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Angiogenesis inhibition on glioblastoma multiforme cell lines (U-87 MG and T98G) by AT-101

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ABSTRACT

Aim: Glioblastoma multiforme (GBM) is the most common glial tumor. Angiogenesis which provides nutrient and oxygen support to proliferating cells play an essential role in GBM development, proliferation and metastasis. The development of antiangiogenetic agents is a promising treatment issue as blood vessels are essential for the vitality of tumor cells. AT-101 is -(−) enantiomer of gossypol having anti-proliferative effects on different cancer cell lines. In this study, the cytotoxicity effect of AT-101 on GBM cell lines (U-87MG ve T98G) was confirmed and also the impact of this drug on VEGF synthesis on these cell lines were evaluated at transcriptional and protein levels.
Materials and methods: U-87 MG and T98 cell lines were treated with increasing concentrations (1 −50 μM) of AT-101 for 72 h. Cytotoxicity was determined by xCELLigence (Roche). Apoptosis was detected by using ELISA and confirmed by caspase assay. VEGF and other proangiogenic factors expression were evaluated by angiogenesis array.
Results: The cell vitality was constantly followed up in the experiment. The cytotoxicity of AT-101 in both cell lines increased significantly at 24 h. The IC-50 values of U-87 MG and T98 were 2.4 and 2.7 μM respectively. The VEGF mRNA levels decreased by 2.7 fold in U-87MG and 3 fold in T98G by treating these cell lines with AT-101 IC50 dose for 24 h.
Conclusions: This study showed that AT-101 suppress VEGF on protein levels at U-87 MG and T98G cell lines and showed AT-101 might be a promising targeted agent in GBM treatment.

1. Introduction

Primary glioblastoma (GBM) is the most common and mortal type of brain cancers. GBM compose 15.1% of all brain and central nervous system (CNS) tumors, incidence increases with age, the median age at diagnosis is approximately 65, with rates highest in 75–84 years. Relative survival estimates are low; 5.1% of patients survived five years postdiagnosis.[1]

The mainstay of treatment remains maximal removal of the neoplastic tissue followed by radiotherapy with concurrent temozolomide and adjuvant temozolomide for 6 months.[2–4] Despite the use of a combined-modality approach, most patients eventually relapse. Treatment decisions must be individualized at this situation, select cases may benefit from second surgery and radiotherapy.[5–7] Nitrosureas, temozolomide rechallenge or bevacizumab may be preferred as systemic treatment options, however, any survival advantage has been shown with these drugs and should be considered as palliative treatments.[8–11]

GBM is the most vascularized glial tumors and VEGF lead the major role in angiogenesis. Targeting angiogenesis seems to be the most ideal therapeutic strategy, however, bevacizumab, a monoclonal antibody against VEGF has not shown survival advantage as single and combination treatments in recurrent GBM patients.[9,12] The intrinsic apoptotic pathway is modulated by BCL-2 family members which have proapoptotic (BAX, BAK) and antiapoptotic features (BCL-2, BCL-XL).[13] In a recurrent GBM patient group,

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upregulation of BCL-2 ve BCL-XL proteins and downregulation of BAX was shown in tumor samples.[14] The antiapoptotic protein BCL-2L12 was found to be expressed in all human primary GBMs independent of previous treatment history and inhibits effector caspase activation. In contrast, BCL-2L12 expression was found to be undetectable in low-grade glial tumors and normal brain tissue.[15]

AT-101 (R-(−)-gossypol acetic acid) is a polyphenolic compound originated from cotton species, a potent enantiomer of gossypol.[16,17] It was demonstrated that gossypol has a cytotoxic effect on several cancer cell lines.[18] Gossypol acts as a BH3 mimetic and induces apoptosis through inhibition of Bcl-2 anti-apoptotic family. It also interacts with mitochondrial caspase pathways.[19]

In our study, we aimed to analyse the cytotoxicity of AT-101 on GBM cancer cell lines and to explore if AT-101 has any effect on VEGF synthesis on protein levels.

2. Materials and methods

2.1. Cell culture and reagents

Human U-87 MG and T98G GBM cells were purchased from ATCC (Manassas, VA, USA). The human U-87 MG cells were cultured in Minimum Essential Medium (MEM, Biological Industries, Haemek, Israel) including 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin (Corning Life Sciences, UK). T98 G cells were cultured in Dulbecco's Modified Eagle's Medium-F12 (DMEMF12; Biological Industries, Haemek, Israel) with 10% heat-inactivated FBS, 1% L-glutamine and 1% penicillin-streptomycin. Both cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

AT-101 was obtained from Ascenta Therapeutics, ABD. AT-101 (10 mM) was prepared in DMSO and filtered through a 0.22 μ filter (TPP, Trasadingen, Switzerland). The stock solutions of AT-101 were stored at −20 °C. The DMSO concentration in the assay was not cytotoxic to both cell lines.

2.2. Real-time assessment of cell proliferation by xCELLigence system

The xCELLigence (Roche Applied Science and ACEA Biosciences, Mannheim, Germany) is a Real-Time Cell Analyzer (RTCA) system that monitors the proliferation of adherent cells. It allows label-free and real-time monitoring. Cell proliferation of GBM cell lines was evaluated as described in our previous study.[20] Briefly, cells were seeded in 75 μL (10^4 cells/well) growth medium. E-Plate was incubated at room temperature for 30 m and then moved to incubator for 72 h incubation. Cell proliferation was monitored every 1 h and recorded by the RTCA instrument. After 72 h incubation, cells were exposed to 25 μL medium containing increasing concentrations of AT-101 (1–40 μM). Control cells were treated with medium-only, DMSO-only or medium + DMSO. The proliferation status of cells was recorded every 2 m for 1 h after AT-101 addition. To determine the long-term effects, cell proliferation was also recorded every 30 m from 1 h to 72 h. The IC_{50} values were calculated via RTCA software (Version 1.2) at 16 h which was determined as the most effective time point.

2.3. Evaluation of the apoptotic effect

The apoptotic effect of AT-101 on human GBM cells was determined by Cell Death Detection ELISA Plus Kit (Roche Applied Science, Mannheim, Germany). This kit evaluates the amounts of fragmented DNA of the apoptotic cells. 10^4 cells were plated onto a 96-well plate in 100 μl of culture medium and treated with the increasing concentrations of AT-101 for 16 h. At the end of incubation, samples were lysed and transferred onto a streptavidin-coated plate. Samples were incubated for 2 h at room temperature with a mixture of peroxidase-conjugated anti-DNA and biotin-labeled anti-histone. After washing steps, 2, 29-azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt was added and absorbance was measured at 405 nm with a reference wavelength at 490 nm (Beckman Coulter, DTX 880 Multimode Reader).

To verify the apoptotic effect of AT-101 on GBM cells, caspase 3/7 activity was also evaluated. Cells were treated with the increasing concentrations of AT-101 for 16 h and Caspase-Glo 3/7 Assay (Promega, Madison, WI) was conducted according to the manufacturer's instructions. GBM cells at 10^4 cells/well density were plated in 100 μl culture medium. Cells were treated with AT-101 (1–40 μM) for 16 h. After incubation, 100 μl of Caspase-Glo 3/7 reagent mixture was added to each sample and incubated for 1 h. Finally, the luminescence of each sample was measured with a luminometer (DTX 880 Multimode Reader, Beckman Coulter, Miami, FL, USA).

2.4. Angiogenic cytokine profiling of AT-101 treated GBM cells

The effect of AT-101 on angiogenic cytokines in GBM cells were evaluated using a Proteome Profiler™ Human Angiogenesis Antibody Array (Catalog # ARY007) (R&D Systems, Abingdon, OX, UK). The cells were treated with the IC_{50} values of AT-101 for 16 h. Then, supernatants of all samples were collected. Before treating with supernatants of the samples, the membranes were blocked with blocking buffer for 1 h. Then the arrays were incubated overnight at +4 °C with the sample supernatants. After incubation, a biotinylated antibody followed by horseradish peroxidase-conjugated streptavidin was added. Chemiluminescence detection of signals was done via the Kodak® Gel Logic 1500 imaging system. The spots were quantified using Koadarray® 2.6 software.

2.5. Statistical analysis

For data analysis and graphs, the GraphPad Prism 5.0 software (CA, USA) was used. All experiments were conducted in triplicate and the results expressed as the mean ± SD. *Student's t-test* was used to compare the results between two groups. Two-way ANOVA followed by Bonferroni post-tests was applied to determine significant differences between different treatments in cancer cells ($p < 0.05$ was considered as significant).

3. Results

3.1. Time and dose-dependent anti-proliferative effects of AT-101 on human GBM cells

As shown in Fig. 1A and B, AT-101 decreased the cell proliferation of both U-87 MG and T98 G cells by a time and concentration-dependent manner. The IC_{50} values were calculated at 16 h time point, which were 2.4 and 2.7 μM for U-87 MG and T98 G cells, respectively.

3.2. Apoptotic effects of AT-101 on GBM cell lines

To examine the induction of apoptosis in response to increasing concentrations of AT-101 at 16 h, we quantified the DNA fragments by using Cell Death Detection ELISA Plus Kit. AT-101 induced DNA fragmentation in both U-87 MG and T98 G cells by concentration-dependent manner at 16 h. As shown in Fig. 2A, there were 5%, 25%, 35% increases in DNA fragmentation in 1, 10 and 20 μM of AT-101 exposed U-87 MG cells, respectively, as compared to untreated controls (*$p < 0.05$). In T98 G cells, there were 7%, 46%, 53%

increases in DNA fragmentation in 1, 10 and 20 μM of AT-101 concentrations, respectively, as compared to untreated controls (*p < 0.05) (Fig. 2B).

Induction of apoptosis was also confirmed via evaluating the caspase 3/7 activity. As shown in Fig. 3A, there were 2-, 4-, and 5-fold increases in caspase 3/7 activity in 1, 10 and 20 μM of AT-101 treated U-87 MG cells after 16 h incubation respectively, as compared to the untreated controls (*p < 0.05) (Fig. 3A). There were 5.5-, 8-, 9-fold increases in caspase 3/7 activity in 1, 10 and 20 μM of AT-101 treated T98 G cells after 16 h exposure respectively, as compared to the untreated controls (*p < 0.05) (Fig. 3B).

3.3. *Changes in angiogenic cytokine levels in GBM cells after AT-101 treatment*

Changes in angiogenic cytokine levels of GBM cells after treatment with AT-101 was also investigated. The angiogenic cytokine secretion levels were different in U-87 MG and T98 G cells after treatment with the IC_{50} values of AT-101. There were 2.6-, 2.7- and 2.5- fold decreases in HGF, IGFBP-1 and VEGF levels, respectively in U-87 MG cells after 16 h AT-101 treatment. In T98 G cells, there were 8.0-, 3.7-, 3.2- and 5.0-fold decreases in Coagulation Factor III, Serpin E1, VEGF and uPA levels, respectively, as compared to untreated control cells (Table 1).

4. Discussion

GBM is the most common and worst prognostic subset of brain tumors. The genetic alterations beneath the disease carry heterogeneity.[21] The most common genetic alterations are EGFR mutation and amplification, PTEN loss, RB1 gene deletion and mutation, NF1 gene mutations. TCGA data has recently identified specific subtypes of patients according to clustered gene mutations with different outcomes.[22] However, the present data do not show a specific target for the disease. One unique feature in this heterogeneity is that these tumors generally are highly vascularized. VEGF-A, the well known proangiogenic factor take the role in new vessel formation which is crucial for the tumor to evolve. VEGF binds to transmembrane receptors of endothelial cells and triggers endothelial cell proliferation, invasion and cell migration and correlates with tumor grade in gliomas.[23,24]

AT-101 by binding the BH3 domain of Bcl-2 family inhibits antiapoptotic features. The results of our study revealed the cytotoxic and antiapoptotic action of the drug on two different GBM cell lines. Other than this, the drug also showed anti-angiogenic effect on GBM. This may be related to Bcl-2 and VEGF molecular interaction. The VEGF release from tumor and endothelial cells increase Bcl-2 expression in microvascular endothelial cells that trigger tumor formation and strengthen angiogenesis. The Bcl-2 also activates NFkB pathway and increase IL-8 expression from proangiogenic cytokines and act as a proangiogenic molecule.[25,26]

After AT-101 exposure in our experiments; VEGF expression reduced to 2-fold and 3.2- fold in U87 MG and T98G cell lines respectively. Endoglin that takes a role in TGF- β signalling pathway and provides the release of angiogenic molecules and VEGF in hypoxic conditions also decreased in both cell lines by AT-101.[27] In a

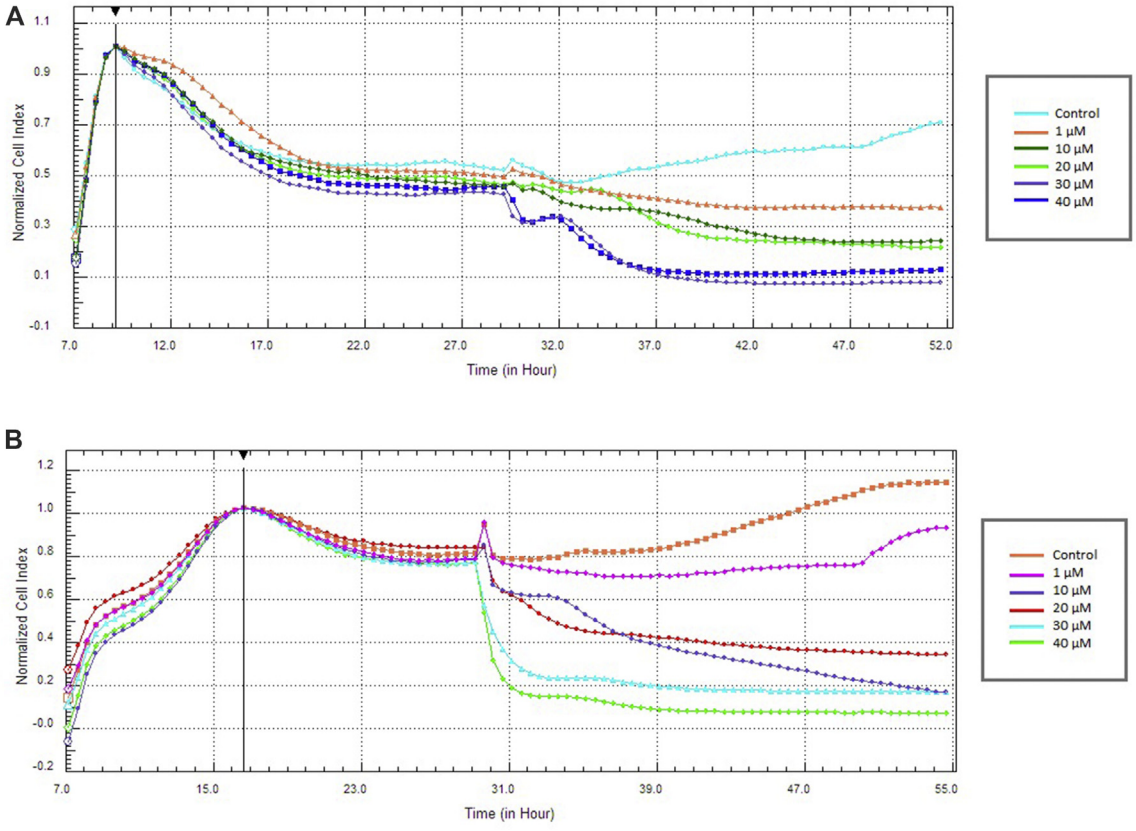

Fig. 1. . a: Dose-response curves of the cell index (CI) of U87 cells exposed to increasing concentrations of AT-101. After seeding 24 h, cells were exposed to 25 μL serum-free medium containing increasing concentrations of AT-101 (1–40 μM). b: Dose-response curves of the cell index (CI) of T98 G cells exposed to increasing concentrations of AT-101. After seeding 24 h, cells were exposed to 25 μL serum free medium containing increasing concentrations of AT-101 (1–40 μM).

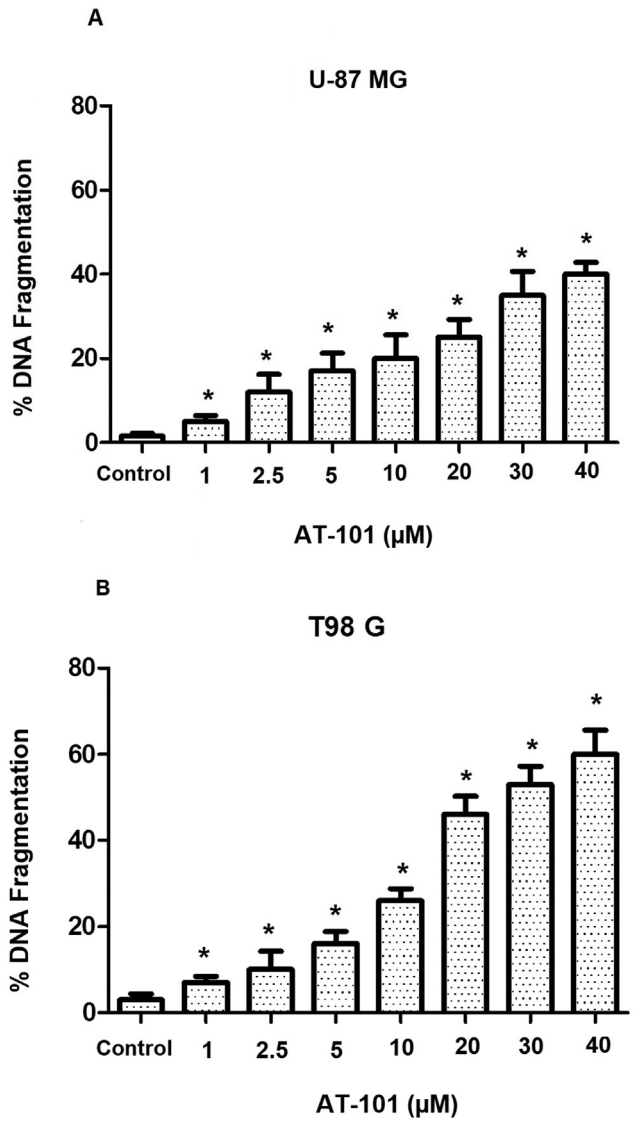

Fig. 2. . a: AT-101 induced DNA fragmentation in U-87 MG cells by concentration-dependent manner at 16 h * $p < 0.05$. b: AT-101 induced DNA fragmentation in T98 G cells by concentration-dependent manner at 16 h *$p < 0.05$.

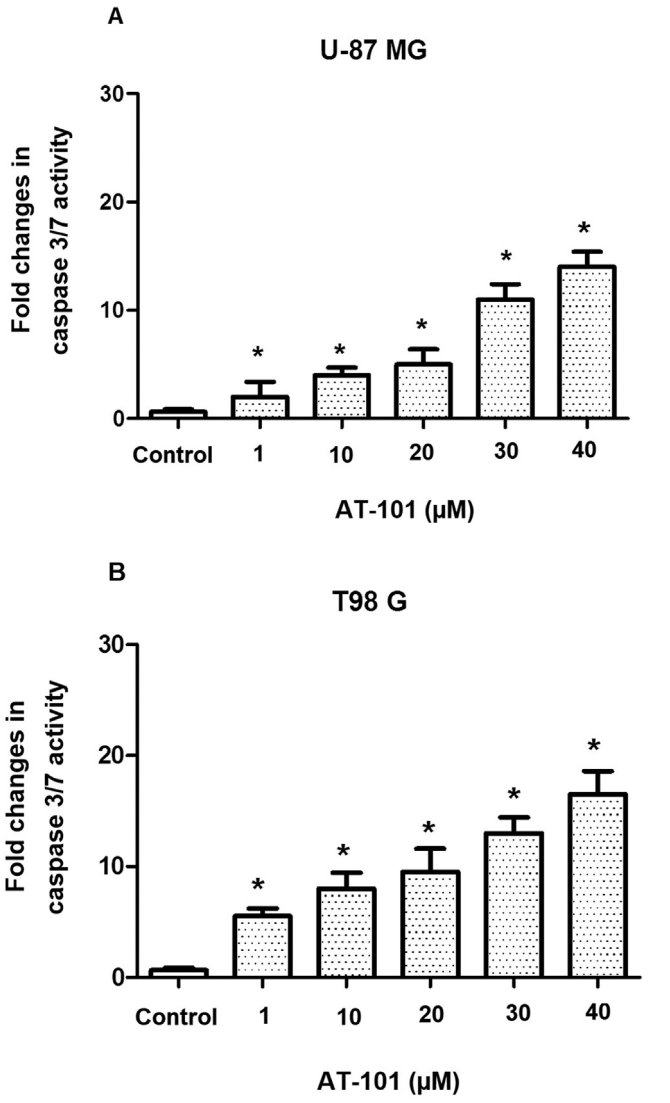

Fig. 3. . a: Caspase 3/7 activity in 1, 10 and 20 μM of AT-101 treated U-87 MG cells after 16 h exposure respectively, as compared to the untreated controls *$p < 0.05$. b: Caspase 3/7 activity in 1, 10 and 20 μM of AT-101 treated T98 G cells after 16 h exposure respectively, as compared to the untreated controls *$p < 0.05$.

recent study, endoglin was shown to be a specific and sensitive marker for evaluation of angiogenesis in GBM.[28]

uPA (urokinase-type plasminogen activator) expression was found to be elevated in GBM and correlates with parenchymal invasion. Binding to its cell surface receptor catalyzes the conversion of plasminogen to plasmin and initiates the proteolytic cascade and breaks down the extracellular matrix.[29,30] uPA levels decreased 2-fold in U87 MG and 5-fold in T98 G cells by AT-101 in our study.

5. Conclusion

AT-101 was shown to decrease VEGF and pro-angiogenetic molecules in GBM cell lines and may be a promising agent for these therapeutic targets. An ongoing phase I clinical trial will clarify the role of gossypol in the adjuvant setting of GBM with radiotherapy and temozolomide combination (NCT00390403). Future studies will clarify the exact role of gossypol in GBM.

Table 1
Changes in angiogenic cytokines after AT-101 treatment.

U-87 MG		T98 G	
Cytokine	Fold change	Cytokine	Fold change
EG-VEGF	−2.0	Coagulation Factor III	−8.0
Endoglin	−2.0	EGF	−2.5
HGF	−2.6	Endoglin	−3.0
IGFBP-1	−2.7	Serpin E1	−3.7
Pentraxin	−2.2	VEGF	−3.2
TIMP-4	−2.0	uPA	−5.0
IL-1 beta	−2.2	IL-8	−3.0
Persephin	−2.0	IGFBP-3	−3.0
uPA	−2.0	Endostatin/Collagen XVIII	−2.0
VEGF	−2.5		

Treatment of GBM cells with the IC_{50} values of AT-101 resulted in different angiogenic cytokine secretion levels in U-87 MG and T98 G cells. Fold decrease of cytokines are shown in the table (EG-VEGF: endocrine gland-derived vascular endothelial growth factor, HGF: hepatocyte growth factor, IGFBP1: insülin-like growth factor 1, uPA: urokinase plasminogen activator EGF: epidermal growth factor).

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