RESEARCH ARTICLE

Modulation of Apoptotic and Akt/PI3K/mTOR pathways to target Glioblastoma Cells using synthetic compound PGEA-AN

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Abstract

Objective: To investigate the anticancer potential of a novel synthetic derivative of a naturally occurring diterpenoid against glioblastoma.

Method: The in vitro study was conducted at the Ojha Campus of Dow University of Health Sciences, Karachi, from February to December 2021, and comprised U87 cells. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine the growth inhibitory effect of 16(R and S) – phenylamino-cleroda3, 13(14) Z-diene-15, 16 olide and standard drug temozolomide against glioblastoma cells, and half-maximal inhibitory concentration was calculated. Microscopy and immunocytochemistry were used to investigate apoptotic morphology and active caspase-3 and B-cell lymphoma 2 (Bcl-2) expression. Quantitative real time polymerase chain reaction was used to investigate the expression of proliferation markers. Data was analysed using SPSS 21.

Results: Both the synthetic derivative and the standard drug significantly inhibited growth of U87 cells ($p<0.001$) with half-maximal inhibitory concentration of 19µM and 185µM, respectively. Apoptotic morphology and upregulation of active caspase-3 protein expression was observed in cells treated with half-maximal inhibitory concentration doses of both the synthetic derivative ($p<0.05$) and the standard drug ($p<0.001$), and Bcl-2 was down-regulated in both the synthetic derivative ($p<0.01$) and the standard drug ($p=0.05$). However, no significant difference was observed in the expression of proliferation markers ($p>0.05$).

Conclusion: The synthetic diterpene derivative PGEA-AN showed growth inhibitory actiity against glioblastoma.

Key Words: Temozolomide, Caspase, Glioblastoma, Diterpenes

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Introduction

Glioblastoma (GBM) transpires from supporting cells, astrocytes, of the brain.[1] GBM covers approximately 80% of all gliomas and about 60% of adult neurological cancers[2] and it has been designated as a highly invasive grade IV tumour by the World Health Organisation (WHO).[3] Worldwide incidence of GBM is <10 per 100,000. Highest incidence is between those aged 55-60 years, but it can occur at any age. About 2.5% of deaths are due to malignant gliomas, making it the 3rd most common cause of cancer-related deaths in individuals aged 15-34 years.[4] Despite several international efforts, GBM treatment is still an exigent task in clinical oncology. Standard treatment regime of GBM is the maximum safe resection followed by radiotherapy and chemotherapy by temozolomide (TMZ), along with effective palliative care to the patients, but the end consequences are still distressing. TMZ is an oral alkylating agent that methylates deoxyribonucleic acid (DNA) at Nitrogen 7 (N7) and oxygen 6 (O6) position on guanine (G) and blocks the growth 2-mitotic (G2-M) phase of cell cycle via disrupting the DNA mismatch repair system.[5] The pathogenesis of GBM is composite, and involves network of interconnected signalling pathways. Flaws of apoptosis are not only a usual incidence in the development of cancer, but also give rise to poor response to medication. There are two main apoptotic pathways, the intrinsic pathway that makes a move via damage to mitochondria, and the extrinsic pathway that takes measure via death receptors on cell surface followed by sequential cell signalling events leading to apoptosis. Caspase-3, an important promoter and executor of apoptosis, is common to both the pathways. B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein and has an important role in the regulation of intrinsic apoptotic pathway. It controls permeability of transition pores of outer mitochondrial membrane by blocking pro-apoptotic proteins, like Bcl-2-associated X protein (BAX) and Bcl-2 antagonist killer 1 (BAK), resulting in inhibition of cytochrome c release from mitochondria and stave off the genesis of apoptosome, activation of caspases and eventually cell demise.[6,7]

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Overexpression of Bcl-2 has also been observed in GBM. Deregulation of Bcl-2 family proteins are responsible for imparting hostility of GBM cells to antineoplastic treatment by disrupting the apoptotic pathways.[6] Another influential signalling cascade disrupted in >75% cases of GBM is protein kinase B-phosphoinositide 3-kinase-mammalian target of rapamycin (Akt/PI3K/mTOR) pathway. It is responsible for maintaining cellular survival, proliferation and metabolism.[8] Identification of new compounds that can target these two pathways is imperative for the treatment of GBM and to overcome treatment resistance.

The 16-Oxo-cleroda -3,13 (14) E- diene -15 oic acid is a bio-privileged diterpenoid of well-reputed medicinal plants polyalthia longifolia and polyalthia longifolia var. pendula, possessing various biological activities.[9-15] Recently, its novel synthetic derivatives, including 16(R and S) – phenylamino-cleroda3, 13(14) Z-diene-15, 16 olide (PGEA-AN), have been prepared[15] of which PGEA-AN has shown to impede the proliferation of neuroblastoma cells by inducing apoptosis.[16] PGEA-AN prominently augmented the expression of tumour protein p53 (P53) and BAX. The compound has also shown to be less toxic than cisplatin.[16] However, to our knowledge, the activity of this novel diterpenoid has not been investigated in GBM yet. The current study was planned to fill the gap in literature by exploring the anticancer potential of PGEA-AN against glioblastoma.

Materials and Methods

The in vitro study was conducted at the Ojha Campus of Dow University of Health Sciences, Karachi, from February to December 2021.

PGEA-AN is a synthetic compound which was prepared by the collaborating chemist from a diterpenoid 16-Oxo-cleroda -3,13 (14) E- diene -15 oic acid isolated from polyalthia longifolia var. pendula.[14,15]. TMZ was obtained from the market (Sigma Chemical Company, St. Louis, United States). Compounds were dissolved in sterile 100% Dimethyl sulfoxide (DMSO) to prepare stock solution of 50mM and stored at -20℃. Stock solutions were diluted in culture media to prepare various test doses.

Human GBM cell line U-87 was acquired from American type tissue culture collection (ATCC, USA). The cells were maintained in complete media with 10% foetal bovine serum (FBS), 1% each of penicillin and streptomycin, sodium pyruvate, L-glutamine and amphotericin B. A haemocytometer (Pericolor HBG, Germany) was used for cell counting. The cells were kept in incubator with humidified atmosphere at 37^0C and 5% of carbon dioxide (CO_2).[17]

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to analyse the effect of test compounds on percentage inhibition of growth in the cells. Briefly, mono-layer of the cells in the flask was trypsinised using trypsin and ≈3000 cells/100µL of complete media was dispensed in 96-well plates. After 24 hours, when the mono-layer was formed, the media was removed, and test concentrations of compounds PGEA-AN and TMZ in complete-media having 2% FBS were added to the cells after incubation for 48 hours at 37°C and 5% CO_2. After the incubation, the media within the 96-well plates was discarded and 200µL of MTT dye 0.5mg/ml was added and incubated for 3 hours. After incubation, the supernatant was removed and 100µL of DMSO was added to the cells. Enzyme-linked immunosorbent assay (ELISA) reader (Bio.Tek, US) was used to record the absorbance at 490nm of wavelength. The assay was performed in triads at least thrice. The percentage of growth inhibition was determined by the formula:

% growth inhibition of cells = 100- [(At - Ab)/ (Ac - Ab)] *100.[17]

The half-maximal inhibitory concentration (IC50) was computed by the equation:

$$IC_{50} = (X_2 - X_1) X (_{50} - Y_1) / (Y_2 - Y_1) + X_1$$

in which X^1 and X^2 denoted maximum and minimum doses to be utilised, and Y^1 and Y^2 denoted the mean percentage of viable cells at the maximum and minimum used doses.[17]

The cells were treated with 10µM, 20µM and 30µM doses of PGEA-AN, and with 30µM, 40µM, 50µM, 100µM, 200µM of TMZ.

Mono-layer of U87 cells in a 6-well plate was treated with IC^{50} of PGEA-AN and TMZ, while DMSO was taken as a vehicle control. After 48 hours, the cells were observed under inverted microscope (Leica, Germany) and the images were captured at 20X magnification.

For analysing the effect of PGEA-AN on the expression of active caspase-3 and Bcl-2, the cells were plated in a 24-well plate and the mono-layer was treated with IC50 dose of PGEA-AN, TMZ and DMSO for 48 hours at 37°C and 5% of CO_2. The cells were treated for 48 hours, and then rinsed with phosphate buffer saline (PBS) and fixed with paraformaldehyde 4%. The cells were then dipped in triton 0.1% and blocked with a blocking solution (ROTI, Block, Germany). The cells were washed with PBS and incubated with rabbit anti-cleaved caspase-3 antibody (Santa Cruz Biotechnologies, Dallas, US) and polyclonal

antibody to Bcl-2 (Cloud-Clone Crop, US) at 4ºC lasting for one night. The following day, the cells were washed with PBS. Secondary antibody (Alexa-Flour, 46 anti-rabbit IgG, Invitrogen/Life Technologies, USA) in PBS was used to incubate the cells for 1 hour at room temperature. The cells were then washed again with PBS, and 4′,6-diamidino-2-phenylindole (DAPI) was used to counter-stain. The stained cells were mounted with mounting media and envisioned under a fluorescent microscope. ImageJ software (National Institutes of Health, US) was used for the quantification of the images.[18]

Ribonucleic acid (RNA) extraction was done using an extraction kit (NucleoSpin RNA Plus, A Takara Bio, Japan) as per the manufacturer's protocol. Briefly, U-87 cells were seeded in 6-well plates and the mono-layer was treated with IC_{50} concentrations of PGEA-AN, TMZ and DMSO for 48 hours. The extracted RNA was checked for its integrity using gel electrophoresis. Concentration and purity of RNA was determined using a spectrophotometer (NanoDrop, Thermo Scientific, USA). Complementary DNA (cDNA) was synthesised from 1µg of template RNA using a kit (Applied Biological Materials, Canada) as per the manufacturer's protocol.

The cDNA was subjected to quantitative real time-polymerase chain reaction (qRT-PCR) (Applied Biological Material, Canada). The primer sequences (Bio Basic Inc., US) used were: Akt (forward,5′-TCTATGGCGCTGAGATTGTG -3′reverse5′CTTAATGTGCCCGTCCTTGT-3′), PI3K (forward5′-GGTTGTCTGTCAATCGGTGACTGT-3′) (reverse5′GAACTGCAGTGCACCTTTCAAGC -3′), mTOR (forward 5′-GCTTGATTTGGTTCCCAGGACAGT-3′) (reverse 5′-GTGCTGAGTTTGCTGTACCCATGT-3′) β-Actin (forward 5′-TGGGCATGGGTCAGAAGGATTC-3′) (reverse 5′-AGGTGTGGTGCCAGATTTTCTC- 3′.

The fold change in the expression of each gene of treated and untreated cells was calculated using 2-delta-delta cycle threshold ($2^{-\Delta\Delta CT}$) method.[19] β-Actin was used as an internal control.[16]

Data was analysed using SPSS 21 software. Data was expressed as mean ± standard error of the mean (SEM) of separate experiments (n≥3) and compared using one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc test. P<0.05 was considered statistically significant.

Results

IC50 for PGEA-AN (Figure 1a) and TNZ (Figure 1b) was 19µM and 185µM, respectively.

The cells in the control group showed normal morphology, like adherent cells with star-shaped body and centrally placed nucleus (Figure 1C.i). In the PGEA-AN group (Figure 1c. ii) and TMZ group (Figure 1C.iii) the cells changed their shape, became rounded, their connections and processes became loose and the size appeared to be small. Cells became unadhered from the flask and neighbouring cells, and detached from the surfaces.

Photomicrograph analysis showed there was significant upregulation of active caspase-3 expression in PGEA-AN ($p<0.05$) and TMZ ($p<0.001$), and the integrated density computed in PGEA-AN was 62.23±3.2 and in TMZ 103.84±5.9. In the control group, it was 31.09±4.2. It was further observed that active caspase-3 expression was elevated significantly in the TMZ group compared to PGEA-AN ($p<0.01$) (Figure 2A-B).

Significant downrigger of Bcl-2 expression was observed in PGEA-AN ($p<0.01$) and TMZ ($p=0.05$) groups compared to the control group. Quantitative expression in PGEA-AN was 19.03±3.34 and it was 57.71±14.8 in TMZ. In the control group it was 137.59±25.6. No significant difference was found in Bcl-2 expression between TMZ and PGEA-AN ($p>0.05$) (Figure 2C-D)

Expression of Akt, PI3K and mTOR messenger RNA (mRNA) was also noted (Figure 3A-C).

Discussion

The present study demonstrated significant apoptotic activity of aniline-derived synthetic compound PGEA-AN against GBM. Dose-dependent cytotoxicity of PGEA-AN was observed against GBM cells and IC_{50} was remarkably lower than the standard TMZ drug, which showed its potential against GBM. It has been reported that deregulated apoptotic pathways are one of the important and well-established causes in the development of cancer and its treatment resistance.[20] The present study demonstrated that active caspase-3 expression was significantly upregulated when exposed to PGEA-AN and TMZ compared to control. Although PGEA-AN-induced upsurge in active caspase-3 was less than TMZ, the effect was produced at a dose much lower than TMZ, which showed its potential against GBM. The dose at which PGEA-AN induced 50% growth inhibition of GBM cells was also quite low compared to the TMZ dose. This supported the idea that there could be a dual mechanism through which PGEA-AN executes apoptosis. It might involve execution of caspase-dependent as well as caspase-independent apoptosis.[21]

Downrigger of anti-apoptotic Bcl-2 marker was observed in TMZ and PGEA-AN group. Results showed more potent

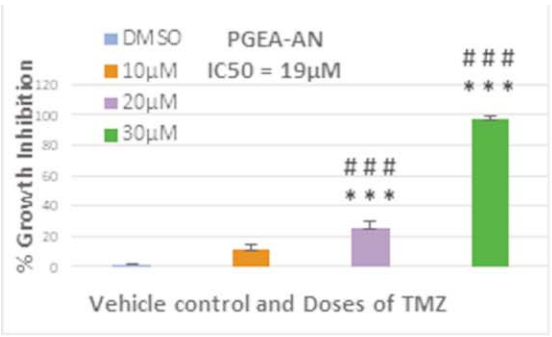

Figure 1 (a)

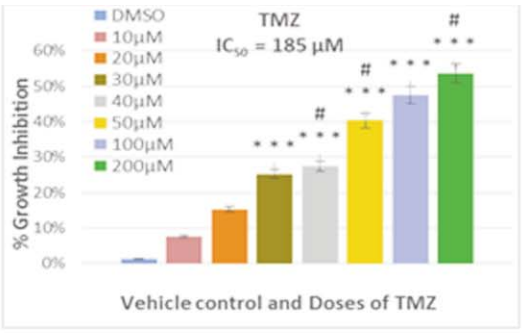

Figure 1 (b)

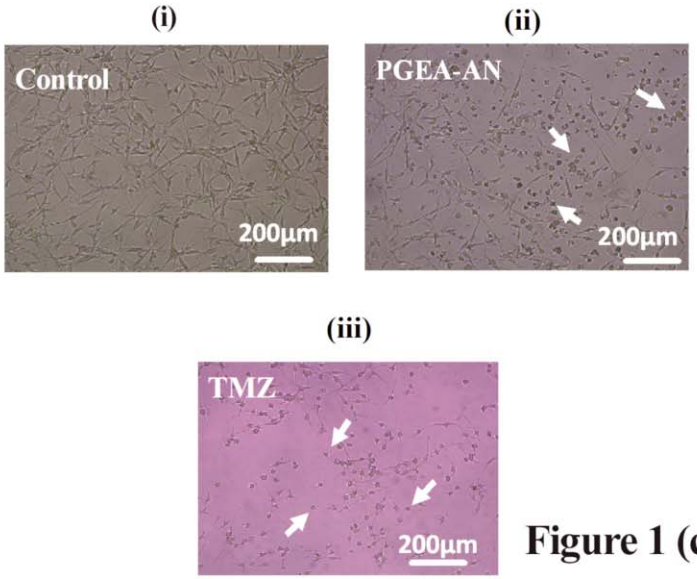

Figure 1 (c)

Figure-1: Effect of 16(R and S) – phenylamino-cleroda3, 13(14) Z-diene-15, 16 olide (PGEA-AN) and temozolomide (TMZ) on growth and morphology of U87 cells after 48 hours of treatment. Bars represents the mean ± standard error of the mean (SEM) of 3 independent experiments. Both PGEA-AN (a) and TMZ (b) inhibited the growth of U87 cells in dose-dependent fashion and half-maximal inhibitory concentration (IC50) was achieved at 19µM and 185µM, respectively. There was significant difference between the treated groups and the control group (***p<0.001). Bonferroni's post-hoc test was applied which revealed a significant difference within different consecutive groups of both PGEA-AN and TMZ. There were significant differences (###p<0.001 and #p<0.05). Representative photomicrographs of the human U87 cells in the control group showed adherent star shaped cells (c i), and apoptotic morphology in PGEA-AN (c ii) and TMZ group (c iii). Cells with apoptotic morphology are indicated by arrow heads. All experiments were performed thrice in triplicates.

effect of PGEA-AN on Bcl-2 at low dose compared to TMZ. It has been reported that PGEA-AN noticeably upregulates pro-apoptotic protein BAX. While it is an established fact that Bcl-2 inhibits apoptosis by antagonising proapoptotic protein BAX, the elevated expression of BAX under the influence of PGEA-AN might be due to the inhibition of Bcl-2. It can be assumed that down-regulation of Bcl-2 might have increased BAX-to-Bcl-2 ratio which leads to cell death via apoptosis through inhibition of mitochondrial pathway.[16] Similar increase in BAX-to-Bcl-2 ratio was also observed in TMZ-treated cells.[22] Several studies have shown the apoptotic activity of terpenes, but at much higher doses compared to PGEA-AN.[23, 24] Further, it has already been reported that it is less nephrotoxic than cisplatin.[16]

Besides apoptotic pathways, there are several other pathways that are also crucial for the maintenance and survival of cells. Among these pathways, Akt/PI3K/mTOR pathway was chosen in the current study as it is well-established that this pathway is disrupted in >75% cases of GBM.[7] Analysis of mRNA expression of target genes of Akt pathway. i.e. Akt, PI3K and mTOR, was also done after PGEA-AN treatment. Results showed no significant difference in the expression of these markers among treatment and control groups. This indicates that there might be no effect of TMZ and PGEA-AN at the mRNA level of GBM. Further, PI3K and mTOR showed apparent surge of expression in the treatment groups, but the results were not statistically significant. In the light of these observations, it can be summed up that TMZ and

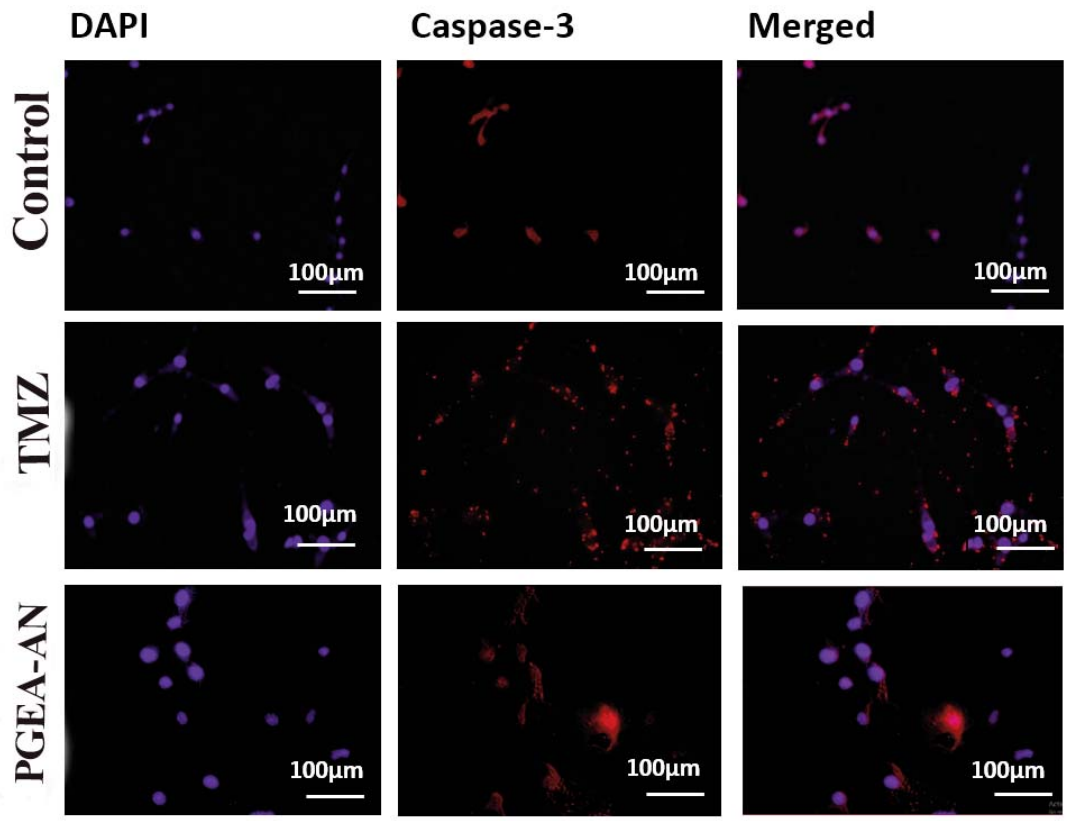

Figure-2 (a)

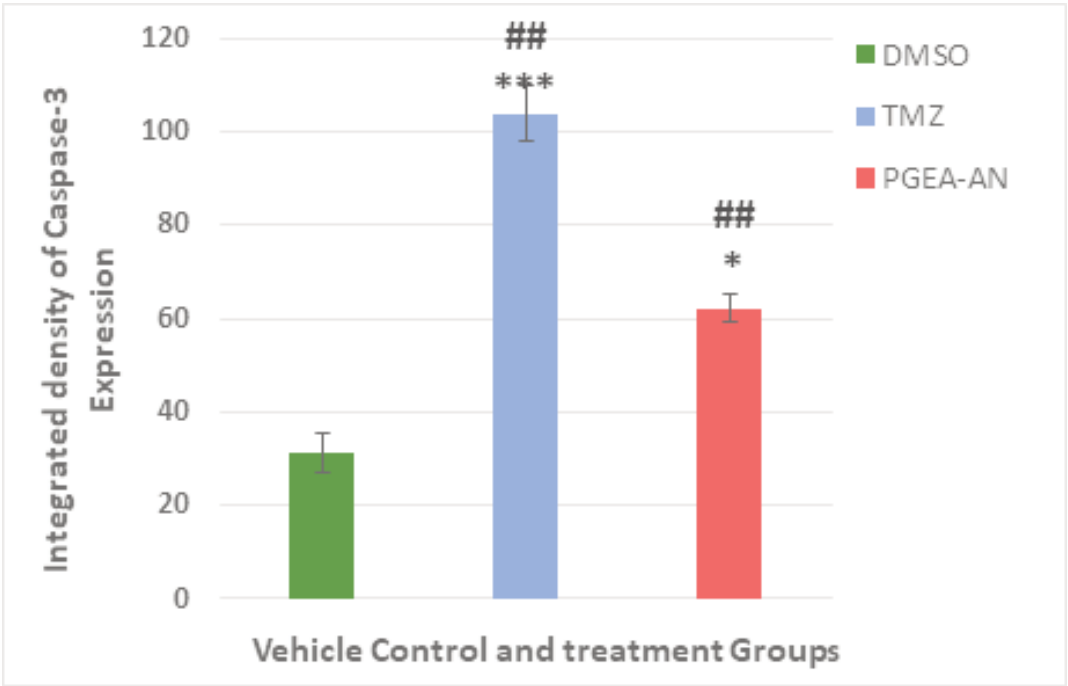

Figure-2 (b)

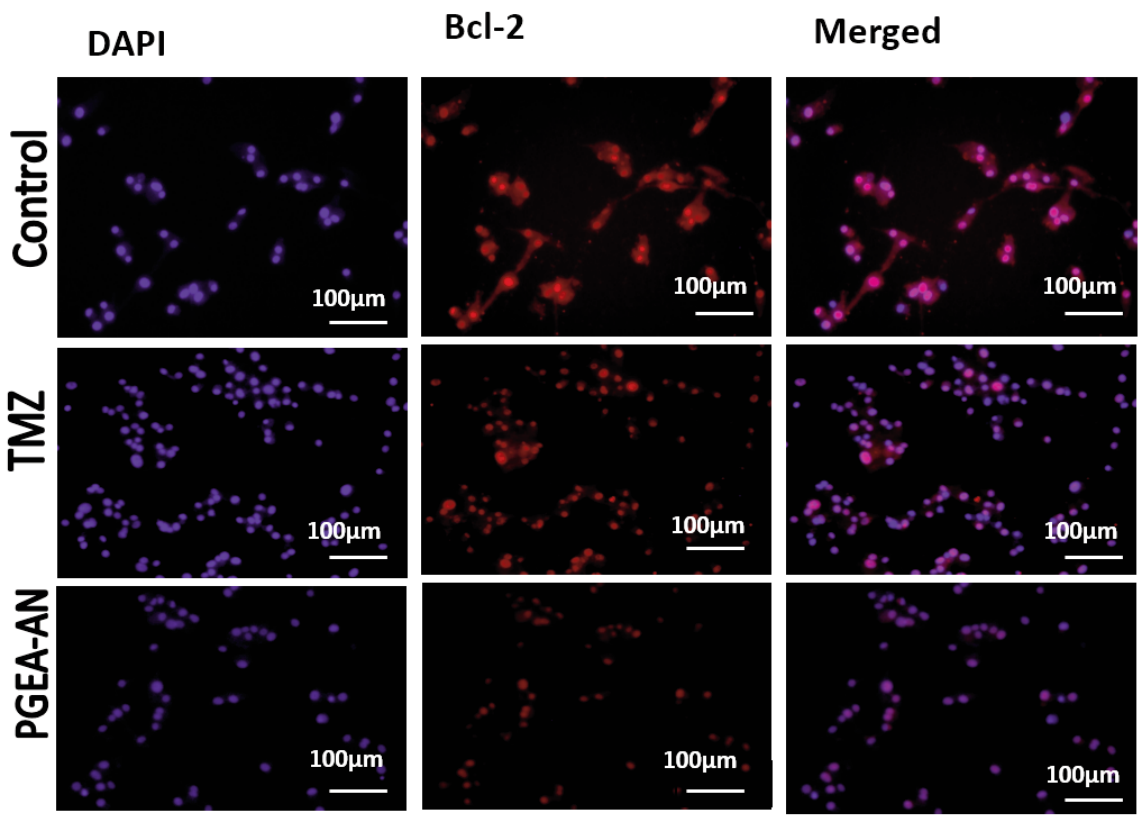

Figure-2 (c)

Figure-2 (d)

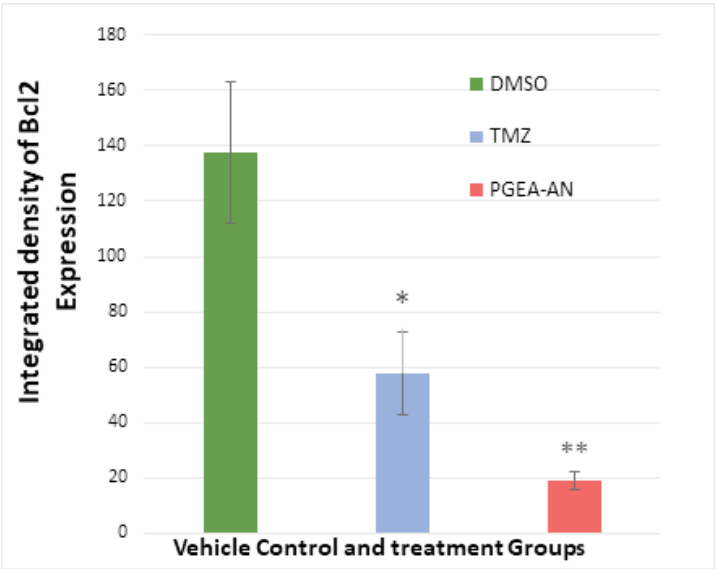

Figure-2 : Representative photomicrographs and bar graphs showing the expression of active caspase-3 and Bcl-2 in U87cells. The human U87 cells were treated with half-maximal inhibitory concentration (IC50) doses of temozolomide (TMZ) and 16(R and S) – phenylamino-cleroda3, 13(14) Z-diene-15, 16 olide (PGEA-AN) for 48 hours. The cells were envisioned by fluorescence microscopy with 20X magnification. (a) Photomicrographs showing noticeable increase in expression of active caspase-3 in both the treatment groups. (b) Graphical representation of active caspase-3 expression showing mean ± standard error of the mean (SEM) of three independent experiments. Active caspase-3 gene expression was significantly increased in the treated groups compared to the control group (*** $p<0.001$ and *$p<0.05$), and between treatment groups (##$p<0.01$). (c) Photomicrographs showing noticeably high expression of Bcl-2 in the control group compared to the treated groups. (d) Graphical representation of Bcl-2 expression showing mean ± SEM of three independent experiments. Bcl-2 gene expression was decreased in TMZ and PGEA-AN groups compared to the control group (*$p=0.05$ and **$p<0.01$). All experiments were performed thrice in triplicates.

PGEA-AN might have no effect on Akt, PI3K and mTOR expression at least at the mRNA level. Previous studies have shown down-regulation of these markers at protein level when treated with TMZ.[25] However, no data is available regarding the effect of PGEA-AN on these markers at mRNA level. These results indicate that PGEA-AN inhibited growth of GBM cells via inducing apoptosis and irrespective of PI3K/Akt/mTOR pathway.

The current study has limitations as it was an in vitro study. In vivo and mechanistic studies are required to validate the anti-GBM effect of PGEA-AN.

Conclusion

PGEA-AN showed growth inhibitory activity against GBM U87 cells via induction of apoptosis, by modulating apoptotic marker caspase-3 and anti-apoptotic marker Bcl-2. Further, PGEA-AN and TMZ did not influence the Akt pathway at the mRNA level.

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Disclaimer: This study was derived from the MPhil Thesis of Dr. Shafea Saad. The title of the thesis is "Modulation of Apoptotic and Akt/PI3K/mTOR pathways to target Glioblastoma Cells using synthetic compound PGEA-AN."

Conflict of Interest: None.

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References

1. 1. Grochans S, Cybulska AM, Simińska D, Korbecki J, Kojder K, Chlubek D, et al. Epidemiology of Glioblastoma Multiforme-Literature Review. Cancers (Basel) 2022;14:2412. doi: 10.3390/cancers14102412.
2. El Atat O, Naser R, Abdelkhalek M, Habib RA, El Sibai M. Molecular targeted therapy: A new avenue in glioblastoma treatment. Oncol Lett 2022;25:46. doi: 10.3892/ol.2022.13632.
3. Louis DN, Perry A, Wesseling P, Brat DJ, Cree IA, Figarella-Branger D, et al. The 2021 WHO Classification of Tumors of the Central Nervous System: a summary. Neuro Oncol 2021;23:1231-5. doi: 10.1093/neuonc/noab106.
4. Hanif F, Muzaffar K, Perveen K, Malhi SM, Simjee ShU. Glioblastoma Multiforme: A Review of its Epidemiology and Pathogenesis through Clinical Presentation and Treatment. Asian Pac J Cancer Prev 2017;18:3-9. doi: 10.22034/APJCP.2017.18.1.3.
5. Hanif F, Perveen K, Malhi SM, Jawed H, Simjee SU. Verapamil potentiates anti-glioblastoma efficacy of temozolomide by modulating apoptotic signaling. Toxicol In Vitro 2018;52:306-13. doi: 10.1016/j.tiv.2018.07.001.
6. Barthel FP, Wesseling P, Verhaak RGW. Reconstructing the molecular life history of gliomas. Acta Neuropathol 2018;135:649-70. doi: 10.1007/s00401-018-1842-y.
7. Valdés-Rives SA, Casique-Aguirre D, Germán-Castelán L, Velasco-Velázquez MA, González-Arenas A. Apoptotic Signaling Pathways in Glioblastoma and Therapeutic Implications. Biomed Res Int 2017;2017:7403747. doi: 10.1155/2017/7403747.
8. Nitulescu GM, Van De Venter M, Nitulescu G, Ungurianu A, Juzenas P, Peng Q, et al. The Akt pathway in oncology therapy and beyond (Review). Int J Oncol 2018;53:2319-31. doi: 10.3892/ijo.2018.4597.
9. Nguyen MV, Han JW, Le Dang Q, Ryu SM, Lee D, Kim H, et al. Clerodane Diterpenoids Identified from Polyalthia longifolia Showing Antifungal Activity against Plant Pathogens. J Agric Food Chem 2021;69:10527-35. doi: 10.1021/acs.jafc.1c02200.
10. Firdous SM, Ahmed SN, Hossain SM, Ganguli S, Fayed MA. Polyalthia longifolia: phytochemistry, ethnomedicinal importance, nutritive value, and pharmacological activities review. Med Chem Res 2022;31:1252-64. Doi:.10.1007/s00044-022-02917-8.
11. Hieu HV, Killari KN, Ranajit SK, Samanth S, Polimati H, Ketha A, et al. A Review on Therapeutic Benefits of Active Chemical Moieties Present in Polyalthia longifolia. Ind J Pharm Sci 2021;83:634-47.
12. Feng Z, Cao J, Zhang Q, Lin L. The drug likeness analysis of anti-inflammatory clerodane diterpenoids. Chin Med 2020;15:126. doi: 10.1186/s13020-020-00407-w.
13. Shamshad S, Versiani MA, Ikram A, Yasmeen K, A Khan R, R Mughal N, et al. Isolation of metallic salts of cytotoxic clerodanes from medicinal plant Polyalthia longifolia var. pendula. Pak J Pharm Sci 2022;35:1691-8.
14. Faizi S, Khan RA, Mughal NR, Malik MS, Sajjadi KE, Ahmad A. Antimicrobial activity of various parts of Polyalthia longifolia var. pendula: isolation of active principles from the leaves and the berries. Phytother Res 2008;22:907-12. doi: 10.1002/ptr.2414.
15. Khan AK, Ahmed A, Hussain M, Khan IA, Ali SA, Farooq AD, et al. Antibiofilm potential of 16-oxo-cleroda-3, 13(14) E-diene-15 oic acid and its five new γ-amino γ-lactone derivatives against methicillin resistant Staphylococcus aureus and Streptococcus mutans. Eur J Med Chem 2017;138:480-9. doi: 10.1016/j.ejmech.2017.06.065.
16. Hussain SS, Rafi K, Faizi S, Razzak ZA, Simjee SU. A novel, semi-synthetic diterpenoid 16(R and S)-phenylamino-cleroda-3,13(14), Z-dien-15,16 olide (PGEA-AN) inhibits the growth and cell survival of human neuroblastoma cell line SH-SY5Y by modulating P53 pathway. Mol Cell Biochem 2018;449:105-1. doi: 10.1007/s11010-018-3347-3.
17. Ashfaque A, Hanif F, Simjee SU, Bari MF, Faizi S, Zehra S, et al. Opuntiol Inhibits Growth and Induces Apoptosis in Human Glioblastoma Cells by Upregulating Active Caspase 3 Expression. Asian Pac J Cancer Prev 2021;22:3607-13. doi: 10.31557/APJCP.2021.22.11.3607.
18. Collins TJ. ImageJ for microscopy. Biotechniques 2007;43:25-30. doi: 10.2144/000112517.
19. Zhang JD, Ruschhaupt M, Biczok R. ddCt method for qRT–PCR data analysis. Citeseer 2013;48:346-56.
20. Neophytou CM, Trougakos IP, Erin N, Papageorgis P. Apoptosis Deregulation and the Development of Cancer Multi-Drug Resistance. Cancers (Basel) 2021;13:4363. doi: 10.3390/cancers13174363.
21. Do BH, Nguyen TPT, Ho NQC, Le TL, Hoang NS, Doan CC. Mitochondria-mediated Caspase-dependent and Caspase-independent apoptosis induced by aqueous extract from Moringa oleifera leaves in human melanoma cells. Mol Biol Rep 2020;47:3675-89. doi: 10.1007/s11033-020-05462-y.
22. Das A, Banik NL, Patel SJ, Ray SK. Dexamethasone protected human glioblastoma U87MG cells from temozolomide induced apoptosis by maintaining Bax:Bcl-2 ratio and preventing proteolytic activities. Mol Cancer 2004;3:36. doi: 10.1186/1476-4598-3-36.
23. Cai J, Zhong YY, Tian S. Naturally occurring davanone terpenoid exhibits anticancer potential against ovarian cancer cells by

inducing programmed cell death, by inducing caspase-dependent apoptosis, loss of mitochondrial membrane potential, inhibition of cell migration and invasion and targeting PI3K/AKT/MAPK signaling pathway. J BUON 2020;25:2301-7.
24. Tarapore RS, Siddiqui IA, Adhami VM, Spiegelman VS, Mukhtar H. The dietary terpene lupeol targets colorectal cancer cells with constitutively active Wnt/β-catenin signaling. Mol Nutr Food Res 2013;57:1950-8. doi: 10.1002/mnfr.201300155.
25. Zhou W, Liu L, Xue Y, Zheng J, Liu X, Ma J, et al. Combination of Endothelial-Monocyte-Activating Polypeptide-II with Temozolomide Suppress Malignant Biological Behaviors of Human Glioblastoma Stem Cells via miR-590-3p/MACC1 Inhibiting PI3K/AKT/mTOR Signal Pathway. Front Mol Neurosci 2017;10:68. doi: 10.3389/fnmol.2017.00068.