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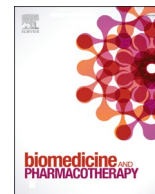
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ABSTRACT

Guanine nucleotide binding protein (G protein) coupled receptor 17 (GPR17) plays crucial role in Glioblastoma multiforme (GBM) cell signaling and is primarily associated with reactive oxidative species (ROS) production and cell death. However, the underlying mechanisms by which GPR17 regulates ROS level and mitochondrial electron transport chain (ETC) complexes are still unknown. Here, we investigate the novel link between the GPR17 receptor and ETC complex I and III in regulating level of intracellular ROS (ROSi) in GBM using pharmacological inhibitors and gene expression profiling. Incubation of 1321N1 GBM cells with ETC I inhibitor and GPR17 agonist decreased the ROS level, while treatment with GPR17 antagonist increased the ROS level. Also, inhibition of ETC III and activation of GPR17 increased the ROS level whereas opposite function was observed with antagonist interaction. The similar functional role was also observed in multiple GBM cells, LN229 and SNB19, where ROS level increased in the presence of Complex III inhibitor. The level of ROS varies in Complex I inhibitor and GPR17 antagonist treatment conditions suggesting that ETC I function differs depending on the GBM cell line. RNAseq analysis revealed that ~ 500 genes were commonly expressed in both SNB19 and LN229, in which 25 genes are involved in ROS pathway. Furthermore, 33 dysregulated genes were observed to be involved in mitochondria function and 36 genes of complex I-V involved in ROS pathway. Further analysis revealed that induction of GPR17 leads to loss of function of NADH dehydrogenase genes involved in ETC I, while cytochrome b and Ubiquinol Cytochrome c Reductase family genes in ETC III. Overall, our findings suggest that mitochondrial ETC III bypass ETC I to increase ROS$_i$ in GPR17 signaling activation in GBM and could provide new opportunities for developing targeted therapy for GBM.

1. Introduction

G-protein-coupled receptors (GPCRs) has emerged as a key player in tumorigenesis, angiogenesis and metastasis. Among several GPCRs, GPR17, a new orphan receptor, has emerged as a potential drug target for many neurological diseases including cancer, multiple sclerosis, Parkinson's disease, Alzheimer's disease, etc. [1]. Specifically, over-expression of GPR17 in glioblastoma (GBM) (Human Protein Atlas: GPR17/pathology), dramatically increases after ischemic conditions.

The GPR17 signalling activation decreases the neurospheres of primary murine GBM cells [2]. GPR17 is also recognised as an important molecular marker for low-grade glioma (LGG), GBM, and for the developmental stages of oligodendrocytes [3]. A better understanding of the role of GPR17 in the molecular basis of GBM formation might provide new avenues for the development of preventive therapies targeting the specific GBM-promoting factors and thereby improve prognosis.

We previously reported that low GPR17 expression is associated with better survival of both LGG and GBM patients. Next-generation

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sequencing data also revealed high level expression of GPR17 in various samples of GBM [4], thus considering it as a notable target for GBM treatment. With the interest in structure-based ligand discovery, we modeled the structure of GPR17 [5]. The model identified novel agonists, AC1MLNKK, T0510.3657 (T0), and CHBC, which selectively activates GPR17 and exhibited better interaction properties than previously known ligand MDL29951[6,7]. Both T0 and CHBC have been identified as a potent GPR17 receptor agonist, causing significant GBM cell death [7]. The elevated level of apoptosis is also linked with intracellular calcium level, change in mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) production [5,7–9]. The correlation between Ca^{2+} and ROS signaling pathways is well established in many pathological conditions [10], including cancer. It is known that Ca^{2+} signaling can influence the cellular ROS, which in turn affects mitochondrial Ca^{2+} and ROS. There is limited data available on the role of GPR17 on modulating ROS and Ca^{2+} signaling. Thus, a deeper understanding of role of GPR17 in redox changes including regulating ROS and mitochondrial function could be an important step towards the individualized treatment of GBM.

ROS are produced during normal or abnormal metabolic processes within the mitochondrial complexes of electron transport chain (ETC) [11,12]. The maintenance of ROS homeostasis is vital for cancer cell survival. Normally, redox systems prevent cell oxidative damage; however in GBM, the cellular redox mechanisms are highly impaired. Many chemotherapeutic agents developed for treating cancer progression are targeted to increase the ROS production and thus induce mitochondrial apoptosis. However, cancer cells develop a ROS-scavenging system to eliminate the over-accumulation of ROS within the cell and mitochondria. Mitochondrial ETC composed of transmembrane protein complexes (CI-CIV) in which, CI and CIII are considered as a major site of ROS production while CII, and CIV are less prone to produce ROS [13]. The combinatorial treatment of low-passage primary patients GBM cell lines overexpressing GPR17, with its ligand and phenolic derivatives induces ROS-mediated apoptosis and thereby inhibits glioma cell proliferation. Although GRP17 activation increases the ROS level in GBM, whether and how mitochondrial complex CI and CIII are involved in regulation of ROS in glioma cells largely remains unknown and unexplored.

We proposed to investigate the role of CI and CIII during the activation of GPR17 receptor in glioblastoma. For this, we used 1321N1 cells derived from U-118MG, a cell line isolated from a human malignant glioma. GPR17 receptor responds to both endogenous purinergic and cysteinyl-leukotriene (CysLT) ligands. The glioma cells, 1321N1 do not express P2Y and CysLT family receptors, thus allowing unbiased evaluation of GPR17 function with no cross-interaction of ligands with other targets [14,15]. The functional role of CI and CIII is assessed by measuring the changes in ROS levels. In this study, we proved that activation of GPR17 signaling upregulated the level of intracellular ROS. We also have shown the changes in ROS level by activating or blocking the GPR17 signal in 1321N1 cells. Upon using ETC CI and CIII inhibitors, we discovered that GPR17 signaling mediates ROS regulated by ETC CIII bypasses CI. Lastly, we demonstrated that in vitro exposure of cultured SNB19 and LN229 cells to GPR17 ligands and CI and CIII blockers regulate ROS through CIII. Collectively, the results offered a model that can be utilized to further understand the role of GPR17 in driving GBM progression via modulating key redox pathways.

2. Materials and methods

2.1. Materials and reagents

Human glioblastoma cell line 1321N1 (astrocytoma, Sigma-Aldrich, St. Louis, MO, cat.no. 86030402) was cultured in Dulbecco's Modified Eagle Medium 4500 mg glucose/L (DMEM; D5796, Sigma-Aldrich, St. Louis, MO, USA) with 10% (v/v) Foetal Bovine Serum (FBS; F1051–100 mL, Sigma-Aldrich, St. Louis, MO), 2 mM sodium pyruvate (S8636,

Sigma-Aldrich, St. Louis, MO, USA) and antibiotic supplements: Penicillin-Streptomycin (100 U/mL) (P4333–100 mL, Sigma-Aldrich, USA) and solubilized Amphotericin B (0.025 mg/mL) (A9528, Sigma-Aldrich, St. Louis, MO, USA). For assay optimization, DMEM with low glucose concentration (1000 mg/L) was used, hereafter referred as low glucose assay medium, whereas 4500 mg/L glucose medium is referred as high glucose assay medium. Cells were maintained in CELLSTAR culture flasks (Greiner Bio-One GmbH, Germany) in incubator at 37 °C in humidified atmosphere of 5% CO_2–95% air. The cell culture was maintained by regular passaging after every 3–4 days utilizing 1X trypsin-EDTA solution (T3924–100 mL, Sigma-Aldrich, St. Louis, MO, USA).

For blocking mitochondrial channels, Antimycin A that blocks ETC channel III (A8674–25MG, Sigma-Aldrich, St. Louis, MO, USA) and Rotenone that blocks ETC channel I (R8875–1 G, Sigma-Aldrich, St. Louis, MO, USA) were used. Dimethyl sulfoxide (DMSO, D2650–5×10ML, Sigma-Aldrich, St. Louis, MO, USA) was used to dilute the compounds·H_2O_2 (H1009, Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control. To activate the hGPR17 known agonist 3-(4,6-dichloro-2-carboxyindol-3-yl)-propionic acid (MDL 29951, Abcam) and antagonist Montelukast Sodium (Abcam) were used. For ROS measurement, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma-Aldrich) was used with required concentrations.

2.2. Confocal microscopy of cellular ROS

The morphology of 1321N1 cells grown in both high and low glucose media was observed with confocal microscope (Nikon-Eclipse Ti-E inverted fluorescence microscope). The cells were cultured in T25-flask with the initial density of 1×10^6 cells and the final density of 2×10^4/well of 96 well was used for measuring H_2DCFDA, which is a ROS sensitive dye. In the cell, the deacetylated H_2DCFDA reacts with ROS which results in the formation of a fluorescent product, 2′, 7′–dichlorofluorescein (DCF) that allows to measure the level of ROS. The cells were incubated for 24 h in both high and low glucose media as described above. Post 24 h of incubation at 37° C, the cells were incubated with 2 μM H_2DCFDA for 45 min at cell culture conditions. Post incubation, cells were centrifuged and resuspended in 400 μL of pre-warmed physiological Phosphate Buffered Saline (PBS), to prevent the interference of phenol red present in high and low glucose DMEM medium with the fluorescent signal of H_2DCFDA [11]. The stained cells were visualized under fluorescent microscopy at 488 nm excitation and 529 nm emission with ND4 ND8 laser. The images were acquired with 40X objective. Acquired images were processed to observe the intracellular (ROSi). All experiments were performed with three biological repeats and technical repeats to acquire statistically significant data.

2.3. Inhibition of mitochondrial CI and CIII and measurement of ROS

The 1321N1 cells were cultured in T25-flask with the initial density of 1×10^6 cells. For treating cells and measurements, the final density of 5×10^4/well of 96 well was used. Cells were incubated overnight in appropriate culture conditions and then treated with the following reagents and blockers with high and low glucose including their controls: H_2O_2 (100 μM), Antimycin (5 μM), Rotenone (150 nM). After 30 mins of incubation, the cells were centrifuged and resuspended in 500 μL complete medium. Cells were stained with 2 μM H_2DCFDA as described above and transferred into 96-well plate. Fluorescence intensity was measured using plate reader (Fluoroskan Ascent FL, ThermoLabsystems) at excitation 485 nm and emission 538 nm.

The fold increase in ROS was calculated by formula (2).

$$Foldincrease = \frac{F_{test} - F_{blank}}{F_{control} - F_{blank}} \tag{2}$$

Where, F_{test} is the fluorescence reading from the treated wells, $F_{control}$ is

the fluorescence readings from the untreated wells, and F_{blank} is the fluorescence readings from the unstained wells.

2.4. hGPR17 expression assay

The GBM cells,1321N1 were seeded in a T-25 flask (Fennokauppa) and transfected at a 70% confluence after 24 h of incubation at 37ºC. In detail, the medium was removed and replaced with fresh DMEM medium (10% FBS and 1% antibiotic) before transfection. After 2 h of incubation, calcium phosphate transfection kit (Sigma-Aldrich) was used for transient transfection of 1321N1 cells. pcDNA3.1-hGPR17 plasmid which is an expression vector for GPR17 was used for transfection. The construction of GPR17 expression vector and the cloning data is already published [16]. For the transfection of 1321N1 cells, 3 μg of pcDNA3.1-hGPR17 plasmid was used (a kind gift from Prof. Dr. Evi-Kostenis, Institute for Pharmaceutical Biology, University of Bonn).

2.5. ROS Measurement at GPR17 agonist and antagonist treatment

Transfected 1321N1 cells were harvested using 1X trypsin-EDTA solution as described before. After trypsinization 5×10^4 density of the cells were used for further treatment (Thermal Fisher Scientific n.d.). Initially, the cells were incubated for 30 mins at 37° C in corresponding conditions: untreated control, Rotenone (150 nM), Antimycin (5 μM), H_2O_2 (100 μM). After incubation, the compounds were removed by centrifugation and resuspended in 500 μL fresh complete medium. hGPR17 agonist MDL 29951 (Abcam), and antagonist Montelukast Sodium with the EC_{50} concentration of 4.9 μM and 4.85 μM, respectively, were added to the resuspended cell and incubated for 2 h at 37 °C in humidified atmosphere. Agonist and antagonist alone treated conditions were also maintained for the comparative analysis of ROS level. H_2DCFDA (2 μM) was added 45 mins before the end of the incubation. Positive controls were stimulated with H_2O_2, Rotenone, and Antimycin while negative controls (only cells and medium) were maintained without staining. Post incubation, cells were centrifuged and resuspended in 400 μL of PBS [17]. For measuring the fluorescence intensity, 5×10^5 cells were used per wells in 96-well plate. Fluorescence intensity was measured using plate reader (Fluoroskan Ascent FL, Thermo Lab systems) at excitation 485 nm and emission 538 nm. The fold increase in ROS was calculated by using formula (2).

2.6. ROS Measurement of SNB19 and LN229 cells

Human glioblastoma cell lines, SNB19 and LN229 are the suitable model that over express GPR17 receptor protein endogenously. Both cell lines were cultured in DMEM complete medium (10% FBS+1% Penicillin-Streptomycin) in incubator at 37 °C in humidified atmosphere of 5% CO2–95% air. For ROS measurement, 70% confluent cells were used. Initially, cells were trypsinized with 1X trypsin+EDTA and suspended into fresh medium. The cell density of 5×10^4 cells per sample were taken for both cell lines and each sample were repeated in triplicates. Cells were initially stimulated with H_2O_2 (100 μM), Rotenone (150 nM), and Antimycin (5 μM) treatment conditions and kept in incubator for 30 mins. The cells were then centrifuged at 3000 rpm at 25 °C and suspended in fresh medium. MDL 29951 and T0 were used as agonist and Montelukast Sodium (4.85 μM) was used as antagonist. Unstimulated cells were counted as negative control, and cells stimulated with H_2O_2, Rotenone and Antimycin were counted as positive controls. For SNB19, agonist MDL29951 and T0 were used with the concentration of 26.33 μM and 76.64 μM, respectively. In case of LN229 the concentration of 41.93 μM and 42.05 μM were used for respective agonists. The above-mentioned concentrations are EC_{50} concentrations obtained from previous experiments [9]. Cells were treated with agonists and antagonist and kept in incubator for 90 mins. After incubation, cells were treated with H_2DCFDA (2 μM) in dark and kept in incubator for 45 mins. After incubation, the cells were centrifuged and washed

with 1X PBS and transferred to black flat bottom 96-well plate to record the fluorescence signals using plate reader at 485 nm excitation and 538 nm emission.

2.7. Isolation of RNA and RNA-sequencing analysis

Both LN229 and SNB19 cells were treated with GPR17 agonists T0 with their respective EC_{50} concentrations. Post 24 h of treatment, total RNA was isolated using the GeneJET RNA Purification Kit following the manufacturer's protocol (ThermoFisher Scientific, Waltham, MA, USA). RNA sequencing was done using Illumina NextSeq 500 (Biomedicum Functional Genomics Unit, FuGU, University of Helsinki, Finland). The sequence analysis pipeline and gene ontology (GO) were performed as described previously [9]. In this analysis a false discovery rate (FDR) adjusted p-value< 0.05 and fold change of 1.5 were set as cutoff values for the selection of differentially expressed genes (DEGs). KEGG enrichment analysis was also performed to find affected signaling pathways (>= twofold cutoff, p-value < 0.05). Specifically, genes involved in ROS pathway, mitochondrial function, mitochondrial ETC complex (I-IV) and mitochondria complex V genes involved in ROS pathway were analyzed to observe the role of GPR17 signaling. In all the experiments, samples were analyzed with biological and technical repeats (n = 6) to obtain statistically significant data.

2.8. Statistical analysis

All experiments described in the present study were performed as with three biological and technical repeats (n = 6). The data were presented as the mean ± standard error of mean. Statistical analyses between two groups were performed by Student's t-test.

3. Results

3.1. High-glucose stimulation evokes the ROSi in 1321N in cells

To determine the effect of glucose on the intracellular oxidative status of 1321N1 cells, ROS level was measured. Before embarking on the investigation of changes in ROS levels induced by activated GPR17 receptor, we first carried out two control experiments to analyse the cellular ROS levels. The GBM cell line, 1321N1 were grown in low glucose and high glucose as described in materials and methods section [18]. Morphological analysis of phase-contrast microscopic image shows that the cells treated with both low and high glucose appeared with bipolar extensions and intracellular granules (Fig. 1A). There are no major morphological changes observed in both conditions. When 1321N1 cells were exposed to 100 μM of H_2O_2, the level of ROS was found to be 0.43-fold lesser in high glucose + H_2O_2 (Fig. 1B) and 0.16-fold lesser in low glucose+ H_2O_2 (Fig. 1C) than the control. Exposing 1321N1 cells with sub-lethal concentrations of H_2O_2 can protect the cells by exhibiting endogenous resistance property [19]. The results showed that Rotenone (ETC CI inhibitor) treated 1321N1 cells under high glucose condition produced 0.53-fold higher level than Antimycin (ETC CIII inhibitor) treated cells (Fig. 1B), while 0.20-fold increase in ROS was observed in Rotenone treated cells than Antimycin in low glucose condition (Fig. 1C). The trend of ROS production in high glucose with H_2O_2, and Rotenone treated conditions was found to be higher than low glucose treated conditions except Antimycin treated cells. Overall, the data indicates that the addition of high glucose is essential for 1321N1 cells to increase the ROS production even in the presence of ETC inhibitors. Hence, high glucose condition was used in all the ROS subsequent measurements performed in the study.

3.2. GPR17 agonist and antagonist induce ROS in 1321N1 cells

Human GPR17 has close structural and phylogenetic relationship between purinergic receptor (P2Y) and cysteinyl leukotriene receptor
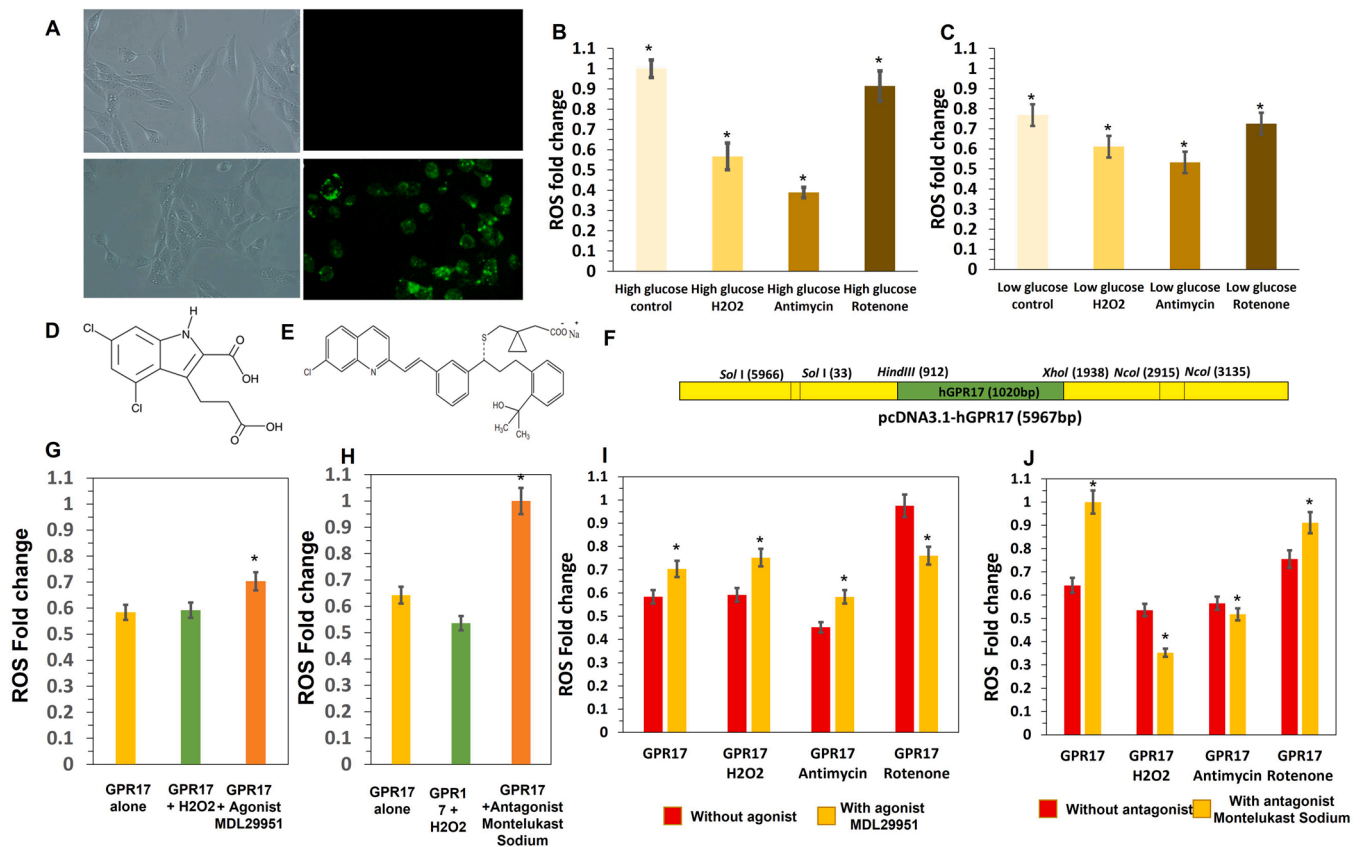
Fig. 1. *GPR17 ligands regulates ROS in GBM cells: A) Phase contrast micrograph of GBM cells 1321N1 grown in low glucose (top left) and high glucose (bottom left). The morphology of cells in either assay media appears similar with corresponding bipolar extensions and intracellular granules. The cells grown in low glucose expressed least level of ROS signals stained with H_2DCFDA (top right); stained cells appeared with very distinct green fluorescence signal (bottom right). B) Fold change in ROS level in 1321N1 cells in the presence of high glucose and (C) low glucose. (D) 2-dimensional chemical structure of GPR17 agonist, MDL29951 and (E) antagonist, Montelukast Sodium. (F) Schematic representation of pcDNA3.1 expression vector with hGPR17 receptor coding gene. (G) Fold change in ROS level in 1321N1 cells in high glucose in presence of GPR17, H_2O_2 and MDL 29951 (H) Fold change in ROS level high glucose in presence of GPR17, H_2O_2 and Montelukast Sodium. (I) Level of ROS in 1321N1 cells transfected with GPR17 in high glucose treated with MDL29951 (agonist) and (J) Montelukast Sodium (antagonist) and ETC complex I and III inhibitors, i.e., Rotenone and Antimycin, respectively. The data were represented with mean $\pm$ S.E.M from n = 6 values with *p < 0.05 (Two-way ANOVA). Statistically significant data were indicated with asterisks and the fold changes were normalised against the control.*

(CysLT) and thus, it is specifically activated by both families of endogenous ligands. Since 1321N1 cells induce ROS generation in high glucose, we further examined the ability of cells exhibiting similar response to GPR17 agonists (MDL 29951) (Fig. 1D) or antagonist (Montelukast Sodium) (Fig. 1E). These two compounds, including H_2O_2, a potent cell permeable oxidant, were characterized for the production of ROS in 1321N1 cells transfected with short isoform of human GPR17 (Fig. 1F). 1321N1 cells were incubated with the ROS sensitive fluorophore H_2DCFDA, followed by the treatment with 4.9 μM and 4.85 μM of MDL 29951 and Montelukast Sodium, respectively·H_2O_2 (100 μM) was used as a positive control. It was observed at high glucose condition, that the presence of GPR17 alone induced ROS to about 0.58-fold, which was similar to GPR17 + H_2O_2 induced 1321N1 cells. Also, it was noticed to about 0.7-fold increased ROS in GPR17 + MDL 29951 treated condition (Fig. 1G) with 1-fold increase in GPR17 + Montelukast Sodium treated condition (Fig. 1H).

3.3. GPR17 ligands regulates ROS through mitochondrial ETC complex III

To identify the involvement of mitochondrial ETC CI and CIII in GPR17 signalling mediated ROS production, we have assessed the ROS levels by using H_2DCFDA fluorophore. As shown in Fig. 1I and Fig. 1J, ROSi was assayed in GPR17 transfected 1321N1 cells following the manufacture's protocol. Expression of GPR17 receptor in transfected

cells did not affects the cellular physiology unless it was activated with an agonist [20]. Fig. 1I represents the levels of ROS fluorescence measured after 1321N1 cells treated with H_2O_2, presence and/or absence of MDL 29951. Stimulation of GPR17-transfected cells with the EC_{50} concentration of an MDL 29951 significantly increased ROS production in all the conditions except Rotenone-treated cells. The data revealed that rotenone treated GPR17 + MDL 29951 induced ROS level to 0.7-fold, which was equivalent to GPR17 treatment alone, whereas antimycin treated GPR17 + MDL 29951 induced to about 0.5-fold ROS (Fig. 1I).

Rotenone-treated GPR17 transfected cells without MDL 29951 displayed the highest ROS fluorescence levels i.e., 1-fold. This suggests that mitochondrial ETC CIII plays crucial role in determining the ROSi level than ETC CI.

Fig. 1 J represents the ROS fluorescence levels in GPR17-transfected cells treated with Montelukast Sodium. The cells in the absence of Montelukast Sodium followed the same trend as in the preceding experiments with agonist but showed a minor increase of ROSi in H_2O_2 and Antimycin-treated cells. Antagonism effect was observed when the Montelukast Sodium incubated cells were treated with H_2O_2, and Antimycin with 0.32- and 0.51 respectively. On the other hand, Rotenone treated Montelukast Sodium incubated 1321N1 cells showed higher fold of ROSi to about 0.9-fold, which was similar to the cells with GPR17 + Montelukast Sodium condition. Thus, the data confirms that ETC CIII determines the ROSi level in 1321N1 cells which was also in

concordance with the agonist treated condition.

Further, to validate the existence of identical mechanism of action of mitochondrial ETC CI/CIII, we have used SNB19 and LN229 GBM cells, that over expresses GPR17 endogenously. In addition to MDL 29951, and Montelukast Sodium, another ligand T0, a GPR17 agonist that was identified as a potential agonist than MDL 29951 was also used. In SNB19 cells, the effects of both agonists MDL 29951 (Fig. 2A) and T0 (Fig. 2B) displayed similar trend as when 1321N1 cells were treated with the MDL 29951. There observed a significant increase of ROSi in MDL 29951 + Antimycin, MDL 29951 + H_2O_2 to about 0.2-and 0.1-fold whereas T0 + Antimycin treated SNB19 cells showed 0.14-fold raise in ROSi with 0.17-fold increase in T0 + H_2O_2 treated cells than their respective controls (Fig. 2A and 2B). Upon Montelukast Sodium treatment, the SNB19 cells with antimycin showed 0.04-fold higher and with H_2O_2 showed 0.14-fold higher ROSi than the control (Fig. 2C). Agonist with rotenone treated SNB19 cells showed decreased ROSi to about 0.17-fold and 0.14-fold than the corresponding controls (Fig. 2A and 2B), whereas antagonist treated SNB19 cells showed decreased ROSi to about 0.14-fold (Fig. 2C).

Interestingly, LN229 cells treated with Rotenone and agonists (MDL 29951 and T0) showed increased ROS level of about 0.26- and 0.09-fold respectively (Figs. 2D and 2E) than the control. Antagonist treated LN229 cells showed higher ROSi in all the treated conditions in the presence of H_2O_2, Antimycin and Rotenone with 0.16-, 0.16-, and 0.18-fold respectively, than the corresponding control (Fig. 2F). Relatively, ROSi level of both the cell lines treated with MDL 29951 + Rotenone was observed to be higher than the H_2O_2 control (Fig. 2A and 2D). The combination of MDL 29951 + Antimycin played a key role in inducing ROS production, as the level of ROS was observed higher than H_2O_2 + MDL 29951 in both the cell lines (Fig. 2A, 2B, 2D and 2E). Compared to agonist and antagonist treated conditions in both cell lines, increased ROS was observed in agonist than antagonist treated condition. Specifically, ROS levels were observed higher in the presence of Antimycin, and agonists (MDL 29951 and T0) when compared to Rotenone, an inhibitor of ETC CI. The obtained data suggests that mitochondrial ETC CIII plays a crucial role than CI in determining the ROSi level in both GBM cell lines which is in consistent with the results obtained from 1321N1 cells.

3.4. Activation of T0-GPR17 signal regulates ROS pathway

Recently, our group have identified T0 as a potential ligand in significantly inhibiting proliferation of LN229 and SNB19 cells than MDL 29,951. Hence, to investigate the genes involved in ROS pathway regulated by GPR17 signaling activation, the GBM cells were treated with the top lead agonist T0, RNA sequencing was performed as described in the materials and methods section. The data was analyzed by performing Principal Component Analysis (PCA) and Hierarchical Clustering Analysis as described previously [9]. More than 20,000 genes were identified from both SNB19 and LN229 cell lines. Collectively, from the comparison of GPR17 treated and untreated condition, 5960 differentially expressed genes (DEGs) were identified with a q-value < 0.05 and fold change > 1.5 by DESeq2 analysis (Supplementary file 1 and 2). It was identified that DEGs were higher in SNB19 cells (3358 DEGs) than in LN229 cells (2602 DEGs). We have also identified about 1372 downregulated DEGs between T0 treated and untreated cells of LN229 cells, while 1806 DEGs were downregulated in SNB19 cells (q-value <0.05). In LN229 cells, about 1230 DEGs were observed to be upregulated while 1552 DEGs were noted upregulated in SNB19 cells (Fig. 3A). About 1031 DEGs including up- and down regulated genes were found common in both LN229 and SNB19 cells. We also identified common DEGs, which were up and downregulated in both cell lines. The overlapping genes between the cell line was found to be higher with 531 downregulated DEGs than the upregulated genes with only 500 DEGs (Fig. 3**B**).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identifies various affected pathways due to T0 agonist treatment. The enrichment analysis of pathways clearly demonstrated that many major signaling pathways are associated with ROS signaling. In detail, pathways affected due to T0 agonist were enriched in KEGG pathways including cell growth and cell death process, which involves cell cycle, p53 signaling pathway and apoptosis. Other than these pathways, most of the affected pathways were related to human disease, predominantly cancer (Fig. 3C). GO annotation analysis also revealed that most of the affected pathways were related to cellular stress, cell migration, cell death and proliferation, DNA damage and repair, and mitochondrial membrane activity and organization due to T0 agonism to GPR17 receptor (Fig. 3D). These data revealed that the GBM cell line might
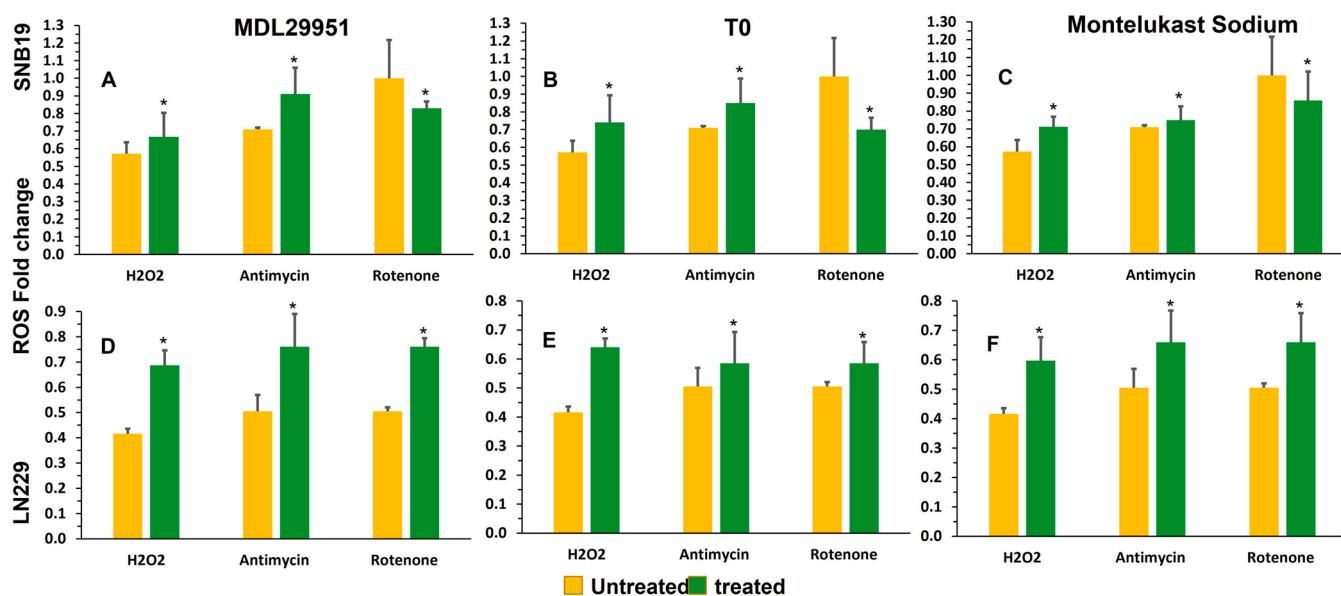

Fig. 2. *GPR17 ligands regulates ROS via mitochondrial ETC complex III. (A) Fold change in ROS of SNB19 cells and (B) LN229 cells treated with Montelukast Sodium antagonist, MDL29951 agonist, T0 agonist, and ETC complex I and III inhibitors,* i.e.*, Rotenone and Antimycin, respectively.* All the experiments were done with mean ± S. E.M from n = 6 values (Two-way ANOVA). Statistically significant data were indicated with asterisks, untreated versus treated samples (* P < 0.05 is considered as significant). The fold changes were normalised against the control.
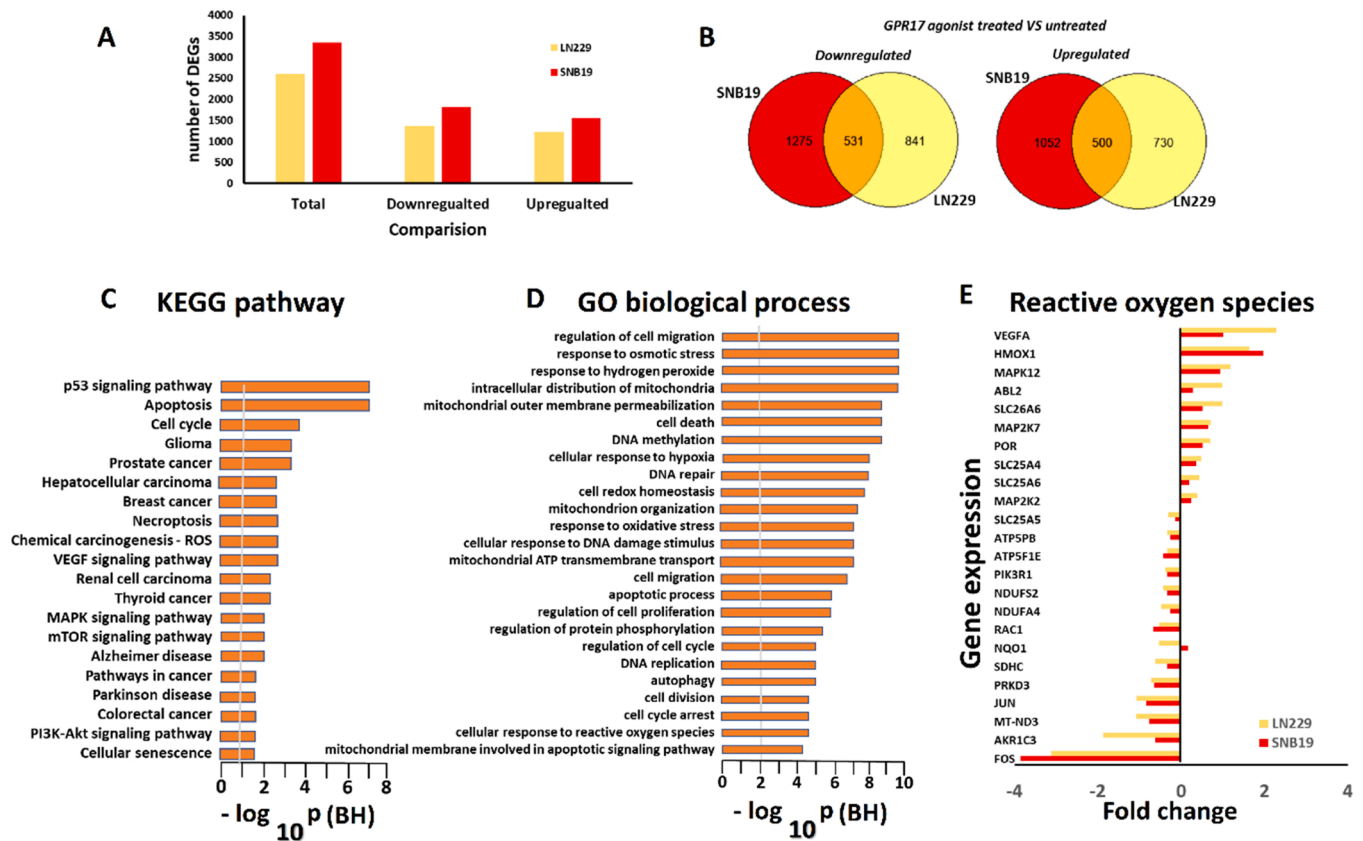
Fig. 3. Activation of GPR17 signal regulates ROS pathway. (A) Graphical representation of identified total DEGs with a q-value < 0.05 and fold change > 1.5 by DESeq2 analysis in LN229 and SNB19 cells treated with GPR17 agonist, T0. (B) Venn diagram of DEGs found in SNB19 and LN229 cells treated with GPR17 agonist. (C) Affected KEGG pathway in LN229 and SNB19 cells treated with GPR17 agonist, T0. (D) Affected GO biological processes found in LN229 and SNB19 cells treated with GPR17 agonist. (E) Identified upregulated and downregulated genes involved in ROS production in SNB19 and LN229 cells treated with GPR17 agonist.

encounter the stress in mitochondria by maintaining and regulating the cellular redox balance.

3.5. GPR17 mediated gene expression profiling in GBM Identifies T0 agonist could be a Novel Therapeutic ligand for GBM

To explore the role of the most important ROS related genes regulated by GPR17 signaling, we further comparatively investigated the DEGs' profiles of both the cell lines (Fig. 3E). Among the various downregulated genes, FOS and AKR1C3 were significantly downregulated in both the cell lines. Other downregulated genes were MT-ND3, Jun, PRKD3, and SDHC. However, VEGFA, HMOX1, and MAPK12 are found to be remarkably upregulated among other upregulated genes. These DEGs' were generally involved in cancer progression and stress related pathways.

ROS is a pivotal role player in controlling the proliferation of glioma cells and the present study revealed that AKR1C3 and Fos have been identified to be highly downregulated due to ROS production (Fig. 3E). Fos belongs to AP-1 (activator protein-1) complex, which is composed of Fos, Jun and ATF protein families. Fos family includes five proteins, c-Fos, FosB, and Fos related antigens Fra-1 and Fra-2 where involvement of c-Fos was found to be in signal transduction, cell differentiation proliferation, cell motility, cancer growth, neuron activation, angiogenesis, invasion, oxidative stress, and metastasis [21]. Mu et al., (2021) studied the knockdown of Fos in HT22 neuronal cells resulting in the increase of oxidative stress and apoptosis. Also, increased Bax/Bcl-2 ratio with higher ATP content are resultants of Fos knockdown, leading to increased neuron apoptosis after oxygen-glucose deprivation (OGD). Fos has been found to be involved in regulatory functions of mitochondria and oxidative stress and thus can be targeted for stroke

therapy [22]. In our present study, Fos has been found to be remarkably downregulated among several genes in both the cell lines, which demonstrates that increased ROS level in the cells was facilitated by T0, a potent GPR17 agonist. AKR1C3 was found as the second highly downregulated gene in SNB19 and LN229 cells (Fig. 3E). Previously, AKR1C3 is found upregulated and identified as a radio-resistant in esophageal cancer and its suppression restored the sensitivity to the ionizing radiation (IR), which is a nonsurgical treatment for most of the tumors [23]. Interaction between IR and water molecules in the cells produce ROS, which reacts with DNA and other macromolecules producing chromosome aberration, gene defects and other damages including mitochondrial damage. Accumulation of DNA damage triggers IR-induced failure of cell division and proliferation that leads to cell apoptosis [24]. Subsequently, Jun is also found to be downregulated, which is a member of AP-1 complex. However, in glioma condition, c-Jun protein is found to be robustly accumulated that contributes to malignancy. Accumulation of c-Jun is not dependent on MAPK activity but can be stimulated by cytoskeleton-dependent pathway [25]. Downregulated Fos, Jun and AKR1C3 revealed the promising role of T0 agonist on GPR17 receptor against tumor progression (Fig. 3E).

Upregulation of VEGF and MAPK12 indicates the cancer progression, and it is a sign of angiogenesis. Downstream activation of vascular endothelial growth factor A (VEGFR A) regulates angiogenesis and vascular permeability, and these physiological responses involve several other effectors including ERK, PI3K, MAPK, FAK, and Rho family GTPases [2]. Tumor angiogenesis primarily relies on VEGFA-driven responses. VEGF is produced by hypoxic tumor cells, endothelial cells (ECs) and tumor associated macrophages (TAMs) [26]. ECs generate low level ROS, functions as signaling molecule to mediate EC proliferation and migration, which contribute to angiogenesis [27]. Thus, T0

upregulates VEGF A in both the cell lines due to increased ROS thereby regulates angiogenesis. Likewise, upregulated HMOX1 is due to the increased ROS level which changes the oxygen tension as it is an oxidant sensitive gene. HMOX1 is widely regarded as the protective mechanism against oxidative tissue injury[3]. Hence, the upregulation of HMOX1 indicates the activation of cell protective mechanism against increased ROS [28] (Fig. 3E).

Analysis of dysregulated mitochondrial genes revealed that genes involved in mitochondrial outer membrane permeabilization (MOMP), and intracellular distribution of mitochondria were found to be downregulated, which indicate the mitochondrial dysfunction due to T0 agonism activity of GPR17 receptor and ROS generation. YWHA gene family belongs to 14–3–3 protein family and they have been implicated in cell signaling, gene transcription, metabolism, neurodevelopment, cell cycle and apoptosis [29]. Dysregulation of YWHA gene family is associated with neurological disorders. Most of the genes from YWHA family have been also found to be upregulated due to T0 agonist activity on GPR17 receptor. Only, YWHAE has been found to be downregulated in both the cell lines and YWHAQ in SNB19 cells alone. Dysregulation of YWHAE gene is associated with impaired neurodevelopment [30]. Upregulation of YWHA gene family demonstrates the increased apoptosis in cells as the genes are involved in inserting protein into the mitochondrial membrane to initiate apoptotic signaling.

SLC25 (solute carrier family 25) facilitates the transport of solutes with different chemical identities across the inner mitochondrial membrane. Among SLC25A genes, SLC25A6 has been found dysregulated in both cell lines and SLC25A36 only in LN229 cells (Fig. 3E). SLC25A6 facilitates the exchange of ADP and ATP between mitochondria and the cytoplasm, and it is known as ADP/ATP carrier (AAC). The knockdown or overexpression of AAC isoforms modulate sensitivity of cells to apoptotic stimuli [31]. Notably, KLF5, RNF126, RNF213, SOD1 and SOD2 genes were upregulated in both cell lines, while KLF9 was upregulated in LN229 cells. All these data from the current study, also validated that the elevated expression of KLF and RNF and SOD family genes might be involved in inducing ROS [32]in GBM cells (Supplementary file 1 and 2). Taken all together, the pathway analysis and biological process studies, revealed that the findings have shown a clear picture on the role of GPR17 receptor regulating the ROS production and thus leading to GBM cell death, where mitochondria played a crucial role in apoptosis induction.

3.6. T0-GPR17 activation disrupts the function of mitochondria and mitochondrial ETC complexes genes

Pathway and network analysis identified the dysregulation of several mitochondrial genes in both the GBM cell lines. The data shows the dysregulation of several genes involved in crucial biological processes including mitochondrial membrane involved in apoptotic signaling, mitochondrial ATP transmembrane transport, mitochondrion organization, mitochondrial outer membrane permeabilization and intracellular distribution of mitochondria (Fig. 4A). TFDP1, YWHAB, YWHAH, YWHAZ that are involved in mitochondrial apoptotic signaling were upregulated in SNB19 cells, whereas YWHAB, YWHAH, YWHAQ and YWHAZ were upregulated in LN229 cells. Genes that are involved in mitochondrial outer membrane permeabilization and intracellular distribution of mitochondria including, BNIP3L, POR, ARMC1, CLUH and LRRK2 were significantly downregulated in both the cell lines. ATAD38, PPRC1, CLUH, EPAS1, ESRRA, LONP1, PRDX3 that are involved in mitochondrion organization were upregulated in both LN229 and SNB19 cells. (Fig. 4A, Supplementary file 1 and 2).

We also have investigated the role of T0-GPR17 on mitochondrial ETC complex I, II, III, IV and V in ROS pathway, the gene expression analysis was performed in both the cell lines (Fig. 4B).

Among dysregulated genes of ETC complex I due to T0 agonist activity on GPR17 receptor, ND3 gene was downregulated in both cell lines. However, most of the genes that belong to the family of NADH dehydrogenase (ubiquinone) 1 beta subcomplex proteins including NDUFS2, NDUFS6, NDUFB1, NDUFB2, NDUFB6, NDUFB9, NDUFAF1, NDUFC2, and NDUFA1 were downregulated only in SNB19 cells. Notably, NDUFS2 was downregulated only in LN229 cells (Fig. 4B). NADH ubiquinone oxidoreductase genes that are located in mitochondrial inner membrane are involved in various mitochondrial functions such as ubiquinone biosynthesis, oxidative phosphorylation and mitochondrial dysfunction.

Among dysregulated genes of ETC complex III, CYB5B gene was found to be downregulated in both cell lines. However, CYB5R2 and CYB5A were found to be downregulated in LN229 cells and UQCRC1 and UQCRQ were found downregulated in SNB19 cells (Fig. 4B). CYB, cytochrome b genes are involved in production of cytochrome b protein and MT-CYB gene has been found associated with GBM. Also, both UQCRC1 and UQCRQ genes played crucial role in redox signaling of many cancers suggesting that upon the activation of GPR17 receptor signaling, the concentration of cellular ROS tightly controlled by ETC complex III related genes UQCRC1 and UQCRQ (Fig. 4B). The data
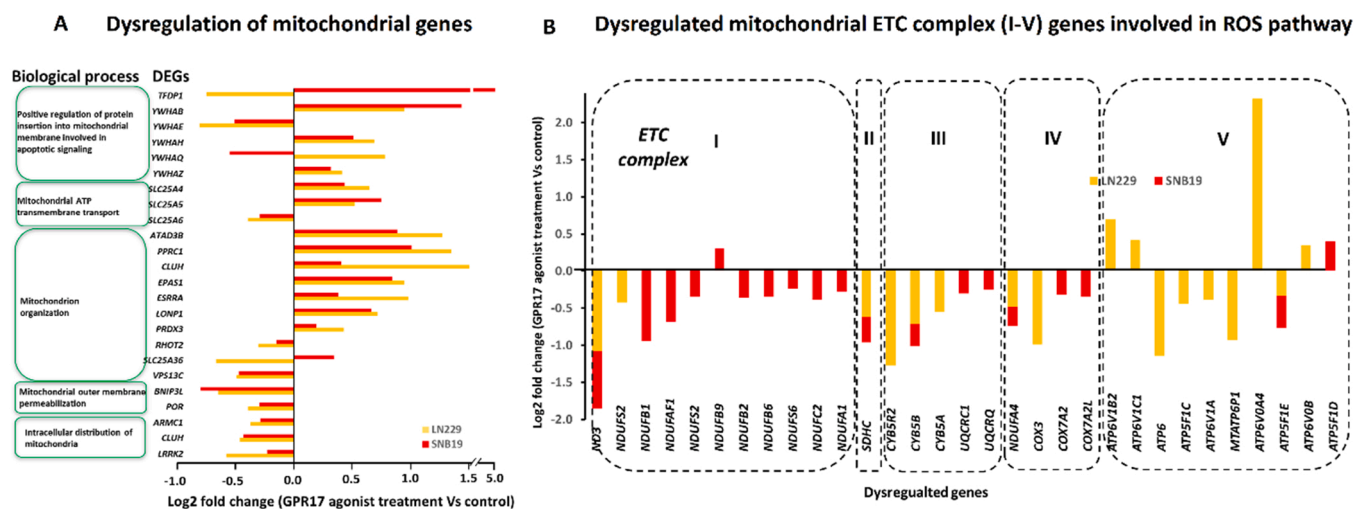

Fig. 4. GPR17 activation disrupts the function of mitochondria and mitochondrial ETC complexes genes. (A) Identified upregulated and downregulated mitochondrial genes in SNB19 and LN229 cells. Genes are categorized according to their functions in mitochondria. (B) Identified upregulated and downregulated genes of mitochondrial ETC complexes of SNB19 and LN229 cells treated with GPR17 agonist, T0.

suggests that pharmacological activation of GPR17 receptor affected not only mitochondrial ETC complex I and III but also II, IV and V. However, the gene expression profile of mitochondrial ETC complex I/III shows that the ETC complex III genes are involved in ROS pathways, which significantly contributed to cell death and proliferation, while ETC complex I genes contribute to mitochondrial function (Fig. 5). These results suggest that GPR17 receptor regulates ROS through the ETC complex I and III, where complex III bypasses complex I, which was also validated by biochemical experiments in GBM cells.

4. Discussion

The present study represents a systematic analysis to examine the role of GPR17 receptor in modulating redox biology in GBM. Specifically, we have reported that GPR17 receptor is a key driver of ROS production driven by ETC complex III. Altered and impaired redox pathways are one of the hallmarks of cancerous cells including GBM [33]. While plethora of studies have attributed numerous pathways that enhance the tumor cells invasiveness in the brain microenvironment as the primary driving forces behind GBM, recent studies have identified a role for cellular redox dysregulation in GBM invasion [33]. ROS generation in tumor cells affects the cell cycle and is involved in tumor

progression and drug resistance in GBM [34]. Here, we have observed that GPR17 receptor drives the modulation of ETC complexes, specifically complex III by increasing ROS production. We also found that induction of GPR17 receptor leads to loss of function of NADH dehydrogenase genes involved in ETC complex I, while cytochrome b and ubiquinol cytochrome c reductase family genes in ETC complex III (Fig. 5).

GPR17 signaling activation and its impact on downstream signaling pathway leads to apoptosis in GBM cells [4,6–8]. GPR17 signaling is not only involved in GBM cell death but also in spinal cord and brain injury [35]. Our previous studies based on the next-generation sequencing data also revealed high level expression of GPR17 receptor in various samples of GBM[4], thus considering it as a notable target for GBM treatment. Our recent analysis on the association between GPR17 expression and overall survival in LGG and GBM revealed that GPR17 expression is correlated with survival, thus it acts as a predictive biomarker for LGG and GBM [1]. In an independent analysis, we found that GPR17-T0 ligand interaction was able to regulate the interaction of phosphoinositide-binding structural (PX) domain and helical peroxisomal membrane targeting (mPTS) domain-containing proteins[36]. Recently, we also found that the activation of GPR17 receptor using T0 agonist leads to the inhibition of cAMP and affected the calcium
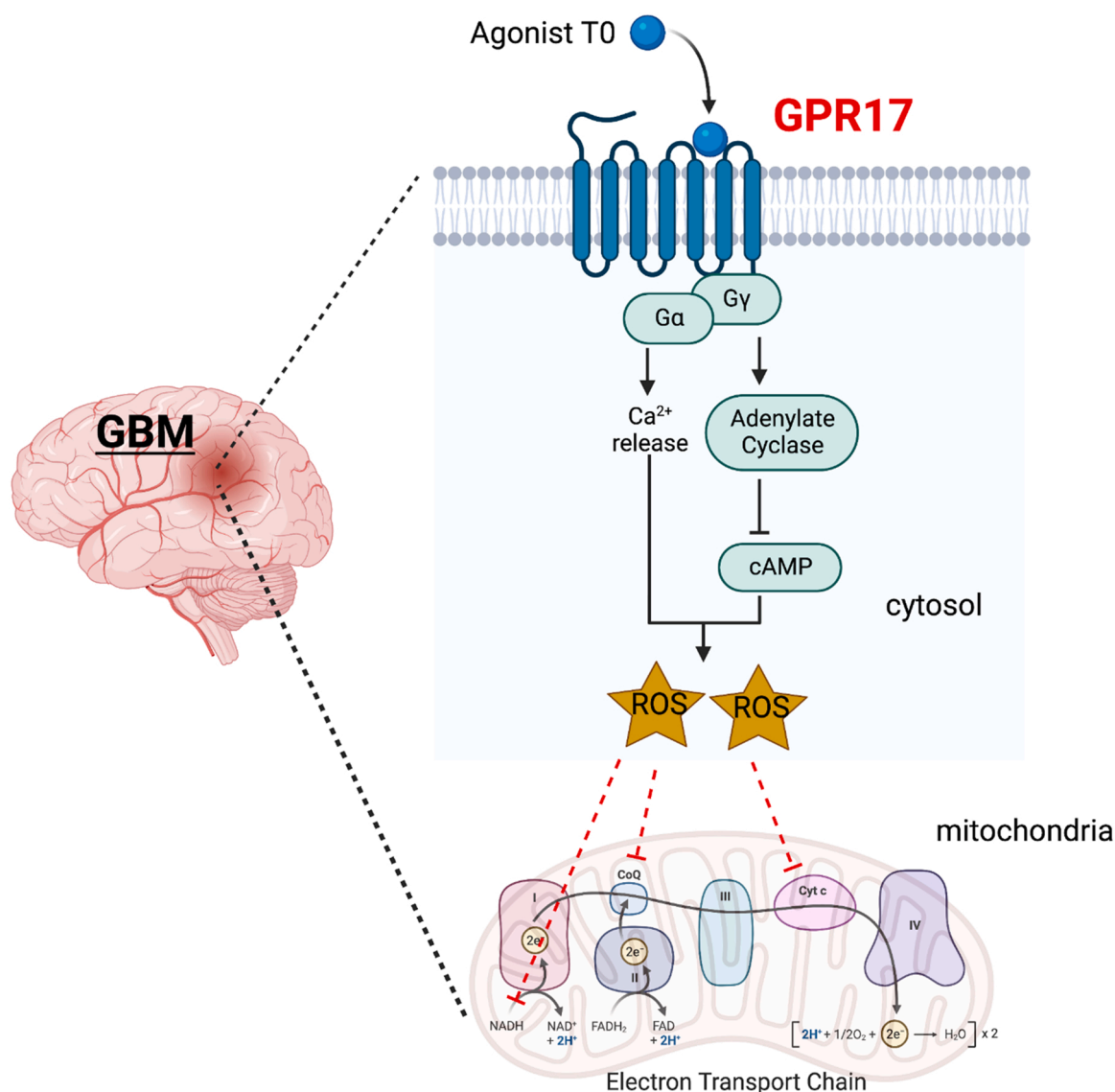

Fig. 5. Diagram depicting the working model of the human GPR17 signaling cascade effects mitochondrial function.

concentration. Notably, the induction of GPR17 receptor also enhanced the cytotoxicity potential in GBM cells and patient tissue-derived mesenchymal subtype GBM cells[4]. Also, T0 in combination with phenolic compounds have decreased MMP and increased ROS in GBM cells [8]. However, the linked mechanism between GPR17 mediated ROS and mitochondria in GBM cell remained unknown.

Increased free radicals, particularly ROS, induce disproportionality in redox homeostasis. ROS trigger cell senescence and apoptosis, consequently limiting further proliferation of the transformed cells and prevents cancer progression [37]. Cancer-suppressive role of ROS is highly dependent on ROS concentration. Complex I and III of mitochondrial ETC high membrane potential, are considered as the predominant point of origin of ROS. ROS disrupts the mitochondrial membrane and opens the mitochondrial permeability transition pore (PTP) and therefore interferes with mitochondrial ETC [38]. We observed that GBM cells treated with high glucose showed elevated ROS level which may be due to the strong dependence on aerobic glycolysis known as Warburg effect. In Warburg effect, the rate of glucose uptake increases dramatically, and lactate is produced despite of fully functioning mitochondria and oxygen [39]. Notably, decreased ROS level was observed when high glucose condition cells were treated with H_2O_2 and Antimycin which can be due to the metabolic alterations that occur in cancer cells to support redox balance and to gain enough energy and building blocks to foster their growth [37,40].

One more reason of this shift is aldo-keto reductases (AKR7A2 and AKR1C3), which are involved in protecting human astrocytoma cells against oxidants like H_2O_2 [41]. The observed results are also in agreement with previous observation in which, high glucose stimulation increases ROS production through the intracellular Ca^{2+} and mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase 1/2 (ERK1/2) [37,40]. We observed that the cells which overexpressed GPR17 protein showed the elevated ROS level due to GPR17 agonistic and antagonistic properties. Similar observations were also reported for adrenergic agonists and antagonists which induces ROS in cardiac muscle cells [42]. Cumulatively, GBM cells treated with Antimycin, and Rotenone depicted that complex III of mitochondrial ETC plays key role in ROS production since the level of ROS was observed to be higher despite of inhibition of complex I through Rotenone.

The gene expression profiles also validated that activation of T0-GPR17 receptor significantly regulated the ROS level via mitochondrial ETC complexes. Also, GPR17-T0 interaction effected various pathways significantly including cell migration and death, mitochondrial membrane permeabilization, intracellular distribution of mitochondria and organization, DNA repair and methylation and cell redox homeostasis [40]. All these pathways are mainly involved in cellular stress and indicating dysfunction of mitochondria due to ROS production induced by GPR17 agonist. Enhanced oxidative stress dysregulates the mitochondrial respiratory machinery [43]. Dysregulated genes of ETC complex I and III due to GPR17 activation indicates the dysfunction of mitochondria due to increased oxidative stress induced by T0, which is GPR17 agonist.

To conclude, our study has showcased the novel role of GPR17 receptor in GBM redox alteration and regulation of ROS through ETC complex I and complex III. Furthermore, we have shown that ETC complex III bypasses complex I in GPR17 driven ROS production in GBM cells. Overall, these findings are an important step in understanding the role of GPR17 in redox reprogramming in glioma as the drivers of the tumor and could be potential prognostic targets in GBM therapies. Collectively, we proposed a model to summarize our findings that the activation of GPR17 receptor inhibits cAMP level, increases calcium and ROS. The GPR17 mediated ROS production tightly controlled by mitochondrial ETC complex III but not I in GBM cells.

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CRediT authorship contribution statement

S.K performed the biological study and data analysis of all experiments. JK permoformed pathway analysis. TR and SK was involved in technical discussion, performed quality checks, and edited the manuscript. SK and AM were involved in the interpretation of the scientific data. MK conceived, developed and managed all studies. All the authors contributed to the manuscript writing.

Declaration of Competing Interest

The authors claim no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114678.

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