

Apoptotic Effects of Temozolomide and Naturopathic Agents upon Glioblastoma Cells

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Abstract

Temozolomide (TMZ), thymoquinone (TMQ), epigallocatechin gallate (EPIGAL) and staurosporine (STAURO) were used as apoptotic inducing agents acting upon the U87-MG (ATCC, HTB15), LN18 (ATCC, CRL2610) and U118-MG (ATCC, HTB14) glioblastoma multiforme (GBM) cell lines. TMZ is the current drug of choice for primary treatment and adjuvant therapy for recurrent GBM. TMQ and EPIGAL are naturopathic agents, while STAURO is a well-studied apoptotic-inducing agent. The degree and time course of apoptosis were measured by flow cytometry techniques capable of detecting changes in mitochondrial function using the fluorescent dye MitoTracker Deep Red. Phosphatidylserine exposure and plasma membrane permeability were detected simultaneously using violet fluorescent reactive dye (VFRD) in combination with AnnexinV-488. The apoptotic effectiveness of the inducing agents TMZ, TMQ, EPIGAL and STAURO were compared with their ability to inhibit invasiveness and degrade Class I major histocompatibility complex (MHC) determinants. Invasiveness was measured *in vitro* by the 3D matrigel spheroid invasion assay. The density of the class I MHC determinants was measured by flow cytometry. Although TMZ is widely used for the chemotherapeutic treatment of GBM, it was determined that the time course for TMZ induced apoptosis was slower than those of TMQ, EPIGAL and STAURO. Unexpectedly, TMZ was ineffective in its ability to inhibit *in vitro* invasiveness and did not degrade the class I MHC determinants as effectively as the other apoptotic inducing agents. The findings raise the question of whether *in vitro* assays of apoptosis and invasiveness are the best measures of the effectiveness of chemotherapeutic agents for the primary treatment and adjuvant therapy of recurrent GBM. The findings also point to the *in vivo* complexity of the efficacy of chemotherapeutic agents whereby preserving the components of natural and acquired immune mechanisms may be more important than the rapid apoptotic effects of the chemotherapeutic agent.

Keywords: Chemotherapeutic agents; Epigallocatechin gallate; Glioblastoma multiforme; Immunosuppression; Invasiveness; Major histocompatibility complex; Naturopathic; Temozolomide; Thymoquinone

Introduction

Therapeutic regimens for glioblastoma multiforme (GBM) and other high-grade gliomas have fallen short of providing effective treatment. Clinical studies comparing chemotherapeutic agents have indicated increased tumor shrinkage and a very slight increase in median survival times, but no evidence for an increase in survival rates [1-4]. More recent clinical studies employing surgery with the use of chemotherapeutic regimens in combination with radiation therapy or by receptor mediated growth hormone deprivation are slightly more effective in increasing median survival times, but again have had limited success in increasing the overall survival rate in patients being treated for primary or recurrent glioblastomas [5-6]. New treatments are diligently being pursued and provide hope for effective treatment [7,8]. However, the difficulties of complete resection, the resistance to radiation and other therapies and particularly the intractable malignant invasiveness still remains at the root of the very poor survival prognosis for patients with GBM and other high grade gliomas. The fact that GBMs are usually malignantly invasive, but in general do not metastasize, is what renders studies about invasiveness particularly relevant to glioblastomas [9]. It is well known that all immunological, radiological and pharmacological treatments of GBMs are treatments that induce cell death by apoptosis. In fact, other than surgery, malignant cell types require a clinical treatment that induces cell death by apoptosis because the cellular debris and inflammation produced by massive necrosis or necroptosis of an extensive tumor would be systemically injurious to the patient. This raises the question of whether effective apoptotic inducing agents also have the property of inhibiting the invasiveness of GBMs. An additional question, is there a

correlation between the ability of a chemotherapeutic agent to induce apoptosis and to inhibit the invasiveness of GMB?

The DNA alkylating agent temozolomide (TMZ) is a second-generation imidazotetrazin that when administered p.o. enters the cerebrospinal fluid and does not require hepatic metabolism for activation. It is the current drug of choice for primary treatment and adjuvant therapy for recurrent glioblastoma multiforme [10-17]. A point of interest is to test the ability of temozolomide to induce apoptosis and inhibit the invasiveness of GMBs as compared to other agents capable of inducing apoptosis.

Staurosporine (STAURO) has been well studied as an apoptotic-inducing agent [18] and is relevant to current explorations regarding the efficacy of small-molecule kinase inhibitors in the treatment of solid cancers including glioblastoma [19]. Other compounds of interest are naturopathic agents. Naturopathic approaches to health care use natural, non-toxic herbs and other agents as supplements to support and encourage healing processes. A fertile source of well-tolerated natural antineoplastic agents has been extracts from botanical preparations, including extracted oils, herbal extracts and teas. Such extracts include the natural products thymoquinone [20-23] and epigallocatechin

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gallate [24-28]. Thymoquinone (TMQ) is the bioactive compound derived from black seed oil (*Nigella sativa*), while epigallocatechin gallate (EPIGAL) is the major polyphenol component of green tea. Both TMQ and EPIGAL are promising antineoplastic agents that are well tolerated at high doses and show low toxicity to non-cancerous cells. STAURO has been well studied and, although not currently a clinically employed drug, it is relevant to current explorations regarding the efficacy of small-molecule kinase inhibitors in the treatment of solid cancers including glioblastoma [29,30]. Although TMQ and EPIGAL are reported to be apoptotic inducing agents [31-36] that cross the blood brain barrier [37-42], their apoptotic effects upon GBMs has not been studied. Also, there are no studies comparing the apoptotic effectiveness of TMZ to the naturopathic agents TMQ and EPIGAL.

As aforementioned, TMZ is the drug of choice in the treatment of GBMs. It would seem that TMZ would be more effective in its ability to induce apoptosis as compared to other agents in the treatment of GBMs. A point of interest is, how does TMZ compare to TMQ EPIGAL and STAURO in their ability to induce apoptosis in established glioblastoma cell lines using a variety of apoptotic indicators?

It is well established that the malignancy of GBMs is in large part due to the propensity of the tumor to invade surrounding brain tissue [43]. Accordingly, of interest is the efficacy of TMZ as compared to TMQ, EPIGAL and STAURO in their ability to inhibit *in vitro* growth and invasiveness of GBMs.

Deficiencies associated with the Class I major histocompatibility complex (MHC) molecules restrict antigen presentation machinery and thus helps tumor cells evade the classical cell-dependent immune responses [44]. It has been reported, that as GBMs proceed through apoptosis, metalloproteinases and serine proteases are activated which degrade cell surface determinants [45,46]. Upon treatment with chemotherapeutic agents, those cells within the tumor that survive the apoptotic process could have a low density of class I MHC molecules on the surface and escape immune surveillance. Thus, an additional question is, how does TMZ compare to TMQ, EPIGAL and STAURO in their ability to degrade HLA-ABC determinants as the glioblastoma cells proceed through apoptosis?

Methods

Cell types and maintenance

All cell lines were obtained from American Type Culture Collection (ATCC). The LN18 cell line (ATCC, CRL-2610) was established in 1976 from a patient with a right temporal lobe glioma [47]. The cells are poorly differentiated, adherent and grow well in culture. The U87-MG (ATCC, HTB15) and U118-MG (ATCC, HTB14) glioblastoma cell lines were first isolated from patients afflicted with brain glioblastoma multiformes by Ponten et al. [48]. All three cell lines are considered Grade IV astrocytomas and are transplantable as xenographs into nude mice [47,49,50]. The cells are anchorage independent and grow well as monolayers. Cells were maintained at 37°C in an atmosphere of 5% CO₂ at 100% humidity in 75cm² flasks in Advanced DMEM (F12) supplemented with the dipeptide L-alanyl-L-glutamine (2mM), non-essential amino acids, penicillin (100 units/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml), HEPES (10 mM) and fetal bovine serum (5%). Cells were sub-cultured by trypsinization (0.25% trypsin, EDTA). Media and supplements were obtained from Gibco.

Apoptotic inducing agents

The apoptotic inducing agents TMZ, STAURO, TMQ and EPIGAL

were prepared to the appropriate concentrations from a DMSO solubilized stock solution where the reaction concentrations of DMSO were less than 0.03%. Apoptosis was induced at 37°C under growth conditions by the addition of TMZ, TMQ and EPIGAL at a reaction concentration of 100 µM, while STAURO was used at a reaction concentration of 1 µM in cells adhered as a monolayer in 24 well flat bottom plates (Costar). The cells were then harvested by the use of enzyme free cell dissociation buffer (Gibco). All apoptotic inducing agents were obtained from Sigma-Aldrich.

Detection of apoptosis

For the mitochondrial function experiments the 24 well plates consisting of a monolayer of glioblastoma cells were allowed to incubate for 8 h after the addition of the inducing agents. Apoptosis was detected by changes in the mitochondrial function that was illustrated by changes in fluorescent intensity of the mitochondrial membrane binding dye MitoTracker Deep Red 633 (Thermo Fisher). The 1 mM stock solution of MitoTracker was diluted and added to the apoptotic and non-apoptotic control cells at a cell concentration of 1 × 10⁶ cells/ml with a MitoTracker reaction concentration of 300 nM. After lifting the cells from the monolayer with enzyme free cell dissociation buffer, the cells were allowed to stain live in suspension at 37°C in Advanced DMEM (F12) growth medium under growth conditions, for 20 min. Cells were then harvested and fixed in 1% paraformaldehyde (Sigma) and subsequently analyzed by flow cytometry.

For apoptosis detected by the Violet Fluorescent Reactive Dye (VFRD) vs. AnnexinV dot plots, 24 well plates consisting of a monolayer of glioblastoma cells were allowed to incubate for 12 h after the addition of the inducing agents. Apoptosis was detected by AnnexinV binding to membrane-exposed phosphatidylserine in combination with VFRD dye (Thermo Fisher). Following washing and centrifugation the apoptotic and non-apoptotic cells were suspended in 1 ml of PBS each at a cell concentration of 1 × 10⁶ cells/ml. 1 µL of reconstituted VFRD dye was added to the 1 ml of cell suspension, which was then incubated on ice for 30 min in the dark. The cells were then washed 2x with 1 ml of PBS and suspended in 1ml of 10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂, pH 7.4 (annexin-binding buffer). Just prior to FACS analysis 1µL of AnnexinV, Alexa Fluor 488 conjugate (Thermo Fisher) was added to the 1 ml violet stained cell suspension. The doubly stained cells were then assayed by flow cytometry.

Flow cytometry

The samples were analyzed on the 4 laser LSRFortessa (BD Biosciences) using FACS Diva 8.0 software for data collection. A minimum of 10,000 events were collected per sample. Data analysis was done using FCS Express 6 Flow software (De Novo Software, Los Angeles CA).

3D Matrigel Spheroid Invasion Assay

The effects upon growth and invasiveness were tested using a 3D spheroid assay as measured by the changes in the radius and volume surrounding the spheroid. Spheroid assays were performed in Corning Costar Ultra-Low attachment 96 well round bottom plates. The plates feature a covalently bound hydrogel layer that effectively inhibits cellular attachment. Prior to the introduction of the low growth factor matrigel, 6,000 glioblastoma cells were introduced into the wells of the plate in 50 µL of medium (0.5% FBS) and incubated overnight to allow spheroid formation. 50 µL of low growth factor matrigel that contained 0.4 mg/ml collagen-1 was neutralized with NaHCO₃ and then introduced into each well. The matrigel and collagen mix was added ice cold to the ice chilled wells containing the glioblastoma spheroids. The plate was then

centrifuged at 200 g for 5 min at 4°C. The cells were then incubated at 37°C to allow polymerization of the matrigel/collagen-1 mixture. Following polymerization, 100 µL of medium (10% FBS) was gently added to the matrigel surface of each well, with or without apoptotic inducing agents. The apoptotic inducing agents were added in the 100 µL addition at twice the concentration of the appropriate reaction concentration in the 200 µL well volume. The plate was then incubated for 5 days at 37°C. Micrographs were taken at 40x with a Nikon Diaphot inverted microscope equipped with Prog Res Capture imaging software and camera. The areas and average diameters of the spheroid images were calculated using Image J software, which was downloaded from NIH.GOV.

Flow cytometry analysis of HLA-ABC determinants on the surface of glioblastoma cells

Apoptosis was induced in cells adhered as a monolayer in 24 well plates. The plates were allowed to incubate for 15 h after the addition of the inducing agents. The cells were then harvested by the use of enzyme free cell dissociation buffer (Gibco). Rabbit purified antibodies for HLA-ABC (Abcam) were used as a primary antibody

at a reaction concentration of 5 µg/ml. Rabbit purified anti-Keyhole Limpet Hemocyanin (anti KLH, Sigma-Aldrich) was used as the primary antibody negative control (5 µg/ml). The secondary antibody was F(ab)₂-goat anti-rabbit IgG conjugated to phycoerythrin at a reaction concentration of 2.5 µg/ml (Jackson ImmunoResearch). Flow cytometry data were then accumulated as described above.

Results

Changes in mitochondrial function as an indicator of apoptosis

The histograms of Figure 1 represent mean fluorescence intensity (MFI) of MitoTracker Deep Red 633 dye versus cell count for U87-MG Cells (Column I), LN18 Cells (Column II) and U118-MG Cells (Column III). The objective was to determine the relative effectiveness of TMZ, EPIGAL, TMQ and STAURO as apoptotic inducing agents.

MitoTracker Deep Red 633 is a membrane potential-dependent fluorescent dye that becomes permanently bound to the mitochondria and remains attached after the cell dies or is fixed. Cells were harvested and labeled with MitoTracker dye and analyzed by flow cytometry as

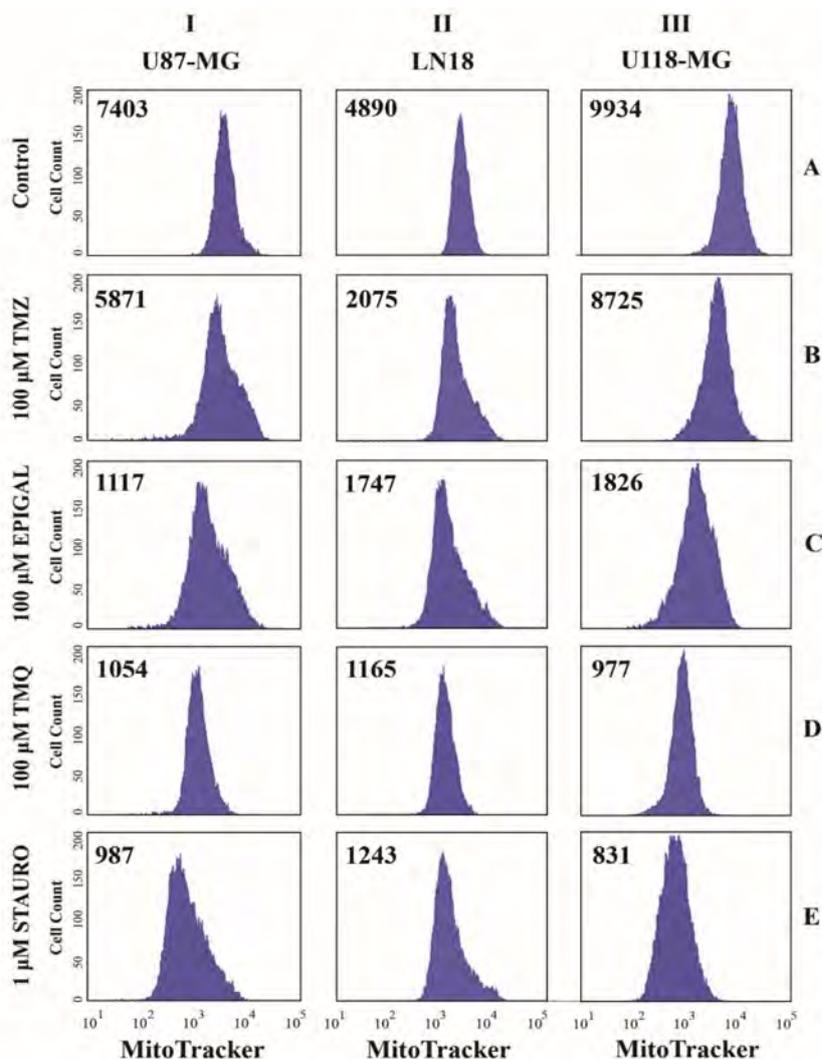


Figure1: Changes in mitochondrial membrane function due to apoptosis.

outlined in the Methods. The DMSO vehicle controls of Figure 1 are represented by the row of panels labeled (A) while the TMZ, EPIGAL, TMQ and STAURO treated cells are represented by the rows of panels labeled (B), (C), (D) and (E) respectively. A decrease in fluorescence intensity indicates a decrease in mitochondrial membrane function, which is a telltale sign that apoptosis is occurring via a mitochondrial pathway. Changes in mitochondrial function are early events in the pharmacological induction of apoptosis. Downstream events of mitochondrial pore activation lead to programmed cell death.

The mean fluorescent Intensity (MFI) values of the vehicle control for the U87-MG, LN18 and U118-MG cells are shown to be 7403, 4890 and 9934 respectively. The treated cells shown in Figure 1 were acted upon with either 100 μ M TMZ, 100 μ M EPIGAL, 100 μ M TMQ, or 1 μ M STAURO on a monolayer of the indicated glioblastoma cell lines for 8 h in 24 well plates. To varying degrees, the MFI of the emissions of the mitochondria bound MitoTracker Red decreases for all of the agents tested. The greatest down shifts in MFI were due to treatment of

the 3 cell types with 1 μ M STAURO (panels of Row E) showing values 987, 1243 and 831, which calculate to be 74%, 87% and 92% decreases for the LN18, U87-MG and U118-MG cells respectively. The 100 μ M TMZ treated cells (panels of Row D) also show a significant downshift in the MFI for all the three cell types. The 100 μ M EPIGAL treated cells (panels of Row C) show less of a downshift, while the smallest down shifts of MitoTracker MFI were shown by the treatment of the cells with 100 μ M TMZ yielding decreases of 12%, 21% and 57% for the U118-MG, U87-MG, LN18 and cells respectively. Taken as a whole, the data of Figure 1 indicate that TMZ is the least effective apoptotic inducing agent with STAURO being the most effective apoptotic inducing agent as measured by changes in mitochondrial function when tested upon the U87-MG, LN18 and U118-MG cell lines *in vitro*.

Changes in cell populations of dot plots as an indicator of apoptosis

Figure 2 consists of dot plots for glioblastoma cells labeled with

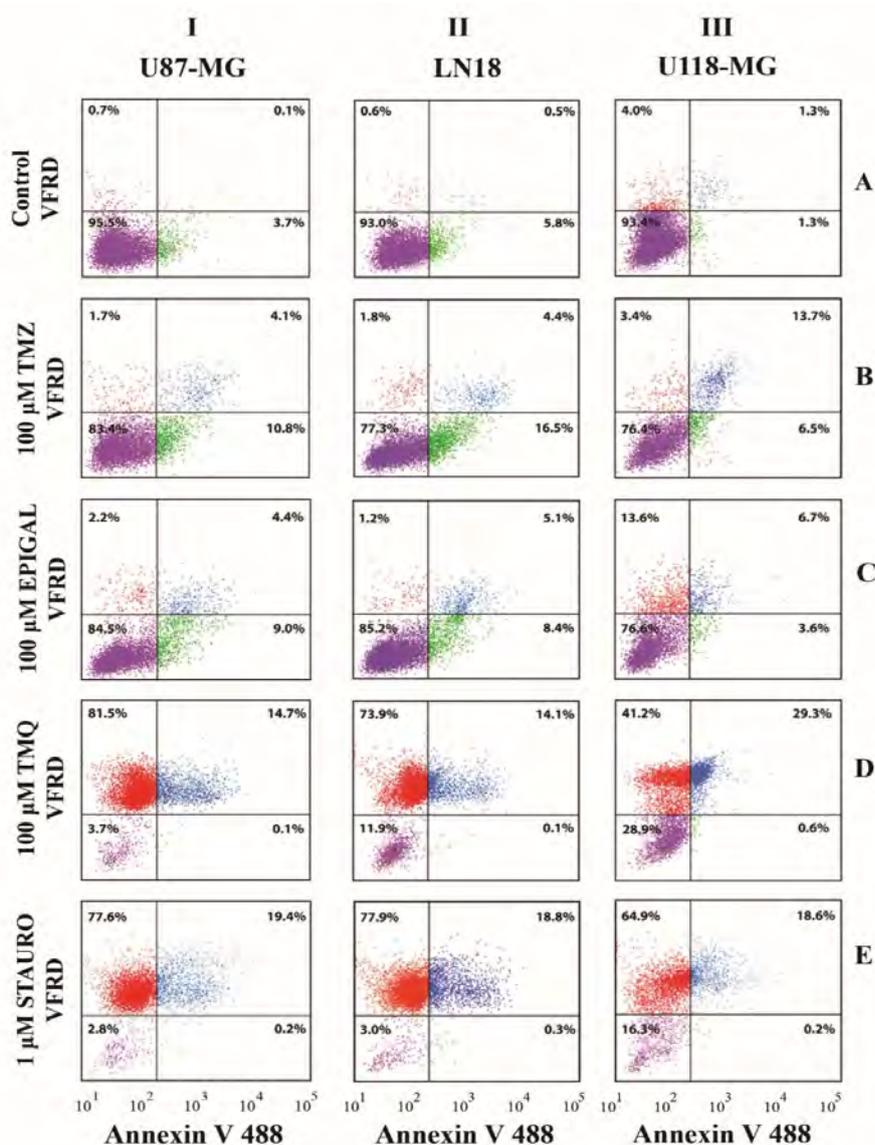


Figure 2: AnnexinV-488 vs. VFRD dot plots.

AnnexinV-488 and VFRD that were analyzed by flow cytometry. The objective was to determine the relative effectiveness of TMZ, EPIGAL, TMQ and STAURO as apoptotic inducing agents.

As described in the Methods, the 24 well plates consisting of a monolayer of glioblastoma cells were allowed to incubate for 12 h after the addition of the inducing agents. Detection of exposure of phosphatidylserine on the outer leaflet of the plasma membrane, which binds the fluorescent dye AnnexinV-488 is one of the classic tests for early and mid-apoptosis. Compromise of the plasma membrane allowing the amine binding dye Violet Fluorescent Reactive Dye (VFRD) to enter the cells is a classical test for mid and late apoptosis. Viable cells are not positive for AnnexinV-488 or VFRD and are neither apoptotic nor necrotic and are represented in the lower left quadrants of each panel; cells in early apoptosis stain for AnnexinV 488, but not VFRD and are in the lower right quadrants of each panel; cells that are in mid to late apoptosis are stained for both AnnexinV-488 and VFRD and are represented in the upper right quadrants of the panels; and cells in very late apoptosis stain positively for VFRD, but not for AnnexinV-488 and are represented in the upper left quadrants of each panel.

The numbers within the quadrants indicate the percentage of cells in each of the quadrants. The apoptotic inducing agents were 100 μ M TMZ, 100 μ M EPIGAL and 100 μ M TMQ illustrated in the panels of Rows (B), (C) and (D) respectively and 1 μ M STAURO shown in the panels of Row E. The non-treated DMSO vehicle control cells are shown in panels of Row A. The apoptotic agents are acting upon U87-MG cells (Column I), LN18 cells (Column II) and U118-MG cells (Column III).

Row (A) of Figure 2 presents the data for the DMSO vehicle controls with greater than 93% of the cells being in the lower left quadrant of the panels for all three cell types (Columns I, II and III) showing low PS exposure and low permeability to the dye VFRD indicating very low levels of apoptosis. The upper left quadrants of the three control panels (Row A) being less than 4% indicate that very few of the control cells are in a late apoptotic or necrotic state.

The TMZ treated cells in Row (B) of Figure 2 show that for all three cell types the majority of the cells are in the lower left quadrants (greater than 75%) with less than 5% of the cells in late apoptosis (upper left quadrants of the panels) with a small population of cells in early and mid-apoptosis (lower right and upper right quadrants of the panels). Thus, following 12 h treatment with 100 μ M of TMZ a small percentage

of the cells are in late apoptosis with less than 22% of the cells being in early to mid-apoptosis.

The data of Row C show that EPIGAL has a very similar dot pattern to TMZ (Row B) for the cell types U87-MG (Column I) and LN18 (Column II) with a slightly higher percentage of cells in the upper left quadrant (13.6%) for the U118-MG cells (Column III). Overall, the dot plots of Row (B) and (C) show that TMZ and EPIGAL are weak apoptotic agents when acting upon a monolayer of U87-MG, LN18 and U118-MG cell lines for 12 h.

The data of Rows (D) and (E) of Figure 2 show that 100 μ M TMZ and 1 μ M STAURO are effective apoptotic inducing agents when acting on the U87-MG, LN18 and U118-MG cell lines for 12 h in a monolayer. This is manifested by a high population of cells in mid and late apoptosis (upper right and upper left quadrants) with a lower population of cells in non-apoptotic and early apoptotic states (lower left and lower right quadrants). In total, the dot plot data indicate that in all three of the cell lines TMZ at 100 μ M was mildly effective as an apoptotic inducing agent, while STAURO at 1 μ M was an effective apoptotic-inducing agent. Likewise, EPIGAL at 100 μ M was relatively ineffective (Row C) as an apoptotic-inducing agent as compared to the TMQ and STAURO (Rows D and E respectively).

Effects of the apoptotic inducing agents upon glioblastoma invasiveness in the 3D matrigel spheroidal assay

Figures 3 to 5 are micrographs of glioblastoma cell lines U87-MG, LN18 and U118-MG that illustrate 3D invasiveness emanating from spheroids embedded in a collagen enriched matrigel [51]. The objective was to determine if the apoptotic inducing agents TMZ, EPIGAL, TMQ and STAURO have the ability to inhibit glioblastoma cell invasiveness. The data of Figures 3 to 5 are representative of a minimum of three spheroidal invasiveness experiments.

Three dimensional spheroidal cell cultures are better approximates of the *in vivo* conditions surrounding solid tumors. Micrographs illustrating spheroid formation of Day 1 resulted from an initial seeding of 6,000 cells. Micrographs were acquired at 40x with a Nikon Diaphot inverted microscope equipped with Prog Res Capture imaging software and camera. Micrographs were taken on Day 1 and Day 5. The micrographs include the untreated control and 100 μ M TMZ, 100 μ M TMQ, 100 μ M EPIGAL and 1 μ M STAURO treated cells. As mentioned in the Methods, the areas of the spheroid images for each micrograph

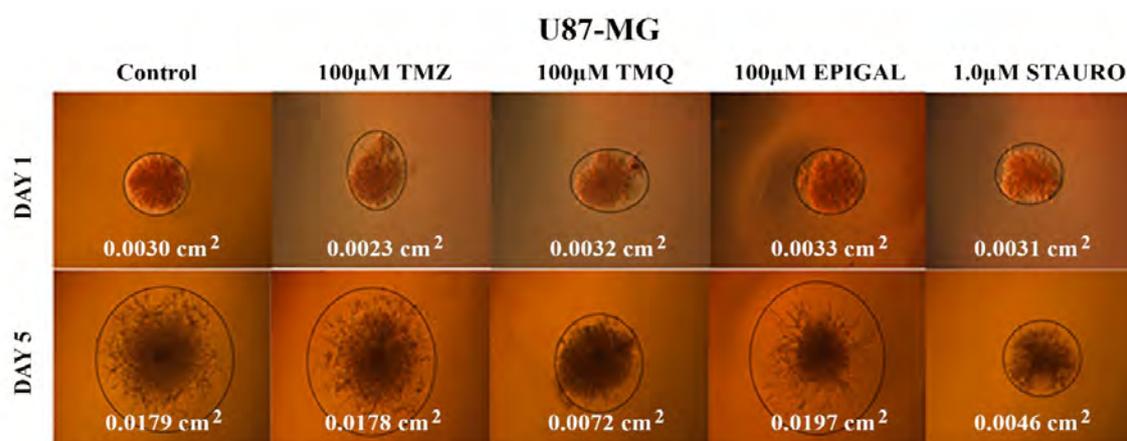


Figure 3: Invasiveness of U87-MG Cell Line on Day 1 and Day 5.

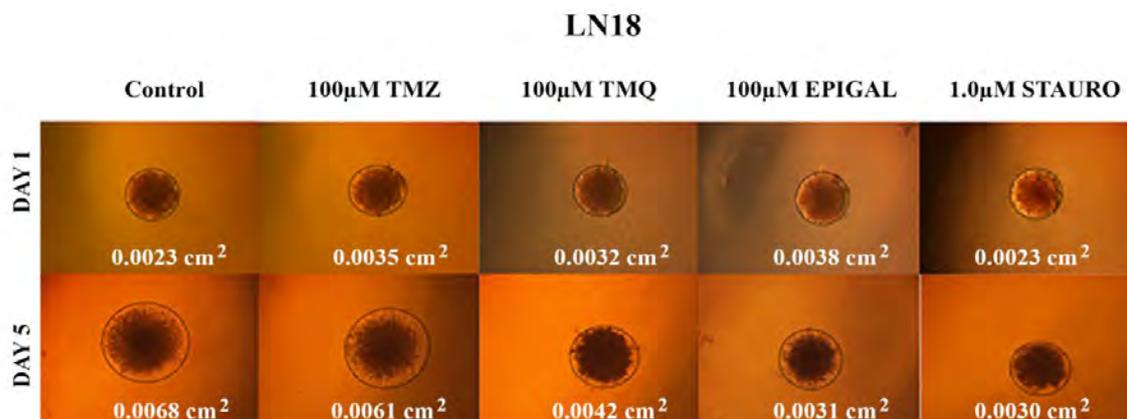


Figure 4: Invasiveness of LN18 Cell Line on Day 1 and Day 5.

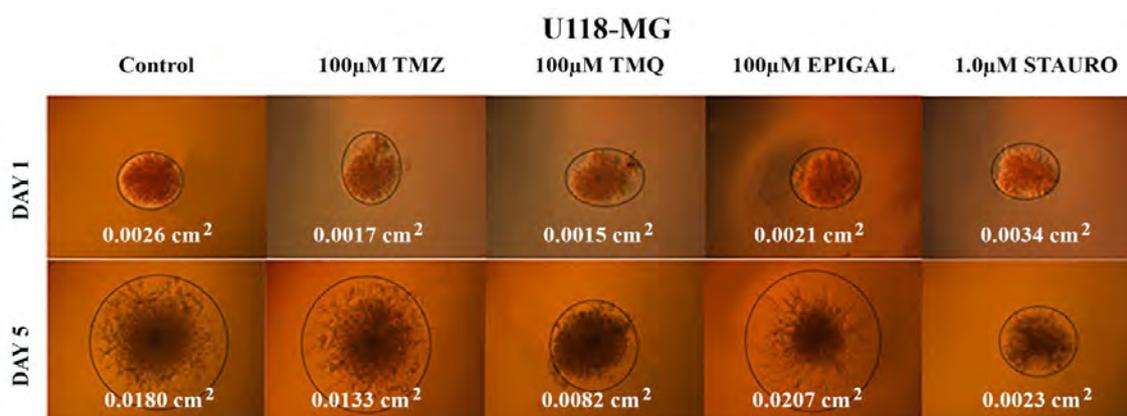


Figure 5: Invasiveness of U118-MG Cell Line on Day 1 and Day 5.

were calculated using Image J software and are located at the bottom of the respective micrographs. The values of the spheroid areas are shown within the micrographs.

The formation of polarized extensions, such as invadopodia emanating from cells within a solid tumor is a hallmark of infiltration through a 3D environment [52]. These protrusive structures formed by migrating and invading cells appear on the leading edges of migrating cells and function to command the direction of the migrating cells. Invadopodia are special F-actin-rich matrix-degrading structures that arise on the surface of the cell membrane. When comparing the control micrographs of Day 1 to the control micrographs of Day 5, all cell types showed an increase in growth and invasiveness over the 5 days.

Figure 3 illustrates how the invasiveness of the U87-MG cell line is affected by 100 μ M TMZ, 100 μ M TMQ, 100 μ M EPIGAL and 1 μ M STAURO. First, looking at the non-treated control (column 1) of the U87-MG cell line it can be seen that the spheroid within the micrograph of Day 5 (row 2 of column 1) is more than 5 times larger in area than that of the spheroid within the micrograph of Day1 (row 1 of column 1). In addition to its dimensional growth, the spheroid of the 5-day control shows long extensive filopodia indicating that the U87-MG cells are highly invasive. The spheroids of the Day 5 micrographs of Figure 3 for 100 μ M TMZ (row 2 of column 2) and 100 μ M EPIGAL (row 2 of column 4) as compared to the spheroid of the Day 5 control

cells (row 2 of column 1) show little difference indicating that TMZ and EPIGAL are not effective inhibitors of invasiveness when acting upon the U87-MG cell line. Comparing the filopodia lengths and areas of the Day 5 spheroids within the micrographs of 100 μ M TMQ (row 2, column 3) and 1 μ M STAURO (row 2, column 5) to the Day 5 control (row 2 of column 1) it can be seen that TMQ and STAURO are effective inhibitors of spheroidal invasiveness.

The spheroidal area within the micrographs of Figure 4 show the control LN18 cells (column 1 of Figure 4) increased 3 fold over the 5 days (column 1, row 2) and manifest short filopodia like extensions emanating away from the outer edge of the spheroid. The spheroid of the Day 5 (column 2, row 2) micrograph of 100 μ M TMZ manifests filopodia and an area similar to that of Day 5 control (column 1, row 2) indicating that TMZ is not an effective inhibitor of invasiveness of the LN18 cell line. The Day 5 micrographs of 100 μ M TMQ (column 3, row 2), 100 μ M EPIGAL (column 4, row 2) and 1 μ M STAURO (column 5, row 2), when compared to the Day 5 control (column 1, row 2), indicate that TMQ, EPIGAL and STAURO have the ability to inhibit invasiveness of the LN18 cells line.

Figure 5 illustrates how invasiveness of the U118-MG cell line is affected by the apoptotic inducing agents. The invasiveness of the non-treated control cells were compared to cells treated with 100 μ M TMZ, 100 μ M TMQ, 100 μ M EPIGAL and 1 μ M STAURO. Looking at the

non-apoptotic control micrographs of the U118-MG cell line it can be seen that the spheroid within the micrograph of Day 5 (row 2 of column 1) is more than 5 times larger in area than that of the spheroid within the micrograph of Day 1 (row 1 of column 1). The spheroid of the 5-day control shows long extensive filopodia indicating that the U118-MG cells are highly invasive. The spheroids of the Day 5 micrographs for 100 μ M TMZ (row 2 of column 2) as compared to the Day 5 spheroid of the control cells (row 2 of column 1) shows that TMZ is not an effective inhibitor of invasiveness when acting upon the U118-MG cell line. The Day 5 spheroid of the U118-MG cells treated with 100 μ M EPIGAL (row 2 of column 4) is actually larger than the U118-MG Day 5 control (row 5 of column 1). Comparing the filopodia lengths and areas of the

spheroids within the Day 5 micrographs of 100 μ M TMZ (row 2 of column 3) and 1 μ M STAURO (row 2 of column 5) shows significant inhibition of invasiveness when compared to the Day 5 control (row 2 of column 1). In comparing their abilities to inhibit invasiveness TMZ, EPIGAL, TMZ and STAURO had similar overall effects upon the U87-MG cells (Figure 3) and the U118-MG cells (Figure 5).

Effect of apoptotic inducing agents upon the degradation of class I histocompatibility cell-surface determinants

The histograms of Figure 6 are plots of HLA Class I expression versus cell count. The intent was to demonstrate that the U87-MG, LN18 and U118-MG cell lines have demonstrable levels of Class I

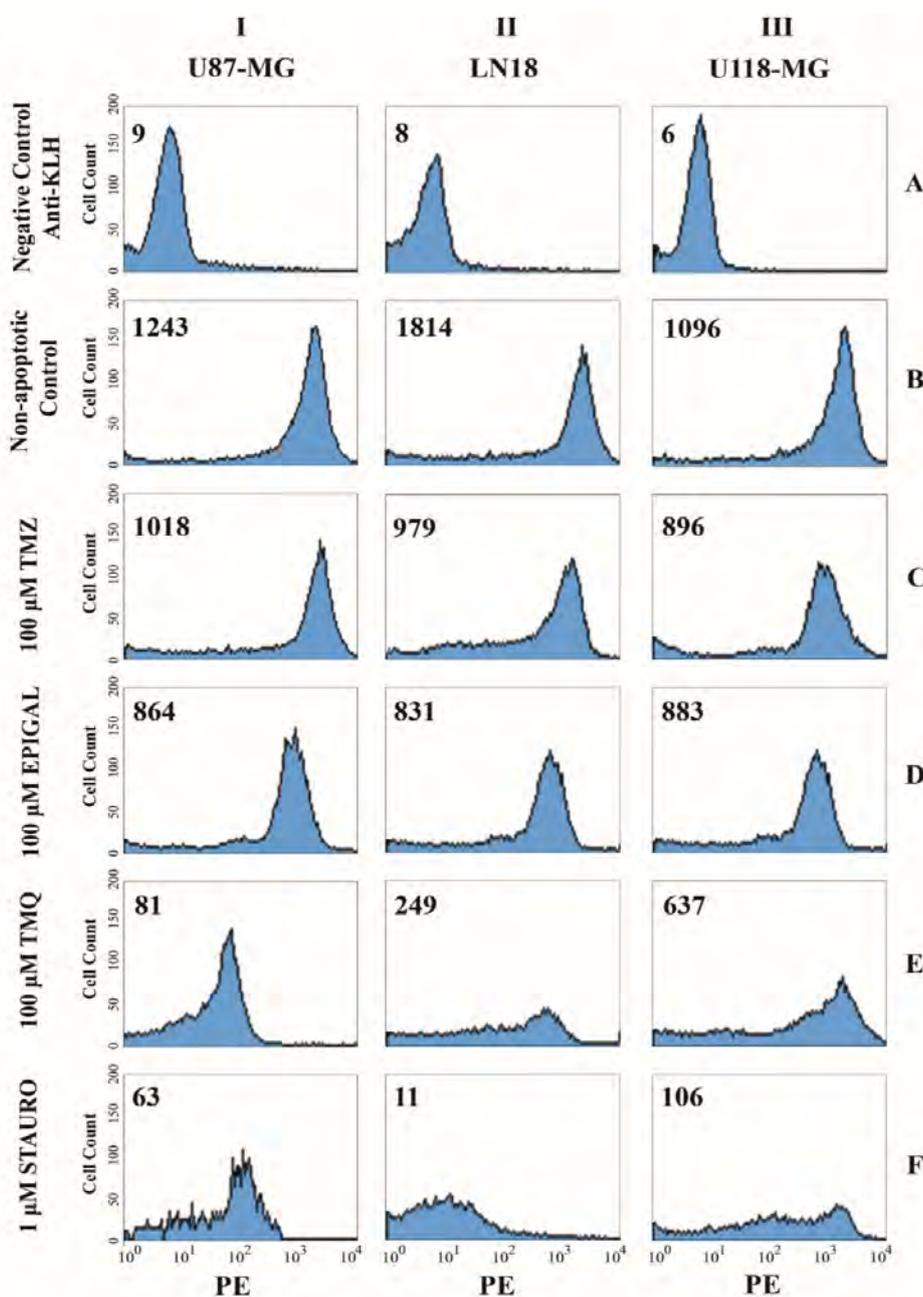


Figure 6: Histograms illustrating the degradation of HLA-ABC determinants as the glioblastoma cells proceed through apoptosis.

histocompatibility leukocyte antigens (HLA-ABC) and to determine the degradation effects of apoptotic inducing agents upon the class I histocompatibility antigens. The values within the panels of Figure 6 indicate the detected values of the phycoerythrin mean fluorescent intensity (MFI) emanating from the surface of the glioblastoma cells for apoptotic and non-apoptotic glioblastoma cells. The determinants on the surface of the glioblastoma cells were detected by mouse anti-HLA-ABC as primary antibody. For all of the histograms of Figure 6 the secondary antibody used was F(ab)₂-goat anti-rabbit IgG conjugated to the fluorescent phycoerythrin. A low MFI indicates a low density of the class I determinants on the surface of the glioblastoma cells.

In Figure 6 Rabbit anti-keyhole limpet hemocyanin (anti-KLH) was used as a non-specific primary rabbit antibody control. The non-binding rabbit anti-KLH controls (Row A) show low MFI values for the U87-MG (Column I), LN18 (Column II) and U118-MG (Column III) cell lines indicating very low binding for the non-specific rabbit antibody for all three-cell types. For Rows B through F rabbit anti-HLA-ABC was used as a primary antibody. Row (B) of Figure 6 shows the MFI for the non-apoptotic controls. The high MFI values within the panels of Row B indicate that the glioblastoma cell lines U87-MG, LN18 and U118-MG in a non-apoptotic state with MFI values of 1243, 1814 and 1096 respectively, have high levels of cell surface Class I histocompatibility antigens.

The data of Figure 6 for Rows (C) to Row (F) illustrate the degradation of the histocompatibility class I determinants as all three glioblastoma cell types proceed through apoptosis. Apoptosis was induced by TMZ (Row C), EPIGAL (Row D), TMQ (Row E) and STAURO (Row F). Examining the MFIs of TMZ treated cells (Row C) compared to the non-apoptotic controls (Row B) it can be seen that 100 μ M TMZ does have a slight ability to induce the degradation of Class I histocompatibility determinants with MFI values of 1018, 979 and 896 which calculate to be 18%, 18% and 46% decreases for the U87-MG, U118-MG and LN18 cells respectively. The losses in class I determinants of the EPIGAL treated cells (Row D) are similar to the TMZ treated cells, but the losses of class I determinants were more complete for the TMQ treated cells (Row E) and the STAURO treated cells (Row F). The TMQ treated cells show decreases of 42%, 86% and 93% while the STAURO treated cells show decreases of 95%, 98% and 99% for the U87-MG, LN18 and U118-MG cells respectively. Comparison the data of Figures 1 and 2 to the data of Figure 6 indicates that the apoptotic effectiveness of the inducing agent is proportional to the apoptotic agent's ability to promote degradation of the cell-surface class I MHC determinants.

Discussion

The treatment of tumors by irradiation or chemotherapeutic agents induces apoptosis. However, within the population of the treated tumors there is invariably a mixture of resistant non-apoptotic cells along with a mixture of cells in different stages of apoptosis [53]. Chemotherapeutic treated glioblastoma multiformes (GBMs) and other high-grade gliomas can readily mutate into therapeutic resistant forms [54] leaving only the defenses of acquired and natural immune responses. A known feature of glioblastoma multiformes (GBMs) is that they are immunosuppressive [55]. Surface cluster determinants are important stimulants of natural immune mechanisms with class I major histocompatibility complexes (MHC) being particularly important to acquired immune defenses against tumors [56]. Apoptotic cells secrete pericellular metalloproteinases and serine proteases capable of degrading cell surface determinants of the apoptotic cells themselves

and determinants of the surrounding non-apoptotic cells within the tumor [46]. To escape immune surveillance, tumor cells have the innate ability to diminish cell surface determinants that stimulate natural and acquired immune mechanisms [57]. Within a tumor, the degradation of immune stimulating surface determinants of neighboring cells by the metalloproteinases and serine proteases secreted from cells that are in late apoptosis can contribute to the tumor's ability to circumvent natural and acquired immune defenses.

In vitro testing of the ability to induce rapid and acute apoptosis or to inhibit invasiveness may not be the best predictors of the *in vivo* efficacy of a chemotherapeutic drug. Temozolomide (TMZ) being the drug of choice in the treatment of GBMs was not the most effective apoptotic inducing agent nor was it the most effective in its inhibition of invasiveness. The *in vitro* mitochondrial function and dot plot tests performed here show that TMZ was not rapid as an apoptotic-inducing agent. The *in vitro* dot plots illustrated that TMZ treated glioblastoma cells had a small population of cells in late apoptosis. Also, by the 3D spheroid test it was determined that the TMZ did not effectively inhibit invasiveness. The data raise the question as to why TMZ is effective as an adjuvant drug in the treatment of GBM. Overall, the data presented here indicate that there may be *in vivo* effects other than those manifested by *in vitro* apoptotic and spheroidal testing that are more relevant with regard to TMZ's clinical effectiveness. Also, in comparing the apoptotic and anti-invasive properties of the naturopathic agents thymoquinone (TMQ) and epigallocatechin gallate (EPIGAL), one would expect TMQ to be a more promising antineoplastic agent than EPIGAL in that TMQ showed a greater ability to induce apoptosis and to inhibit invasiveness of the glioblastoma cell lines. However, EPIGAL, a well-tolerated extract from tea, does manifest benefits in the treatment of malignant tumors [24-28] implying that molecules related to EPIGAL cannot be discounted as having the potential to be well-tolerated anti-neoplastic agents.

The antineoplastic mechanisms of TMZ are quite complex [58]. TMZ is spontaneously hydrolyzed to the active compound 5-(3-dimethyl-1-triazenyl) imidazole-4-carboxamide (MTIC) which is capable delivering a methyl group to purine bases of DNA (O6-guanine; N7-guanine and N3-adenine) causing base pair mismatches that can arrest the cells in various stages of mitosis and eventually trigger cytotoxicity and apoptosis [59]. Tumor cells possess mismatch-repair mechanisms such as O⁶-methylguanine-DNA methyltransferase (MGMT), which is a DNA-repair protein that removes methyl groups from the O⁶ position of guanine [60]. It is likely that there are additional repair mechanisms yet to be identified. TMZ is rendered more effective when administered in repetitive doses at a high frequency [61]. It is also rendered more effective when administered with an agent that inhibits the methyltransferase action of MGMT [62]. TMZ does not require enzymatic conversion in the liver to the active component MTIC [63] and both MTIC and unconverted TMZ pass the blood brain barrier, which may be a factor as to why TMZ is more effective in the treatment of GBMs than carmustine and other alkylating agents. However, a possibility that is raised here is that the clinical effectiveness of TMZ may be in part related to it being less toxic and less acute in its apoptotic properties. Stated more explicitly, if GBM cells treated with TMZ are arrested sufficiently and slowly progress through apoptosis and do not affect the immune stimulating capacity of surrounding cells within the GBM tumor, it may permit immune surveillance mechanisms to remain intact longer.

The many difficulties in developing a successful pharmaceutical agent are well known which include being well tolerated with few

contraindications. These and other concerns are standard considerations in drug development that pharmaceutical houses pay great attention to. They are mentioned here to call attention to the fact that in drug development the question of whether chemotherapeutic agents have immunosuppressive effects has been somewhat neglected. Apoptotic glioblastoma cells have very different characteristics than non-apoptotic glioblastoma cells. The ability to induce acute apoptosis may not be the best predictor of a clinically effective anti-neoplastic drug. What is proposed here is that cells in late apoptosis can exacerbate the immune suppression of glioblastoma patients and may interfere with the sustained effectiveness of natural and acquired immune defenses.

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