Antiproliferative effect of GTS-21 in glioblastoma cells

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Abstract. Glioblastoma multiforme (GBM) is the most common malignant brain tumour in adults. The poor prognosis and short median overall survival of patients with GBM is associated with resistance to therapy after surgical and adjuvant treatment. The expression of various acetylcholine receptors (AChR) in GBM has been widely reported. The present study aimed to investigate the expression of cholinergic system-related genes in primary GBM and to explore the antiproliferative effect of 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21) in GBM cell lines. Therefore, the expression of 28 genes associated with the cholinergic system was detected using a customized RT^2 Profiler PCR Array in 44 GBM and 5 healthy control brain tissue samples. In addition, the activity of GTS-21, an alpha 7 subunit nicotinic AChR (α7 nAChR) agonist, and that of α-bungarotoxin (α-BTX), an α7 nAChR antagonist, was determined in primary and established GBM cells. Therefore, the A172, U87 and G28 cell lines and primary GBM cells were treated with GTS-21, ACh or nicotine. Cell viability was evaluated using MTT assay at 24, 48 and 72 h following cell treatment with the corresponding compounds. The results revealed that the expression of cholinergic system-related components was notably downregulated, except that of cholinergic receptor nicotinic alpha 7 subunit (*CHRNA7*), in primary GBM and U87 cells. However, the dominant-negative duplicate form of *CHRNA7* was also downregulated. Furthermore, A172 and G28 cells exhibited a heterogeneous gene expression pattern. Additionally, GTS-21 inhibited the proliferation of GBM cells in a dose- and time-dependent manner. Interestingly, treatment with α-BTX restored the proliferation of U87 cells, but not that of A172 and G28 cells. Collectively, the findings of the present study suggested that GTS-21 may inhibit the proliferation of GBM cells and may therefore serve as a novel therapeutic approach to the treatment of GBM, which warrants further investigation.

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Abbreviations: GBM, glioblastoma multiforme; CSC, cancer stem cell; NSC, neural stem cell; Ach, acetylcholine; AChR, acetylcholine receptor; nAChR, nicotine acetylcholine receptor; MGMT, O^6-Methylguanine-DNA-methyltransferase; CHAT, choline acetyltransferase; CHRM, cholinergic receptors muscarinic; GTS-21,(3E)-3-[(2,4-dimethoxyphenyl)methylene]-3,4,5,6-tetrahydro-2,3'-bipyridine; AD, Alzheimer's disease; BChE, butyrylcholinesterase

Key words: glioblastoma, 3-(2,4-dimethoxybenzylidene) anabaseine, acetylcholine receptors, bungarotoxin, nicotine receptors

Introduction

Glioblastoma multiforme (GBM) is the most common type of malignant tumour of the brain in adults (1). There are currently no curative treatments for this tumour entity, thus resulting in a median survival of 14 months (1,2). This could be due to the heterogeneity in terms of genetic and epigenetic alterations (3), which triggers GBM tumourigenesis. Cancer stem cells may be considered as a therapeutic target for the treatment of GBM (4). Furthermore, it has been suggested that the tumour environment can maintain GBM growth, resistance to conventional therapy and immune escape (4,5). Paez-Gonzalez *et al* suggested that neuronal networks could regulate the proliferation and differentiation of normal neural stem cells in the developed brain (6).

Although they have been recognized as crucial components of central and peripheral neurotransmission, acetylcholine (ACh) and its receptors (AChR) are evolutionarily conserved and are also broadly expressed in several other non-neuronal cell types, including bronchial epithelial, glial, pulmonary vascular, ovarian and cancer cells (7,8).

Nicotinic AChRs (nAChRs), as the main regulators of complex inhibitory and stimulatory networks, mediate the production and release of growth, angiogenic and neurogenic factors in cancer cells (7,9). Furthermore, it has been reported that nAChRs regulate the activation of intracellular signalling pathways in a cell type-specific manner (10).

Emerging evidence has suggested that alpha 7 subunit (α7) nAChRs (11) regulate the cholinergic anti-inflammatory pathways (12,13). Among these pathways, a study showed that ACh dose-dependently inhibited the TNF-α and IL-6 pathways in human macrophages (14). α7 nAChRs are characterized

by the presence of the genetically duplicated fusion gene *CHRFAM7A* (15,16). The chimeric gene product, dup α7, interacts with the α7 subunits to form a dominant-negative and dysfunctional receptor. The hybrid, resulting from the fusion of the partial duplication of *CHRNA7* with *FAM7*, *CHRFAM7A*, is expressed in the brain and peripheral tissues (15-18).

The present study aimed to investigate the role of ACh, nicotine, α-bungarotoxin (α-BTX) and 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21) in inhibiting the proliferation of primary GBM cells and GBM cell lines, namely A-172, G-28 and U-87.

Materials and methods

Patient characteristics. The data and samples of the patient sample used in our previous study were reclaimed The samples are collected between 01/2006 and 12/2012 (Departments of Neurosurgery and Neuropathology, University of Giessen, Germany) (19). At the time of diagnosis, the mean age of the 44 patients with GBM was 57.4±15.7 years (f:m=13:31), while the progression-free survival (PFS) time of the patients was 16 months. In the present study, all cases received a gross total resection. The overall survival (OS) of patients with *MGMT* promoter methylation was longer (OS, 23 months; range, 14.8-29.2) compared with those without *MGMT* promoter methylation (OS, 11 months; range, 5.5-16.5) (19). Inclusion criteria was diagnosed GBM, exclusion criteria were missing consent, missing files and Karnofsky-Index < 80. All patients provided written informed consent prior to enrolment in the study (Table I).

Table I. Patient characteristics.

Characteristic	Value
Total cases, n	44
Age at diagnosis, years	57.4
Male, n	31
Female, n	13
Survival methylated, months	22
Survival unmethylated, months	11
Resection, n	41
Missing data, n	3

Chemicals and cell lines. Nicotinehydrogentatrate (cat. no. N3260) and ACh were purchased from Sigma-Aldrich (Merck KGaA). GTS-21 (cat. no. 4557) was obtained from Tocris Bioscience, while α-BTX (cat. no. B1601) was purchased from Thermo Fisher Scientific, Inc. The A172, G28, U87 cell lines were purchased from ATCC. U87 cell line is a glioblastoma of unknown origin (HTB-14).

Tissue specimens from patients with histologically confirmed GBM were immediately collected after surgery. To isolate cells for the primary cell cultures, the tissues were finely chopped in DMEM (Dubelcco's modified Eagle's medium) and trypsinized using an 0.05% EDTA/trypsin solution. The trypsin reaction was stopped by adding 10 ml DMEM and to obtain a single cell suspension, the minced tissues were passed through a 60-μm cell strainer (Sigma-Aldrich; Merck KGaA). Subsequently, cells were washed with PBS (Phosphate buffered saline). Following centrifugation, the cell pellet was resuspended in DMEM and the cells were then seeded into 25 cm^2 culture flasks (Greiner Bio-One GmbH) and cultured in an incubator at 37˚C and 5% CO_2. Then, following the appropriate number of passages, the cells were expanded to 175 cm^2 flasks (Greiner Bio-One GmbH). For cell cryopreservation, the cells were first washed with PBS, centrifugated, resuspended in DMSO (Merck KGaA) and finally stored in liquid nitrogen. Frozen cells were thawed in a 37˚C-water bath and were then cultured as adherent monolayers in 25-cm^2 flasks. The clinicopathological characteristics of patients are listed in Table I.

RNA isolation and cDNA synthesis. For total RNA isolation from frozen specimens, the RNeasy Lipid Tissue Mini Kit® (Qiagen GmbH) was used. To ensure proper tissue dissociation, the specimens were treated with a lysis reagent, provided by the kit, for 2 min in a bead mill at 5,000 rpm. The RNA concentration was measured with NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific Inc.). cDNA synthesis was carried out using 0.5 μg total RNA/sample as a template with the RT^2 First Strand Kit (Qiagen GmbH).

Gene expression analysis. For the simultaneous analysis of 31 genes, including 28 target genes and three housekeeping genes, namely *RPL13A*, *TBB* and *GAPDH*, a Custom RT^2 Profiler PCR Array was used, containing lyophilized primers for each target gene (CAPH12576; cat. no. 330231; Qiagen GmbH). The analysis was carried out on the StepOnePlus quantitative PCR cycler (Thermo Fisher Scientific, Inc.) using the RT^2 SYBR®-Green Mastermix (Qiagen GmbH) according to the manufacturer's recommendations. For the analysis, the qPCR results from all samples were merged and uploaded into the online analysis tool provided by the manufacturer. The online tool calculates the ΔΔCq-based fold change in the expression of each target gene normalized to that of the housekeeping genes.

MTT assay. To evaluate cell survival and proliferation, a MTT assay was performed following treatment of cells with GTS-21 (Tocris Bioscience), ACh or nicotine (both from Sigma-Aldrich; Merck KGaA). Briefly, cells were seeded into 96-well plates at a density of 10^4 cells/well. All cell lines were treated with 6.25, 12.5, 25 or 50 μM GTS-21, ACh or nicotine. The negative control cells for the GTS-21 group were treated with 0.1% DMSO, while those of the ACh and nicotine groups with 0.1% H_2O. The MTT reagent (Sigma-Aldrich; Merck KGaA) was diluted in DMEM at a ratio of 1:10. Following treatment of cells with the corresponding compounds for 24, 48 and 72 h, the culture medium was removed and the cells were supplemented with 200 μl of MTT-DMEM solution and incubated at 37˚C in an atmosphere of 95% air and 5% CO_2 for an additional 90 min. Subsequently, the cells were lysed in 150 μl isopropanol/hydrochloric acid solution (Sigma-Aldrich; Merck KGaA) for 15 min and the absorbance value in each well was measured using a microplate reader at a wavelength of 562 nm, with a reference wavelength of 630 nm.

Statistical analysis. Statistical analysis was implemented using SPSS 20 (IBM Corp.). The comparison of different

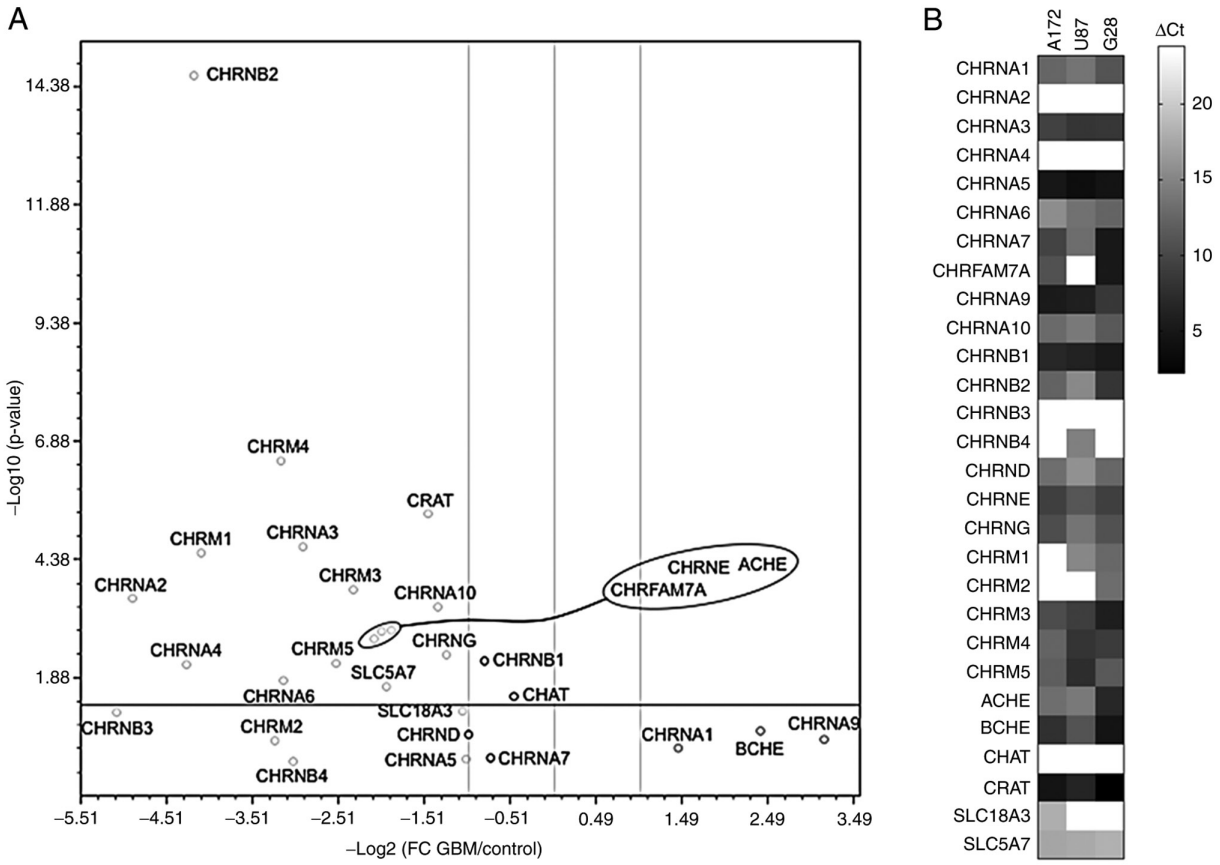

Figure 1. Gene expression of the cholinergic system in primary GBM tissue and cell lines. (A) Volcano plot visualization of the comparison of GBM (n=44) vs. healthy tissue (n=5). The blue horizontal line represents the P-value threshold of 0.01. Vertical lines represent the log2 fold change threshold of -1/1 respectively, with no change indicated by the middle line. (B) Heat map visualization of the normalized ΔΔCq [$Cq_{gene\ of\ interest}$ - mean (Cq_{GAPDH}+Cq_{TBP}+Cq_{RPL13A})] cholinergic system gene expression levels in A172, U87 and G28 cell lines. A lower value of ΔΔCq indicates a high expression and vice versa. GBM, glioblastoma multiforme. Red indicates upregulated genes and green represents downregulated genes.

concentrations of GTS-21 and α-bungarotoxin were performed using Kruksal-Wallis-test (post hoc: Bonferroni). P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± SEM.

Results

Cholinergic system-related gene expression is markedly deregulated in GBM. Using a custom-made qPCR array covering the majority of components of the cholinergic system, the expression levels of these genes were assessed in 44 tissue samples obtained from patients with primary GBM and compared with those in five tissue samples from healthy subjects (Fig. 1A). The results demonstrated that the expression of the majority of genes encoding the receptor subunits was significantly downregulated (fold changes, $\log_2$ >-1; P<0.01). Additionally, the gene expression levels of *ACHE* and those of the dominant-negative form of *CHRNA7* and the duplicate fusion gene product of *CHRFAM7A* were also significantly suppressed. In addition, the expression levels of three genes, namely *CHRNB1*, encoding the nicotinic β1 receptor subunit, *CHAT*, encoding choline acetyltransferase and *CHRNA7*, encoding the nicotinic α7 receptor subunit, remained unchanged.

The expression levels of the cholinergic system-related genes were also determined in the established GBM cell lines A172, U87 and G28 (Fig. 1B). *CHRNA2* was not expressed in any of the three cell lines examined. By contrast, *CHRNA1*, *CHRNA9* and *BCHE* were expressed in all cell lines. The most significant differences in the gene expression profile between primary GBM cells and cell lines were observed in *CHAT* expression, as *CHAT* was not expressed in A172, U87 and G28 cells compared with primary GBM cells. This finding could be attributed to the loss of ACh synthesis. Despite the similarities, the cell lines exhibited quantitative and qualitative heterogeneity in the expression of several genes, including *CHRM2*, which was expressed in G28, but not in A172 and U87 cells and the fusion gene product *CHRFAM7A*, which was not expressed in U87 cells.

Overall, the aforementioned findings revealed significant differences in the expression of cholinergic system-related genes in GBM. More specifically, the expression of *CHRNA7* remained unchanged in all cell lines examined, indicating that these cell lines may serve as important tools for evaluating the role of *CHRNA7* in GBM. Furthermore, the heterogeneity in the expression of *CHRFAM7A* could contribute to a better understanding of the participation of the dominant-negative regulator in the cholinergic system.

GTS-21, but no other cholinergic agonists, inhibits GBM cell proliferation. Subsequently, the present study aimed to investigate the effect of the activation of the α7-composed

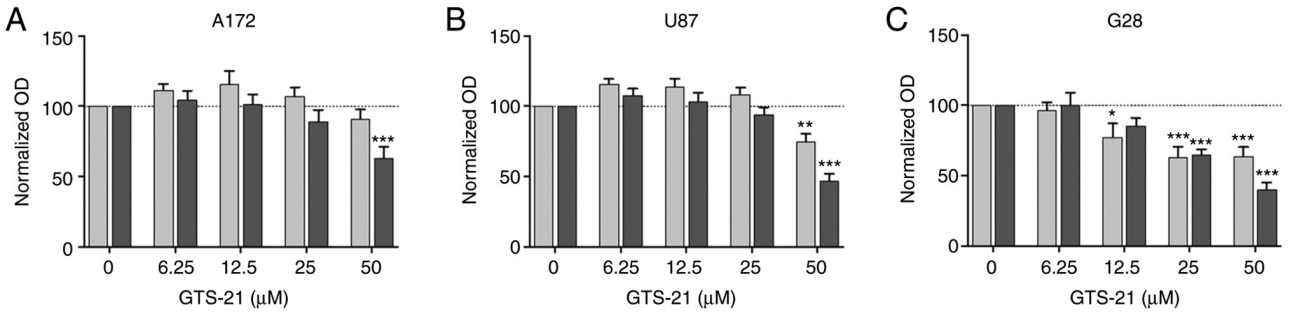

Figure 2. GTS-21 impairs glioblastoma multiforme cell line proliferation in a time- and dose-dependent manner. (A) A172, (B) U87 and (C) G28 cells were incubated with different concentrations of GTS-21 for 24 h (light grey) or 48 h (dark grey). The MTT assay was performed afterwards and individual values were normalized to the DMSO treated control group. The horizontal line depicts the baseline. Data are presented as the mean ± SEM (n=5) *P<0.05, **P<0.01 and ***P<0.001 vs. the DMSO treated group. GTS-21, 3-(2,4-dimethoxybenzylidene) anabaseine; OD, optical density.

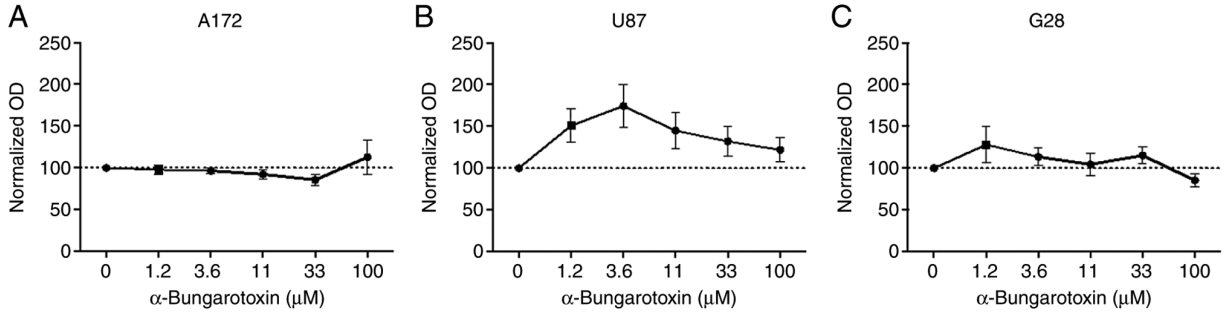

Figure 3. Coincubation with α-bungarotoxin reestablishes the proliferation of GTS-21 treated U87 cells. (A) A172, (B) U87 and (C) G28 cells were incubated with increasing concentrations (1.2-100 μM) of α-bungarotoxin in the presence of GTS-21 (50 μM). An MTT assay was subsequently performed and individual values were normalized to that of the untreated control group. Data are presented as the mean ± SEM (n=4). GTS-21, 3-(2,4-dimethoxybenzylidene) anabaseine; OD, optical density.

nAChR on cellular proliferation. Therefore, the three GBM cell lines A172, U87 and G28 were stimulated with increasing concentrations of GTS-21, a well-known partial agonist of α7 and α4β2 subunits, for 24 and 48 h. Since both α4 and β2 subunits were not expressed in the current cell model, GTS-21 served as a sole α7 agonist. Cell treatment with 50 μM GTS-21 significantly attenuated the proliferation of all cell lines at 48 h (Fig. 2). However, the extent of reduction differed among the different GBM cell lines (A172 cells, 63.3±7.5%; G28 cells, 39.4±5.3%; U87 cells, 47.2±4.8%). Additionally, GTS-21 exerted an antiproliferative effect on G28 cells even after treatment with 12.5 μM GTS-21 for 24 h (Fig. 2C). However, treatment of cells with equimolar concentrations of cholinergic agonists, namely nicotine and ACh, had no effect on cell viability (Fig. S1). In summary, pharmacological stimulation of the α7 receptor subunits with GTS-21 reduced GBM cell proliferation.

α7 subunit affects the antiproliferative capacity of GTS-21. To investigate whether the antiproliferative effect of GTS-21 was associated with the α7 subunit, GBM cells were co-treated with 50 μM GTS-21 and increasing concentrations of α-BTX (1.2-100 μM), an antagonist of the homo-pentameric α7 receptor, for 48 h. The results showed that treatment of A172 and G28 cells with α-BTX had no effect on their proliferation capacity (Fig. 3A and C). However, the proliferation rate of U87 cells treated with 3.6 μM α-BTX was notably increased (174.6±21.71%; Fig. 3B). This finding indicated that the proliferative effects of GTS-21 could be abolished by α-BTX only in U87 cells.

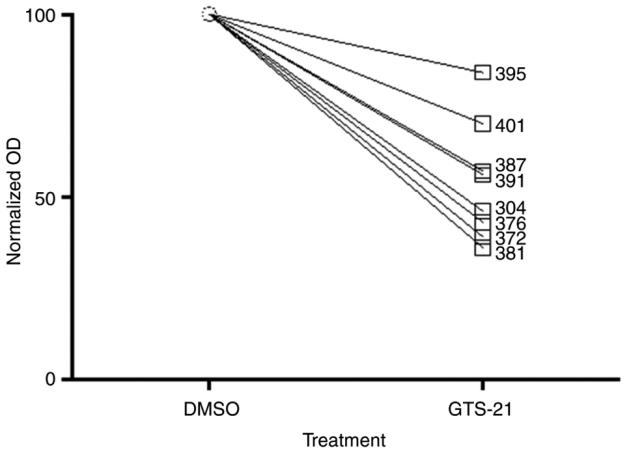

Figure 4. GTS-21 treatment inhibits proliferation of cultured primary GBM cells. A total of 8 primary GBM cell lines (395, 401, 387, 391, 304, 376, 372 and 381) were treated with GTS-21 (50 μM) for 48 h, after which cell proliferation was assessed using an MTT assay. The OD of each sample was normalized to the corresponding DMSO-treated control sample. Data are presented as the mean ± SEM (n=4). GBM, glioblastoma multiforme; GTS-21, 3-(2,4-dimethoxybenzylidene).

GTS-21 inhibits the proliferation of primary GBM cells. A total of eight primary GBM cell cultures were treated with

6.25, 12.5, 25 or 50 μM GTS-21 for 48 h. The proliferation rate of primary tumour cells treated with different doses of GTS-21 was significantly reduced compared with A172, G28 and U87 cells (Fig. 4), suggesting that all primary cells responded to treatment. The median inhibition rate (loss of proliferation) in primary GBM cells was 51%, ranging between 36 and 84%.

Discussion

Normal brain function depends on the presence and interaction between diverse neurotransmitters and neuropeptides. Emerging evidence has demonstrated that neuropeptides and neurotransmitters, as well as their cognate receptors, are expressed by astrocytes (20). Therefore, the ligand-receptor-mediated cellular responsiveness could also be considered as an adjunctive therapy approach to GBM. However, persistent invasiveness and resistance to standard therapy remain the major challenges in the development of more effective treatment options for GBM.

Nevertheless, novel therapies are urgently needed to improve PFS and OS of patients with GBM. Bavo *et al* (21) showed that the activation of nAChR, composed of α7 and α9α10 subunits, could promote GBM cell proliferation. Another study demonstrated that the decreased expression levels of *CHRFAM7A* were associated with the onset of Alzheimer's disease, supporting its role in pathological conditions. By contrast, *CHRFAM7A* was upregulated in schizophrenia and bipolar disorders (11). Furthermore, previous study (17,22) demonstrated that *CHRFAM7A* was a stoichiometric dominant-negative regulator of a7 nAChR. Instead, no mutations have been identified in GBM occurring at the CHRNA7 loci, supporting its normal expression in these cancer cells (23). To the best of our knowledge, the current study was the first to investigate the effect of *CHRNA7*, encoding α7 nAChR, and *CHRFAM7A* on three GBM cell lines, GBM tissues and normal brain tissues (control).

Pucci *et al* (24) reported the effect of α7 nAChR on the proliferation of GBM cells. In the current study, the potential role of GTS-21 as a partial agonist of α7 nAChR was investigated in GBM cell lines. The results revealed that the proliferation ability of all cell lines was significantly attenuated following treatment with GTS-21. However, the optimal concentration for inhibiting the cell proliferation differed among the three cell lines tested, with G28 cells being the most sensitive, since G28 cells showed the highest expression of *CHRFAM7A*. Interestingly, antagonizing the aforementioned strong effect by means of cell treatment with α-BTX, showed that the cell proliferation was only restored in U87 cells, possibly due to a lack of *CHRFAM7A* expression.

Furthermore, the expression of *CHRNA1* and *CHRNA9*, encoding the a1 and a9 subunits of nAChR, and that of *BCHE*, encoding the ACh-degrading enzyme, butyrylcholinesterase, was notably upregulated. This finding confirmed the previously reported findings, showing that GBM cells could express the α7 and α9α10 subunits of nAChRs (21). A previous study also demonstrated that *CHRNA1* and *CHRNA9* upregulation was associated with shorter OS in patients with GBM (10).

The results suggested that the upregulation of α7 nAChR in heterologous cell lines could suppress the cell proliferation. It has been reported that α7 nAChR is upregulated in the GBM vessel endothelium, GBM cells and tumour-associated macrophages, thus suggesting that α7 nAChR could be a potential target for the treatment of GBM (25). However, the effect of GTS-21 was only abrogated in U87 cells, which did not express *CHRFAM7A*, following treatment with α-BTX, suggesting that GTS-21 could act in an α7 nAChR-independent manner.

The limitation of our study is absence of data related to TERT and IDH mutation and very small population.

In conclusion, the present study showed that GBM cell treatment with GTS-21, an agonist of the α7 receptor, attenuated their proliferation. However, treatment with α-BTX restored the proliferation of U87 cells pre-treated with GTS-21. These findings suggested that GTS-21 may be considered as a novel treatment approach to GBM, due to its α7 nAChR-independent mechanism of action. However, further *in vivo* studies are required to elucidate the underlying mechanism of action and potential therapeutic application of GTS-21.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAK designed the current study, wrote and reviewed the data and literature of the manuscript, and analyzed the data. BK collected and analyzed the data. FU performed statistical analysis and reviewed the data and literature of the manuscript. MAW and MKFB analyzed the data. PDF analyzed the data, performed statistical analysis and reviewed the data and literature of the manuscript. FPS and HG designed the current study and reviewed the data and literature of the manuscript. All authors read and approved the final manuscript.MAK and FU confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The current study was approved by the local Ethics Committee Justus-Liebig-University Giessen (application no. AZ 07/09). All research followed the international and national regulations in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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