Valproic Acid Induced Differentiation and Potentiated Efficacy of Taxol and Nanotaxol for Controlling Growth of Human Glioblastoma LN18 and T98G Cells

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Abstract Glioblastoma shows poor response to current therapies and warrants new therapeutic strategies. We examined the efficacy of combination of valproic acid (VPA) and taxol (TX) or nanotaxol (NTX) in human glioblastoma LN18 and T98G cell lines. Cell differentiation was manifested in changes in morphological features and biochemical markers. Cell growth was controlled with down regulation of vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), nuclear factor-kappa B (NF-κB), phospho-Akt (p-Akt), and multi-drug resistance (MDR) marker, indicating suppression of angiogenic, survival, and multi-drug resistance pathways. Cell cycle analysis showed that combination therapy (VPA and TX or NTX) increased the apoptotic sub G1 population and apoptosis was further confirmed by Annexin V-FITC/PI binding assay and scanning electron microscopy. Combination therapy caused activation of caspase-8 and cleavage of Bid to tBid and increased Bax:Bcl-2 ratio and mitochondrial release of cytochrome c and apoptosis-inducing factor (AIF). Upregulation of calpain and caspases (caspase-9 and caspase-3) and substrate degradation were also detected in course of apoptosis. The combination of VPA and NTX most effectively controlled the growth of LN18 and T98G cells. Therefore, this combination of drugs can be used as an effective treatment for controlling growth of human glioblastoma cells.

Keywords Apoptosis · Glioblastoma · Nanotaxol · Taxol · Valproic acid

Introduction

Glioblastoma is the most common and deadliest malignancy, accounting for nearly 40% of primary brain tumors in adults with a mean survival of less than 1 year following diagnosis [1]. It is a heterogenous and highly vascularized brain tumor characterized by poorly differentiated neoplastic astrocytes that avoid apoptosis due to P-glycoprotein (P-gp) mediated multi-drug resistance [2]. Development of new therapeutic strategies that induce differentiation and apoptosis, provide anti-angiogenic activity, and overcome multi-drug resistance is urgently warranted.

Valproic acid (VPA), a histone deacetylase inhibitor, has been shown to induce differentiation and anti-angiogenic and anti-proliferative activities in several cancers, including both P-gp positive and negative cell lines [3]. The multimodal efficacy of VPA with other anti-cancer drugs could be useful for successful treatment of glioblastoma. While the anti-cancer efficacy of taxol (TX) is well established in ovarian, head-and-neck, bladder, breast, and lung cancers [4], recently TX has also been shown to provide potent therapeutic effects in experimental brain tumor [5, 6].

Because of its low aqueous solubility, TX is less permeable to the blood–brain-barrier (BBB) [7]. Besides, the commercial formulation of 6 mg/ml (7 mM) TX in 1:1 mixture of the solvents Cremophor EL® (CrEL, polyoxyethylated castor oil) and ethanol has systemic toxicity, side effects, and short time stability, and is associated with hypersensitivity reactions [8]. TX is a substrate for the P-gp
Many attempts have been made to reformulate TX-bound nanoparticles, which are natural or synthetic polymers of lipids or metal containing vehicles of dimensions <100 nm and hold promise as the effective drug delivery carriers across the BBB [12]. ABI-007, also known as ‘Abraxane’, is a Cremophor-free, albumin-bound 130-nm nanoparticle form of TX that has shown anti-cancer activity in human lung (H522), breast (MX-1), ovarian (SK-OV-3), prostate (PC-3), and colon (HT29) tumor xenografts [13]. The delivery of TX as Abraxane has been found to overcome insoluble nature and solvent related toxicities of TX [14]. The clinical potency of Abraxane in metastatic breast cancer [15] and advanced non-small cell lung cancer patients is well established [14], but the therapeutic potency of Abraxane or nanotaxol (NTX) in glioblastoma has not yet been explored.

In this study, we investigated the anti-tumor efficacy of VPA, TX, or NTX alone and in combination in multi-drug resistant glioblastoma LN18 (with overexpression of P-gp) and T98G (with less expression of P-gp) cell lines [2, 16–19]. The combination of VPA and TX or NTX was found to markedly down regulate several survival signals and angiogenesis markers, induce differentiation, and activate mitochondria-dependent pathways for apoptosis in both LN18 and T98G cell lines.

Materials and Methods

Reagents

VPA and primary β-actin IgG antibody (monoclonal clone AC-15) were obtained from Sigma Chemical (St. Louis, MO). TX (paclitaxel) was procured from Bristol-Myers Squibb (Princeton, NJ). NTX (Abraxane) was obtained from Abraxis Oncology (Schaumburg, IL). The colorimetric MTT assay kit was procured from Millipore Chemicon (Temecula, CA). AnnexinV-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit for detection of apoptosis was purchased from BD Biosciences (San Jose, CA). Wright staining kit was bought from Fisher Scientific (Middletown, VA). Other primary IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Depending on the primary antibody used was either horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG (ICN Biomedicals, Aurora, OH). Enhanced Chemiluminescence (ECL) Plus reagent was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ).

Cell Culture and Treatments

Human glioblastoma LN18 and T98G cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Both LN18 and T98G cells were separately grown in monolayer to sub-confluency in 75-cm² flasks containing 10 ml of DMEM and RPMI 1640 (Mediatech, Herndon, VA), respectively, and supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 1% penicillin, and 1% streptomycin (GIBCO, Grand Island, NY) in a fully-humidified incubator containing 5% CO₂ and 95% air at 37°C. Cells were allowed to starve for 24 h in RPMI 1640 or DMEM with 2% FBS, 1% penicillin, and 1% streptomycin and thereafter maintained in this low-serum condition during the treatments. Freshly prepared stock solutions of VPA, TX, and NTX were made in serum-free medium just prior to treatment. Dose–response studies were carried out to determine the suitable doses of the drugs for the inhibition of cell growth, induction of differentiation, and cell death. Cells were treated with 0.5, 1, 2, or 4 mM of VPA for 72 h followed by treatment with either 50 nM TX or 50 nM NTX for 24 h alone and in combination.

MTT Assay

The differential sensitivities of LN18 and T98G cells to VPA, TX, or NTX either alone or in combination were determined using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay. We used different doses of VPA to induce differentiation whereas a fix dose of either TX or NTX to induce apoptosis. The rationale was to reduce cell proliferation with induction of differentiation and increase apoptosis using combination of drugs in glioblastoma cells. For MTT assay, both cell lines were first exposed to 0.5, 1, 2, and 4 mM VPA for 72 h. At 48 h post-exposure of VPA, cells were treated with 50 nM TX or NTX for another 24 h and in combination with VPA (total treatment for 72 h). Cells were then exposed to MTT reagent for 4 h at 37°C after which isopropanol was used to dissolve the formazan crystals. The optical density (OD) was recorded at 570 nm in a microplate reader. Percent growth inhibition was determined as [(1-(OD of treated cells/OD of control cells))×100. All experiments were performed in triplicates. Cell viability data were analyzed using Compusyn software (ComboSyn, Paramus, NJ) to generate a combination index (CI). Conventionally, CI > 1 indicates antagonism, CI = 1 indicates additive effect, and CI < 1 indicates synergism [20].
Wright Staining for Examination of Morphological Features of Apoptosis

Both LN18 and T98G cells were treated with 2 mM VPA for 72 h as monotherapy and at 48 h post-exposure of VPA, 50 nM TX or NTX was added alone separately or in combination with VPA for another 24 h (total treatment for 72 h). The cells (adherent and non-adherent) from each treatment were centrifuged and sedimented on the culture dish and washed twice with phosphate-buffered saline (PBS, pH 7.4). The cells were fixed with 95% (v/v) ethanol and dried before Wright staining. The morphology of apoptotic cells were detected under the light microscope. Cells were considered apoptotic with characteristic features such as chromatin condensation, cell-volume shrinkage, and membrane-bound apoptotic bodies [18].

Cell Cycle Analysis

LN18 and T98G cells were seeded in a 6-well plate at a density of $1 \times 10^5$ cells per well and incubated for 24 h in low-serum medium prior to addition of drugs. Cells were untreated (control, CTL) and treated with 2 mM VPA for 72 h as monotherapy and at 48 h post-exposure of VPA, 50 nM TX or NTX was added alone separately or in combination with VPA for another 24 h (total treatment for 72 h). Cells were trypsinized and harvested by centrifugation. Flow cytometric analysis of cell cycle was performed by staining of permeabilized cells with PI for DNA content. Cells were re-suspended in PBS, fixed with 70% ethanol, labeled with staining solution (0.05 mg/ml PI, 2 mg/ml RNase A, 0.1% TritonX-100 in PBS), and incubated for 30 min at room temperature (RT) in darkness. DNA content was then analyzed using an Epics XL-MCL Flow Cytometer (Beckman Coulter, Fullerton, CA), as we reported recently [21].

Flow Cytometric Analysis of Apoptosis

The percentage of cells undergoing apoptosis was quantitatively determined by Annexin V-FITC/PI binding assay. LN18 and T98G cells were seeded in 6-well plate at a density of $1 \times 10^5$ cells per well and incubated for 24 h in low-serum medium prior to addition of drugs. Cells were untreated (CTL) and treated with 2 mM VPA for 72 h as monotherapy and at 48 h post-exposure of VPA, 50 nM TX or NTX was added alone separately or in combination with VPA for another 24 h (total treatment for 72 h). After treatments, both adherent and non-adherent cells were harvested and double-stained using the Annexin V-FITC/PI staining kit according to the manufacturer’s protocol. Cells were washed with cold PBS, resuspended in 1× binding buffer (0.1 M HEPES/NaOH, pH 7.4, 1.4 M NaCl, 25 mM CaCl$_2$), stained with Annexin V-FITC/PI, and incubated for 15 min at RT in the darkness. Cells were analyzed for Annexin V-stained apoptotic population using an Epics XL-MCL Flow Cytometer (Beckman Coulter, Fullerton, CA), as per our previous reports [20, 21].

Morphological Analysis by Scanning Electron Microscopy

Both LN18 and T98G cells were grown to sub-confluency on 13-mm round cover slip and incubated in low-serum medium for 24 h before addition of drugs. Cells were either untreated (CTL) and treated with 2 mM VPA for 72 h as monotherapy and at 48 h post-exposure of VPA, 50 nM TX or NTX was added alone separately or in combination with VPA for another 24 h (total treatment for 72 h). After the treatments, cells were fixed with 2.5% glutaraldehyde in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 1 h at RT. Cells were post-fixed in 1.0% osmium tetroxide (OsO$_4$) for 10 min and washed in 0.1 M phosphate buffer (pH 7.2). Dehydration was done in ascending concentration of ethanol. Cover slips were dried with the critical point apparatus using liquid CO$_2$ as transition fluid. The specimens were cold sputter coated with gold and observed in a JEOL 6,300 V Scanning Electron Microscope (SEM) at an accelerating voltage of 15 kV, as we reported earlier [21].

Protein Extraction and Western Blotting

For Western blotting, total proteins were extracted following homogenization of cells, quantitated spectrophotometrically, denatured in boiling water for 5 min, and resolved by SDS-polyacryl amide gel electrophoresis and then electroblotted to the polyvinylidene (PVDF) membranes. The blots were incubated with a primary IgG antibody followed by incubation with an alkaline HRP-conjugated secondary IgG antibody. Subsequently, specific protein bands were detected by HRP/H$_2$O$_2$-catalyzed oxidation of luminol in alkaline conditions using the ECL system and autoradiography [18, 22].

Statistical Analysis

Results were analyzed using Minitab® 15 Statistical Software (Minitab, State College, PA). Data were expressed as mean ± standard error of mean (SEM) of separate experiments ($n \geq 3$) and compared by one-way analysis of variance (ANOVA) followed by Fisher’s post hoc test. Significant difference between CTL and a treatment was indicated by *$P < 0.05$, or **$P < 0.001$; and between TX and VPA + TX, or NTX and VPA + NTX was indicated by #$P < 0.05$. 
Results

Treatments Decreased Cell Viability

The growth inhibitory effect of VPA, TX, and NTX either alone or in combination on LN18 and T98G cells were determined (Fig. 1). VPA monotherapy was used at different concentrations such as 0.5, 1, 2, and 4 mM for 72 h while 50 nM TX or 50 nM NTX was used alone for 24 h. For combination studies, the anti-proliferative effect was determined by treating both cell lines with VPA (0.5, 1, 2, and 4 mM) for 48 h and then either 50 nM TX or 50 nM NTX was added in presence of VPA for another 24 h (total treatment for 72 h). VPA inhibited cell growth in both cell lines (Fig. 1a). Treatment with VPA dose-dependently decreased the cell viability in LN18 and T98G cells. The percentages of cell viability in LN18 and T98G cells after treatment with 50 nM TX or NTX for 24 h were also decreased (Fig. 1a). Most importantly, treatment with VPA + TX, or VPA + NTX showed significant reduction in cell viability when compared with TX or NTX monotherapy (Fig. 1a).

We used a variety of doses of VPA but a fixed dose of either TX or NTX and found that higher doses of combination of drugs showed more cell death than lower doses. But our aim was to use particular doses of the drugs, which would induce cellular differentiation, inhibit cell proliferation, and arrest cell cycle to trigger apoptosis. VPA (2 mM) and TX or NTX (50 nM) alone showed the best efficacy, VPA (2 mM) + TX (50 nM) produced the best synergistic efficacy (CI = 0.791 in LN18 cells and CI = 0.98 in T98G cells), and VPA (2 mM) + NTX (50 nM) also showed the best synergistic efficacy (CI = 0.45 in LN18 cells and CI = 0.49 in T98G cells) (Fig. 1a) and therefore, these treatments were finally selected for further studies for inducing differentiation and apoptosis.

Fig. 1 VPA, TX, and NTX alone and combination therapy decreased cell viability and increased apoptotic features in LN18 and T98G cells. Treatments: 0 control (CTL), 1 CTL (untreated) or monotherapy (0.5 mM VPA, and 50 nM TX or NTX) or combination therapy (0.5 mM VPA followed by either 50 nM TX or 50 nM NTX), 2 CTL (untreated) or monotherapy (1 mM VPA, and either 50 nM TX or 50 nM NTX) or combination therapy (1 mM VPA followed by either 50 nM TX or 50 nM NTX), 3 CTL (untreated) or monotherapy (2 mM VPA, and either 50 nM TX or 50 nM NTX) or combination therapy (2 mM VPA followed by either 50 nM TX or 50 nM NTX), and 4 CTL (untreated) or monotherapy (4 mM VPA, and either 50 nM TX or 50 nM NTX) or combination therapy (4 mM VPA followed by either 50 nM TX or 50 nM NTX). Treatment schedule with monotherapy: VPA for 72 h, TX or NTX for 24 h; and with combination therapy: VPA for 48 h and then TX or NTX in presence of VPA for 24 h (total treatment for 72 h). a MTT assay was used to assess cell viability following monotherapy and combination therapy. b Wright staining to evaluate the morphological features of differentiation and apoptosis
Induction of Morphological and Biochemical Features of Differentiation and Apoptosis

Wright staining revealed that exposure to VPA inhibited cell proliferation and induced morphological features of differentiation such as long and thin projections of the cells (Fig. 1b). Western blotting showed the biochemical features of astrocytic differentiation with upregulation of the glial fibrillary acidic protein (GFAP) and down regulation of the inhibitor of differentiation 2 (ID2) in both LN18 and T98G cells (Fig. 2a, c, d). Uniform β-actin expression served as an internal control. Increase in GFAP, an astrocyte-specific intermediate filament protein, is a biochemical marker of differentiation in glioblastomas. The treatment with VPA + TX caused upregulation of GFAP expression in T98G cells only whereas VPA + NTX significantly increased GFAP expression in both LN18 and T98G cells, when compared with control or monotherapy (Fig. 2a, c, d).

Inhibition of Angiogenic, Survival, and Multi-Drug Resistance Markers

Vascular endothelial growth factor (VEGF) is a major regulatory factor in tumor angiogenesis and predominantly

![Table and graphs showing Western blotting results and densitometric analysis of protein levels in LN18 and T98G cells.](image)
overexpressed in glioblastoma [23]. Western blotting was used to examine any effect of the single or combinatorial treatment on VEGF expression. Both single and combination therapies were found to potently inhibit VEGF expression in both LN18 and T98G cells (Fig. 2b–d).

Another genetic alteration associated with glioblastoma is amplification of epidermal growth factor receptor (EGFR) gene, resulting in overexpression of EGFR protein. EGFR amplification has been shown to increase glioblastoma proliferation and invasion [24]. We assessed expression of EGFR by Western blotting. Use of VPA + TX was found to significantly reduce EGFR expression whereas VPA + NTX totally abolished EGFR expression in both LN18 and T98G cell lines (Fig. 2b), compared with all other treatments (Fig. 2c, d).

The levels of several anti-apoptotic proteins were determined by Western blotting (Fig. 2b). Treatment with the combination of drugs (either VPA + TX or VPA + NTX) showed dramatic down regulation of the survival factors such as NF-κB, total-Akt, and p-Akt in both LN18 and T98G cell lines (Fig. 2b). Treatment with VPA + NTX most effectively inhibited expression of the survival factors (e.g., NF-κB, total-Akt, and p-Akt) in both cell lines (Fig. 2c, d).

Untreated LN18 cells (harboring overexpression of P-gp) showed high level of P-gp expression. Treatment with 2 mM VPA alone and especially combination of drugs (either VPA + TX or VPA + NTX) remarkably down regulated the P-gp expression in LN18 cell line (Fig. 2b, c). No detectable level of P-gp was observed in untreated T98G cells (harboring very little expression of P-gp). Use of VPA + NTX was more effective than that of VPA + TX in down regulating MDR expression.

Flow Cytometric Analysis of Cell Cycle

Flow cytometric analysis was performed to examine the cell cycle distribution patterns in LN18 and T98G cells following treatments (Fig. 3). Flow cytometric data revealed that treatment with VPA caused a small increase in sub G1 phase, a decrease in G0/G1 phase, and an increase in G2/M population in both cell lines. Treatment with TX showed a significant increase in sub G1 phase and down regulated G0/G1 population with a significant increase in G2/M phase in both LN18 and T98G cell lines. A significant increase in sub G1 phase and a decrease in both G0/G1 and G2/M populations in both cell lines (Fig. 3a) were seen due to treatment with NTX. Combination therapy (either VPA + TX or VPA + NTX) most effectively increased the sub G1 apoptotic phase. Notably, the use of VPA + NTX caused more apoptosis than the use of VPA + TX in both cell lines (Fig. 3b).

![Fig. 3](image-url)
Flow Cytometric Detection of Apoptosis by Annexin V-FITC/PI Binding Assay

Annexin V-FITC/PI double-labeling technique was used to understand the nature of cell death (apoptotic or necrotic) after the treatments (Fig. 4). Monotherapy with VPA, TX, and NTX induced apoptosis in LN18 and T98G cells (Fig. 4a). But the combination therapy showed a significant increase in apoptosis, when compared with either treatment alone. Flow cytometric data revealed that combination therapy (either VPA + TX or VPA + NTX) most significantly increased apoptosis in both glioblastoma cell lines (Fig. 4b). However, use of VPA + NTX showed greater induction of apoptosis than the use of VPA + TX in both cell lines. Annexin V-FITC/PI staining confirmed the induction of cell death through phosphatidylserine externalization and indicated the mode of cell death was apoptosis and not necrosis.

Scanning Electron Microscopy to Detect Fine Morphological Changes

Scanning electron microscopy of both LN18 and T98G cells after treatment with VPA showed heterogeneity of cytoplasmic prolongations and microextensions, including blebs and filopodia formation (Fig. 5). On the other hand, combination therapy (either VPA + TX or VPA + NTX) most effectively induced certain features of apoptosis.
including membrane blebbing, perforations, loss of membrane integrity, nuclear fragmentations, cell shrinkage, and formation of apoptotic bodies (Fig. 5).

Activation of Caspase-8 and Proteolytic Cleavage of Bid to tBid

In order to determine the changes in apoptosis related proteins in both LN18 and T98G cells following treatments, we performed Western blotting (Fig. 6). Uniform β-actin expression served as a loading control. Compared with control and monotherapy, treatment with VPA + NTX caused substantial increase in the 20 kD active caspase-8 fragment in both cell lines. Active caspase-8 cleaved Bid as a substrate for generation of tBid (Fig. 6a) and its translocation to mitochondria for induction of cell death. Thus, this combination therapy was capable of inducing apoptosis partly via extrinsic pathway in both LN18 and T98G cell lines.

Induction of Apoptosis with an Increase in Bax:Bcl-2 Ratio

The Bcl-2 family of proteins plays a key role in regulation of mitochondrial permeability during apoptosis via intrinsic pathway. The levels of expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were examined by Western blotting to determine the changes in Bax:Bcl-2 ratio (Fig. 6b). Expression of β-actin, considered as a loading control, was uniform in all treatment groups. Cells treated with TX, NTX, and VPA + TX showed an increase in Bax expression and decrease in Bcl-2 expression in both cell lines (Fig. 6c). Use of VPA + NTX most dramatically increased Bax expression and decreased Bcl-2 expression, when compared with control or single treatments. Combination therapy significantly increased the Bax:Bcl-2 ratio in both cell lines. An increase in the Bax:Bcl-2 ratio can trigger release of several pro-apoptotic molecules from mitochondria for activation of intrinsic caspase cascade for apoptosis.

Activation of Mitochondrial Pathway of Apoptosis

An alteration in the permeability of the outer mitochondrial membrane causes the release of various pro-apoptotic molecules, including cytochrome c and AIF, which promote the mitochondrial pathway of apoptosis (Fig. 7). We used β-actin as cytosolic loading control and expression was almost uniform in all treatment groups. Cells treated with TX, NTX, VPA + TX, or VPA + NTX showed an increase in cytosolic levels of cytochrome c in both LN18 and T98G cell lines (Fig. 7a). Single treatment with VPA showed upregulation of cytosolic cytochrome c in T98G cells but not in LN18 cells (Fig. 7a). Mitochondrial release of cytochrome c is a well known pre-requisite for formation of apoptosome and activation of caspases for apoptosis. Treatment with NTX alone or VPA + NTX generated the cytosolic cleaved AIF fragments in both LN18 and T98G cells (Fig. 7a) and thereby facilitated apoptosis.

Upregulation of Cysteine Proteases

Western blotting was used to determine expression of proteases such as calpain and caspase-9 in both LN18 and T98G cells following single (VPA, TX, and NTX) and combination (either VPA + TX or VPA + NTX) treatments (Fig. 7b). Level of β-actin expression remained uniform in all treatments and was used as an internal control. Single or combination therapy increased expression of calpain, a Ca^{2+}-dependent cysteine protease, and also expression and activation of caspase-9 in both glioblastoma cell lines (Fig. 7b). Further, activation of caspase-
3 was detected following combination therapy in both cell lines (Fig. 7b). Caspase-3, the major executioner caspase [25], causes cleavage of cellular substrates to promote morphological and biochemical features of apoptosis.

Increase in Proteolysis

Calpain and caspase-3 can be simultaneously activated for apoptosis in glioblastoma cells [18]. We found the increases in calpain and caspase-3 activities in the formation of calpain-specific 145 kD spectrin breakdown products (SBDP) and caspase-3 specific 120 kD SBDP, respectively, in both LN18 and T98G cells following combination therapy (Fig. 7c).

The increase in caspase-3 activity was further examined in the cleavage of inhibitor of caspase-3 activated DNase (ICAD) (Fig. 7c) that would then release CAD from the CAD/ICAD complex followed by CAD translocation to the nucleus for DNA fragmentation. Our results showed that single therapy (VPA, TX, and NTX) or combination therapy (either VPA + TX or VPA + NTX) markedly caused cleavage of ICAD in LN18 and T98G cells (Fig. 7c). Thus, degradation of ICAD further confirmed an increase in caspase-3 activity in apoptosis.

Taken together, the results of our investigation showed induction of differentiation, suppression of angiogenesis, survival, and multi-drug resistance markers, activation of proteolytic pathways, and cleavage of specific substrates for apoptosis in both LN18 and T98G cells following combination therapy (either VPA + TX or VPA + NTX). Based on our experimental results, we provided a schematic model to show the sequence of molecular events in these pathways after the treatments of human glioblastoma cells (Fig. 8).
In this investigation, we explored the efficacy of the combination therapy (either VPA + TX or VPA + NTX) in two multi-drug resistant glioblastoma cell lines LN18 (increased P-gp expression) [2] and T98G (reduced P-gp expression) [16, 17]. This is the first report on a novel combination therapy (either VPA + TX or VPA + NTX) for human glioblastoma LN18 and T98G cells. Earlier findings reported that VPA reduced cell proliferation, induced cytotoxicity in human glioblastoma cells, and increased GFAP expression for astrocytic differentiation [26]. To observe the therapeutic effects, most of the preclinical cell culture studies used a high dose of VPA that could develop new derivatives of VPA having more potent activity in lower dose than the parent VPA. The current study for the first time demonstrated that either VPA + TX or VPA + NTX down regulated ID2 expression in both cell lines. The ID family of helix-loop-helix proteins is involved in cellular proliferation and angiogenesis and
The VEGF family and its receptors are known to act as the central signaling pathway in glioblastoma angiogenesis [23]. Amplification of EGFR gene often occurs in glioblastoma resulting in overexpression of EGFR, a transmembrane tyrosine kinase receptor responsible for glioblastoma proliferation and invasion [24]. Our combination therapy using VPA + TX or NTX showed potent anti-angiogenic activity by down regulating expression of VEGF and EGFR. VPA is a potent inhibitor of tumor angiogenesis and caused significant decrease in VEGF expression in colon adenocarcinoma cell line [28]. TX down regulates VEGF expression in LN18 cells [29] and other glioblastoma U138MG and U251MG cells [30]. Our combination therapy showed the anti-angiogenic potential by down regulating expression of VEGF and EGFR in glioblastoma cells. A previous study reported synergistic efficacy of combination of pegylated-interferon-alpha (PEG-IFN-α) and TX in glioblastoma U87MG cells due to inhibition of proliferation and angiogenesis [31]. Similarly, a Phase II clinical trial of TX and topotecan with filgrastim (also known as G-CSF) showed therapeutic effects in glioblastoma patients [32], indicating importance of TX in the combination therapy to control growth of glioblastoma.

Aberrant or overexpressed Akt signaling is a major event in glioblastoma. Phosphorylation of Akt (p-Akt) results in activation of Akt kinase activity, which has high potential to deregulate cell cycle, induce cell proliferation, avoid apoptosis, and stimulate cell survival through upregulation of NF-κB [33]. So, NF-κB down regulation leads to apoptosis [34]. Combination therapy using VPA + TX and especially VPA + NTX showed down regulation of survival signals and anti-apoptotic factors due to suppressed expression of total-Akt, p-Akt, and NF-κB in both LN18 and T98G cells. TX in combination with all-trans retinoic acid decreased expression of p-Akt and NF-κB to promote apoptosis in U87MG xenografts [6], implicating the importance of combination therapy for suppression of survival pathways in glioblastoma.

Down regulation of P-gp expression was observed in both LN18 and T98G cells following combination therapy, indicating VPA could work as an inhibitor of P-gp to facilitate TX and NTX induced cytotoxicity by overcoming multi-drug resistance. Our study is in agreement with the previous reports that demonstrate Elacridar [35] and Valspodar [10], the strong inhibitors of P-gp, are highly effective in increasing the delivery of TX across the BBB and significantly reducing tumor volume in glioblastoma xenografts. Recently, an oral combination therapy using the P-gp inhibitor HM30181A and TX significantly regressed tumor volume by inhibiting TX efflux in animal model of glioblastoma [11], thereby lending support to the possibility of either VPA + TX or VPA + NTX as the potential combination therapy for treatment of glioblastoma.
to VPA treatment, supporting a previous finding where VPA treatment induced stellate and flattened morphological appearances in glioblastoma cells [26]. The combination therapy (either VPA + TX or VPA + NTX) showed characteristic features of apoptosis including membrane asymmetry and blebbing, cell shrinkage, and apoptotic body formation in both LN18 and T98G cells.

Treatment with either VPA + TX or VPA + NTX was found to activate caspase-8 for Bid cleavage, indicating activation of extrinsic or death receptor pathway for apoptosis. Earlier studies reported that VPA alone [38] and in combination with IFN-γ [39] induced apoptosis through activation of caspase-8, Bid cleavage, and alterations in expression of Bax and Bcl-2. TX alone [6, 30, 40] or in combination with all-trans retinoic acid [41] caused caspase-8 activation for Bid cleavage to generate tBid and trigger apoptosis. Caspase-8 mediated cleavage of Bid provides a link between death receptor and mitochondrial pathways of apoptosis. An increase in the Bax:Bcl-2 ratio is a key determinant step for inducing the release of several pro-apoptotic molecules from the mitochondria. In our current study, VPA + TX and more importantly VPA + NTX caused significant upregulation of Bax with concomitant down regulation of Bcl-2 in both LN18 and T98G cells to cause an increase in the Bax:Bcl-2 ratio, which appeared in line with previous studies where TX induced apoptosis by phosphorylation and inactivation of Bcl-2 [42] and caused upregulation of Bax and down regulation of Bcl-2 in LN18 cells [29] and also in U138MG and U251MG cells [30].

Translocation of tBid and upregulation of Bax are associated with mitochondrial release of cytochrome c in the cytosol [18], which is a prerequisite for formation of apoptosome leading to activation of caspase-9 and caspase-3 for apoptosis [43]. Previously, TX alone or in combination with all-trans retinoic acid was reported to dramatically increase the level of cytosolic cytochrome c in LN18 cells as well as in T98G and U87MG xenografts [1, 6, 29]. Mitochondria can also induce caspase-independent apoptosis through release of AIF into the cytosol [44]. In this study, we showed that combination therapy with VPA + TX and more importantly VPA + NTX increased the expression of active fragment of AIF in the cytosol. Both tBid and Bax translocation to mitochondria can change mitochondrial outer membrane permeabilization, resulting in the release of cytochrome c in the cytosol for activation of caspases. AIF is a mitochondrial membrane integral protein that can be cleaved by active calpain for formation of a soluble apoptogenic form to exert caspase-independent apoptosis [45].

In our study, VPA + NTX was more effective than VPA + TX to increase the expression of caspase-9, calpain, and caspase-3 activities (shown in the formation of 145 kD SBDP and 120 kD SBDP, respectively) and also caspase-3 mediated ICAD cleavage were found in both LN18 and T98G cell lines. The activation of these proteases and their substrate cleavage leading to apoptosis were previously reported in glioblastoma LN18 [26, 29, 46], U138MG and U251MG cells [30], and T98G and U87MG xenografts [1, 6, 29].

Taken together, results from the current investigation showed that VPA + TX and most notably VPA + NTX induced differentiation, suppressed angiogenic and survival factors, inhibited multi-drug resistance, and activated calpain and caspase proteolytic cascades for induction of apoptosis in human glioblastoma cells (Fig. 8). Thus, the combination of VPA + NTX could be further explored as a potential therapeutic strategy for treatment of glioblastoma in preclinical as well as in clinical settings in the near future.

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