACC-3
	R:5-TATCCGCAGACACTCTCCA-3
SMAD2	F: 5-GGGACTGAGTACACCAAATACG-3
	R: 5-TACCTGGAGACGACCATCAA-3
SMAD3	F: 5-CCTGAGTGAAGATGGAGAAACC-3
	R: 5-GGCTGCAGGTCCAAGTTATTA-3
TGF-B1	F: 5-CGTGGAGCTGTACCAGAAATAC-3
	R: 5-CTAAGGCGAAAGCCCTCAAT-3

Caspase-3 and caspase-9 activation analysis

Apoptosis was assessed following the manufacturer's guidelines by using a caspase-3 and caspase-9 colorimetric assay kit from BioVision, CA, USA. The assay identifies DNA fragmentation in the cytoplasm of apoptotic cells. To detect apoptosis, U87 cells were distributed into 96-well plates at a density of $5x10^5$ cells per well and incubated for 24 h. At the end of the incubation, the cells were treated with inhibitory concentrations of rhein. The cells were then collected and combined with 50 µL of lysis buffer, followed by a 10-min incubation on ice. Subsequently, 50 µL of 2X reaction buffer was added to each cytoplasmic fraction. Lastly, 5 µL of caspase-3 substrate Asp-Glu-Val-Asp (DEVD)-p-nitroaniline (pNA) and caspase-9 substrate Ac-Leu-Glu-His-Asp (LEHD)-pNA were incorporated into the protein cell lysate of each well. Incubation at 37˚C for a duration of 2 h was carried out for all samples. Following this incubation period, absorbance readings were taken at 405 nm using a microplate reader (Bio Rad Laboratories, CA, USA). The alteration in caspase-3 and caspase-9 activity was calculated by dividing the measurements from the samples treated with rhein by those from the untreated control samples.

Statistical analysis

All findings were expressed as the mean ± standard deviation (SD). The GraphPad Prism software (version 10.0.2, GraphPad Software, La Jolla, CA) was employed to conduct a comparative analysis between the control and treatment groups using Student's t-test and one-way ANOVA test.

Results

Rhein inhibits the cell viability on U87 GBM cells. The concentrations and time periods of rhein on U87 GBM cells were evaluated using the XTT assay. Figure 2 demonstrates that administering 5-100 µM of rhein to the U87 GBM cell line for 24 and 48 h resulted in a dose- and time-dependent reduction in cell viability. The XTT assay showed that rhein treatment for 24 h (IC_{50} 10 µM) resulted in significant cell viability against the control cells. According to this result, the concentration of 10 µM was chosen as an effective dose in the subsequent analysis (Figure 2).

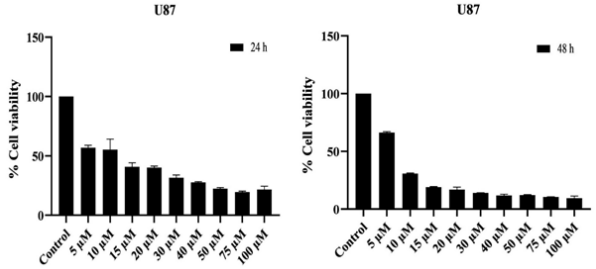

Figure 2. The cytotoxic effect of rhein on U87 GBM cell line. The cells were treated with control and rhein (5-10-15-20-30-40-50-60-75-100 µM) for 24 and 48 h. The XTT cell proliferation assay was used for the detection of IC_{50} values. The dose and control groups were subjected to least three independent experiments.

Rhein supressed the colony formation in U87 GBM cells. The colony analysis results showed that the IC_{50} dose of rhein significantly suppressed the colony formation capacities of U87 GBM cells after the treatment. The colony numbers were 582 ± 15.56 for the control group and 151±10.08 for the rhein-treated group (***p<0.001) (Figure 3).

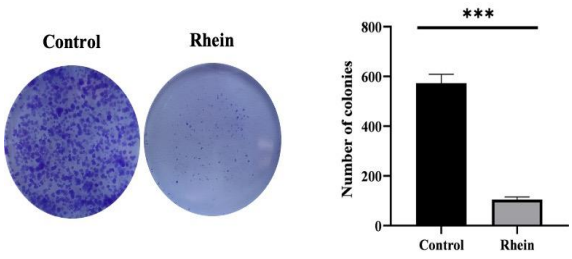

Figure 3. Rhein inhibits cell viability and colony formation of U87 GBM cells. The figure shows the colony formation of the U87 GBM cells treated with rhein for 48 h. The untreated cells were used as a control. The effect of rhein on colony formation is presented by comparing it with the control value. The results are presented as the mean ± standard deviation (std), with a sample size of 5 (n=5), and a significance level of ****P*<0.001.

Rhein promoted apoptosis through TGF-β mediated pathway. The impact of rhein on cell death in U87 GBM cells was assessed using RT-qPCR analysis. After the treatment of rhein, the relative expression levels of apoptosis-related genes (*CASP3*, *CASP8*, *CASP9*, *BAX,* and *BCL-2*) were analyzed using RT-qPCR. In the gene expression results, the expression levels of *CASP3* (12.55 ± 0.8, p=0.00073), *CASP8* (2.94 ± 0.68, p=0.0016), *CASP9* (3.48 ± 0.75, p=0.00024), *BAX* (5.39 ± 0.55, p=0.00042), and *TGF-β1* (4.44 ± 0.65, p=0.00018) genes were significantly increased after the rhein treatment. In addition, *BCL-2* (-1.54 ± 0.46, 0.00031), *SMAD2* (-2.1 ± 0.7, p=0.0165), *SMAD3* (-1.04 ± 0.51, p=0.033), and *TIMP1* (-1.17 ± 0.6, p= 0.0037) genes were significantly decreased after the rhein treatment (Figure 4).

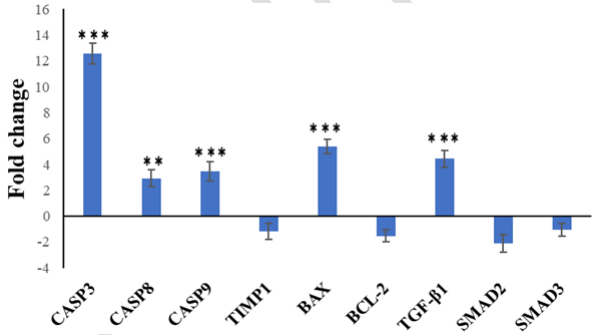

Figure 4. The bar graph presents the fold changes in the expressions of the selected genes, indicating the means of the significant fold changes compared to the control (**P*<0.05, *** *P*<0.001).

Rhein modulates caspase-3 and caspase-9 activity in U87 GBM cells. To investigate the involvement of caspase-3 and caspase-9 in the apoptosis induced by rhein, the enzymatic activities of effector caspase (caspase-3) and initiator caspase (caspase-9) were examined. The findings revealed an increase in the activities of caspase-3 and caspase-9 following treatment with rhein. Specifically, the activities of caspase-3 and caspase-9 increased ~2 fold and ~2.5 fold, respectively, at a concentration of 10 µM compared to the control (Figure 5).

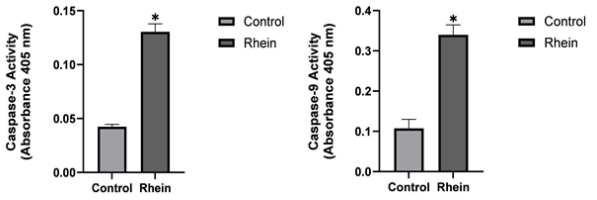

Figure 5. The activity of caspase-3 and caspase-9 after the rhein treatment. The colorimetric ELISA assay was employed to measure the activities of caspase-3 and caspase-9. These activities were then normalized to control cells and represented as a fold change. Consequently, there was an elevation in the activities of caspase-3 and caspase-9 following administration of rhein when compared to the control. Data are presented as mean ± standard deviation (std) with a sample size of 3 (n=3) and a significance level of **P*<0.05.

Discussion

Rhein, an anthraquinone metabolite common in *Rheum* species, is an important metabolite used in pathological conditions such as inflammation, diabetes, osteoarthritis, and bacterial infections (Moldovan et al., 2000; Hu et al., 2019). Recent evidence has proven that rhein exerts potent antitumor effects in different cancer cell lines (Yang et al., 2019; Chen et al., 2020 ; Wei et al., 2022). The current research was investigated antiproliferative, apoptotic and antimetastatic effects of rhein in the U87 GBM cell line. First, the results of this study showed that rhein suppressed U87 cell proliferation in time- and dose-dependent manner. The IC_{50} dose that half of the maximal inhibitory concentration value was detected as 10 µM at 24 h (Figure 2). In the literature, there are limited studies about the antiproliferative effect on U87 cells of rhein. There are only two studies investigating the antiproliferative activity of rhein on U87 cell lines. One of these studies determined the IC_{50} dose of rhein as 40 µM in 72 h in glioblastoma cell lines (T98G, U87, and U251) (Chen et al., 2020). The other study detected the IC_{50} dose of rhein lysinate (the salt of rhein) by MTT assay as 160 µmol/L at 48 h. In this study, the detection of lower doses and times in higher cytotoxic activity may be due to the fact that the XTT assay is more sensitive than the MTT assay. In addition, they determined a high cytotoxic effect of rhein-piperazine-dithiocarbamate hybrids 3 synthesized from rhein against A549, PC-9 and H460 cell lines at low dose (IC_{50}= 10.81-23.78 µg/mL) (Wei et al., 2022). This finding, which is similar to the presented study, confirms the cytotoxic activity of rhein in U87 GBM cells (Figure 2).

TGF-β plays a pivotal role in a myriad of cellular processes, including cell proliferation, migration, apoptosis, embryogenesis, and tissue homeostasis, serving as a double-edged sword in the context of

cancer development and progression (Hata & Chen, 2016). The complexity of the TGF-β signaling pathway, regulated by its ligands, type 1 and type 2 receptors, and Smad proteins, unfolds through both SMAD-dependent and SMAD-independent mechanisms. Upon ligand binding, SMAD 2 and 3 undergo phosphorylation, forming heteromeric complexes with SMAD 4 that translocate to the nucleus to modulate the expression of target genes (Yu & Stamenkovic, 2000). In pancreatic cancer, TGF-β's role oscillates between tumor suppression in the early stages to tumor promotion in the advanced stages, largely influenced by the tumor stage and microenvironment (Yang et al., 2021). This duality extends to its ability to induce apoptosis in various cell types, including prostate cells, hepatocytes, and B lymphocytes, showcasing the pathway's intricate involvement in cancer biology (Shen et al., 2017; Yang et al., 2021). In the present study, upon administering a toxic dose of rhein to U87 GBM cells, a notable upregulation of the *TGF-β1* gene expression by 4.44 fold was observed, signifying an activation or enhancement of the TGF-β signaling pathway. In the pathogenesis of glioblastoma, increased expression of TGF-β1 can modify the cellular microenvironment to support tumor progression or trigger the apoptotic pathway to promote the death of tumor cells. The increase in *TGF-β1* gene expression by rhein suggests a mechanism by which this molecule activates tumor-suppressive properties, thereby encouraging the death of glioblastoma cells. Given the lack of research specifically addressing rhein's efficacy against cancer cells in relation to the TGF-β pathway, our findings have been compared with existing studies to provide context. Zhu and colleagues' study revealed that rhein dose-dependently inhibits the mRNA expression and protein production of plasminogen activator inhibitor-1 (PAI-1) in endothelial cells induced by TGF-β1 (Zhu et al., 2003). When compared to the findings of this study, it is possible to suggest that rhein can modulate the TGF-β signaling pathway in both normal and cancerous cells, and its effects on this pathway may vary depending on the cell type. Furthermore, the research of Guo and colleagues demonstrated that rhein inhibited cell hypertrophy and extracellular matrix **(**ECM) accumulation mediated by TGF-β1, suggesting a renoprotective effect of rhein, possibly through inhibiting the overexpression of TGF-β1 (Guo et al., 2001). This evidence, alongside our findings, suggests that rhein's ability to modulate the TGF-β pathway extends beyond cancer cells to include protective effects in renal tissues, highlighting the compound's broad therapeutic potential. Our results, showing rhein's modulation of TGF-β pathway components in cancer cells, complement Guo et al.'s observations by illustrating the versatile impact of rhein across different cell types and pathological conditions. Besides, the expressions of *SMAD2*, *SMAD3*, and *TIMP1* genes were downregulated, with respective fold changes of -2.1, -1.04, and -1.17. The observed decrease in the expression of *SMAD2* and *SMAD3* under rhein treatment may indicate the promotion of cell death through non-canonical mechanisms of the TGF-β signaling pathway. The decrease in *SMAD2* and *SMAD3* expressions could be mitigating the pro-tumorigenic effects of TGF-β in the later stages of cancer. This mechanism, consistent with the cytotoxic effects of rhein observed in U87 GBM cells, could contribute to the suppression of tumor growth and metastasis via the TGF-β pathway. This situation suggests that targeting the TGF-β pathway could be a potential approach in the treatment of cancer types such as glioblastoma. Moreover, the decrease in *TIMP1* gene expression might have significant effects on the remodeling of the ECM and tumor invasion (Rojiani et al., 2015). TIMP1 functions as an inhibitor of matrix metalloproteinases (MMPs), preventing tumor cells from crossing the ECM. However, the reduced expression of TIMP1 could promote the remodeling of the ECM and potentially make tumor cells less invasive. This could contribute to the antimetastatic properties of rhein and aid in suppressing the progression of glioblastoma.

Apoptosis can be induced by mitochondria-mediated and death receptor-mediated pathways. These pathways lead to the activation of effector caspases such as caspase-3 and caspase-8. In terms of the apoptotic mechanism, TGF-β increases the expression levels of antiapoptotic protein BCL-2 and proapoptotic caspase-3 and caspase-8 (Yang et al., 2019). A study investigating the apoptotic effect of rhein in HepaRG cells showed that levels of BAX, cleaved caspase-3, -8, -9, and PARP increased while BCL-2 decreased (You et al., 2018). It has also been reported that rhein induces mitochondrial apoptosis in a caspase-dependent manner in PANC-1 and MIAPaCa-2 cell lines, characterized by the downregulation of *BCL-2*, *BCL-xL*, *survivin,* and *XIAP*, and upregulation of cleaved caspase-3, -9 and PARP (Liu et al., 2022). Moreover, Tang et al. (2017) showed that rhein triggers apoptotic and autophagic mechanisms, correlating with changes in the expression of *CASP3*, *BAX*, *Beclin-1,* and *BCL-2* genes in rat F98 glioma cells. In the present study, treatment with rhein caused an increase in the mRNA expression levels of *CASP3* (12.55 fold), *CASP8* (2.94 fold), *CASP9* (3.48), and *BAX* (5.39) in U87 cells. However, with a decrease in *BCL-2* (-1.54 fold) mRNA level, rhein triggered the induction of mitochondria-mediated apoptosis. Furthermore, a significant increase in the concentrations of caspase-3 and caspase-9 proteins compared to the control provides confirmatory evidence for gene expression analysis. In conclusion, the ability of rhein to induce apoptosis could be considered a potential therapeutic strategy in the treatment of aggressive cancer types such as glioblastoma. This highlights the development of new approaches in cancer therapy by targeting apoptotic pathways as well as modulating the tumor microenvironment.

There are no existing findings in the literature regarding the effect of rhein on the colony-forming

capacity of glioblastoma cells. However, it has been reported that rhein inhibits colony formation in colorectal (Zhuang et al., 2019; Zhang et al., 2021), and lung (Yang et al., 2019; Liu et al., 2022) cells. In a study conducted on human NSCLC cell lines, it was demonstrated that rhein inhibits the STAT3 signaling pathway and increases the expression level of the proapoptotic protein *BAX* while decreasing the expression level of the antiapoptotic protein *BCL-2*. Furthermore, a colony formation assay similarly confirmed that rhein promotes apoptosis in human NSCLC cell lines, thereby inhibiting growth and proliferation (Yang et al., 2019). In another study assessing the anticancer activity of rhein against colorectal cancer (CRC) cells, cell viability and anchorage-independent colony formation assays showed that rhein inhibits the mTOR signaling pathway, demonstrating anticancer activity against CRC (Zhang et al., 2021). Similarly, a study by Liu and colleagues (2022) showed that rhein suppresses the proliferation and migration of lung cancer cells via the Stat3/Snail/MMP2/MMP9 signaling pathway. Lastly, a study conducted by Zhang and colleagues revealed that rhein inhibits the AKT/mTOR signaling pathway in oral cancer cells, inducing the accumulation of reactive oxygen species (ROS) and cell apoptosis (Zhang et al., 2023). In the current research, the findings from the colony analysis indicated that the ability of U87 cells to form colonies was significantly reduced following treatment with rhein. The colony formation ability and cell viability assay collectively suggest the potential antiproliferative activity of rhein on U87 cells. These findings show an antimetastatic property by causing an increase in *TGF-β1* level with a decrease in rhein *TIMP1* level, stimulating mitochondria-mediated apoptosis, and supporting apoptosis mediated by SMAD-independent pathway.

In conclusion, considering all these data, the fact that rhein exhibits antiproliferative, apoptotic, and cytotoxic activities by modulating TGF-β and apoptosis pathways makes it valuable for detailed studies regarding its clinical use.

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