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REVIEW ARTICLE

From epidemiology and neurometabolism to treatment: Vitamin D in pathogenesis of glioblastoma Multiforme (GBM) and a proposal for Vitamin D + all-trans retinoic acid + Temozolomide combination in treatment of GBM

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Abstract

Here we review tumoricidal efficacy of Vitamin D analogues in glioblastoma multiforme (GBM) and potential synergisms with retinoic acid and temozolomide based on epidemiological and cellular studies. Epidemiological data suggest that winter birth is associated with higher risk of GBM, and GBM debulking in the winter enhanced mortality, which may relate with lower exposure to sunlight essential to convert cholecalciferol to Vitamin D. Comparative studies on blood bank specimens revealed that higher prediagnosis levels of calcidiol are associated with lower risk of GBM in elderly men. Supplemental Vitamin D reduced mortality in GBM patients in comparison to nonusers. Expression of Vitamin D Receptor is associated with a good prognosis in GBM. Conversely, Vitamin D increases glial tumor synthesis of neutrophins NGF and NT-3, the low affinity neurotrophin receptor p75NTR, IL-6 and VEGF, which may enhance glioma growth. Antitumor synergisms between temozolomide and Vitamin D and Vitamin D with Vitamin A derivatives were observed. Hence, we hypothesize that Calcitriol + ATRA (All-Trans Retinoic Acid) + Temozolomide – CAT combination might be a safer approach to benefit from Vitamin D in the management of high-grade glial tumors. Adding acetazolomide to this protocol may reduce the risk of pseudotumor cerebri, as both Vitamin D and Vitamin A excess may cause intracranial hypertension; this approach may provide further benefit as acetazolomide also exhibits anticancer activity.

Keywords Glioblastoma · Glioma · Vitamin D · Vitamin a · Temozolomide

Introduction

Gliomas constitute the most frequent brain neoplasms in humans and their common forms include astrocytoma, oligodendroglioma, and ependymoma (Elmaci and Altinoz 2016). These malignancies are grouped as grades I–IV based on World Health Organization (WHO) criteria. WHO grade II or III gliomas exert an invasive character, tendency to up-grade, and are associated with poor long-term prognosis. In adults, Grade IV or glioblastoma multiforme (GBM) is the most frequently encountered malignant brain tumor and can either origin de novo (primary glioblastoma) or via progression from low-grade gliomas (Elmaci and Altinoz 2016). GBMs are characteristically invasive and diffusely invade the normal cerebral parenchyma, which hinders total surgical resection and contributes to the poor prognosis. Further, GBM has a poor response to current treatments and have a median survival of 14 months (Elmaci and Altinoz 2016). While many compounds are synthesized and tested for treatment of these grave malignancies, another plausible strategy would be harnessing the potential of known molecules in combinatorial approaches. Here we review studies demonstrating that the lack of sunlight exposure negatively influences glioma prognosis, and that Vitamin D analogues induce glioma

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cell death in vitro and enhance survival in glioma patients in a pilot Phase-II trial. However, as seen with other anticancer approaches, Vitamin D could potentiate glioma stem cells and angiogenic cytokines in cell culture. Vitamin D receptors heterodimerize with Retinoid X Receptors, and ATRA (all-trans retinoic acid), which robustly enhances tumoricidal activity of Vitamin D analogues in GBM. Hence, we believe a triple approach employing Vitamin D + Retinoids+Chemotherapy (eg. temozolomide would be a safer and more potent strategy. Table 1 summarizes epidemiological and clinical clues suggesting Vitamin D's antineoplasticity in GBM pathogenesis and progression. Table 2 summarizes the molecular mechanisms of the potential antineoplasticity of Vitamin D analogues in GBM. Lastly, Table 3 summarizes Vitamin D regulation of cytokines, growth factors, and neurotrophins, which may jeopardize its antineoplastic potential and even cause potential harm in GBM patients. The potential for Vitamin D to induce proteins that may stimulate GBM growth contributes to our rationale that it may be detrimental as a single agent therapy.

A short description of Vitamin D and its neurometabolism in brain

Vitamin D constitutes a group of liposoluble secosteroids (steroids in which one of the bonds in the steroid rings is broken) which regulate intestinal absorption of calcium, magnesium, and phosphate in addition to other biological functions. Synthesis pathways of Vitamin D derivatives are demonstrated in Fig. 1. In the human skin, ultraviolet light converts Provitamin D_3 (7-dehydrocholesterol) to Vitamin D_3 via an intermediate isomer, Previtamin D_3 (Garcion et al. 2002). In addition, Vitamin D_2 (ergocalciferol) and Vitamin D_3 (cholecalciferol), can be consumed in the diet via plant or animal products, respectfully. Neither Vitamin D_3 nor Vitamin D_2 harbor sufficient biological activity, hence they should be converted to the endocrinologically-active form defined as 1,25-dihydroxyvitaminD (1,25$(OH)_2D_3$, calcitriol). In the liver, Vitamin D_2 or D_3 is converted to 25-hydroxyvitaminD, which can be measured from the serum to determine a person's Vitamin D status. In the kidneys, calcidiol is further hydroxylated by 1α-hydroxylase to form the bilogically active form of Vitamin D, calcitriol (1,25-dihydroxyvitaminD) (Garcion et al. 2002). Vitamin D forms are hydrophobic and able to be transported in blood via carrier proteins. The half-life of calcidiol is several weeks, while the half-life of calcitriol is a few hours. Calcitriol exerts negligible toxicity at low concentrations (<1000 μg/day), but hypercalcemia-associated toxicities may occur when consumed in excess (Bak et al. 2016). In the body, most organs convert calcidiol to calcitriol, as needed. Indeed, relatively recent studies demonstrated that the mitochondrial enzyme 1α-hydroxylase, which catalyzes the formation of calcitriol in the kidney, also exists in the extrarenal sites including skin, lymph nodes, colon (epithelial cells and parasympathetic ganglia), pancreas (islets), adrenal medulla, brain (cerebellum and cerebral cortex), and placenta (decidual and trophoblastic cells) (Zehnder et al. 2001).

In addition to its well-known effects to regulate mineral and skeletal homeostasis, calcitriol blocks the growth of osteosarcoma, melanoma, mammary, and colon cancer cells, and triggers differentiation of myeloid leukemia cells of both murine and human origin (Naveilhan et al. 1994). Many of the biological roles of calcitriol occur via its features to act as a hormone. When calcitriol binds to the Vitamin D Receptor (VDR), the activated calcitriol-VDR complex migrates into the cell nucleus and binds to specific DNA sequences. These sequences are Vitamin D responsive elements (VDRE) that trigger transcription of the target genes (Naveilhan et al. 1994). In addition to

Table 1 Epidemiology of Vitamin D antineoplasticity in glioma

Epidemiological Findings	Source
Winter birth increases risk of gliomas	Brenner et al. 2004
Winter birth increases risk of brain neoplasia	Mainio et al. 2006
Risk of gliomas fluctuate in different seasons and is highest in January	Koch et al. 2006
Winter season of surgery associates with higher risk of death	Hakko et al. 2009
25-OH-D_3 levels correlate negatively with risk of glioma in children with low birth weight	Bhatti et al. 2015
Calcidiol's higher blood levels correlate with lower risk of glioma from 2 years before diagnosis	Zigmont et al. 2015
Glioblastoma (GBM) patients consuming Vitamin D as a separate supplement had lower mortalities	Mulpur et al. 2015
Expression of Vitamin D Receptor in GBM tissues correlates with better survival	Salomon et al. 2014
Phase II-trial with 0.04 microg/kg alfacalcidol in GBM patients. 3 out of 11 patients responded with very prolonged survival	Trouillas et al. 2001

Table 2 Vitamin D molecular antineoplasticity in glioma

Molecular Findings	Source
Calcitriol induces of the low affinity neurotrophin receptor $p75^{NTR}$	Naveilhan et al. 1996a
Calcitriol induces P53 and GADD45	Baudet et al. 1996a
Calcitriol reduces PMP22/gas3, SPARC/osteonectin, MAP1C/Dynein Heavy Chain, S100β and Aldolase C	Baudet et al. 1998
Calcitriol induces Cysteine-Rich Protein, MGP, β-Tubulin, mortalin	Baudet et al. 1998
Calcitriol reduces Tenascin-C	Alvarez-Dolado et al. 1999
Cholecalciferol induces ceramide pathway	Magrassi et al. 1998
Calcitriol induces p21, p27 and p57 and reduces Cyclin D1	Salomon et al. 2014
Calcitriol synergies with temozolomide in eliciting autophagic death of GBM cells	Bak et al. 2016

intrinsic transactivation properties as homodimers, VDR may associate with receptors of the thyroid hormone or retinoic acid X receptor to activate transcription as heterodimers (Davoust et al. 1998). VDR belongs to the Steroid-Thyroid Receptor Superfamily (STRS). Nuclear localization of calcitriol was defined in major target tissues such as skin and bone, but also unexpectedly in parathyroid and pituitary glands, and in the pancreas (Naveilhan et al. 1994).

In the 1980s, the putative target tissues of calcitriol have expanded prominently from its classic target (Neveu et al. 1994a). Increasing evidence suggests specific actions of calcitriol in the brain; metabolites of Vitamin D exist in the cerebrospinal fluid and in specific nuclei of the brain, and calcitriol treatment increases enzyme activity of choline acetyltransferase. In primary glial cells, VDR expression allow calcitriol to upregulate expression of 25(OH) Vitamin D_3 24-hydroxylase and Vitamin D catabolization (Neveu et al. 1994a). It was initially thought that the supply of cerebral calcitriol was solely dependent on the calcitriol in blood circulation, however, further studies revealed the existence of cerebral enzymes that catalyze calcitriol synthesis (Zehnder et al. 2001; Garcion et al. 2002). It was further demonstrated that microglial cells in culture produce calcitriol from its precursor (Garcion et al. 2002).

Using exogenous vitamin D3 avoids the problem of hypercalcemia as vitamin D_3 and 1,25-Dihydroxyvitamin D_3 (calcitriol) exert equivalent anticancer activity

Above, we underlined that calcitriol exerts anticancer activity, yet it may cause hypercalcemia-associated toxicities when consumed in excess. Since 1α-hydroxylase also exists in extrarenal sites, Swami et al. presumed that Vitamin D_3 will be converted to calcidiol in the body and then to calcitriol locally in the cancer's micromillieu where it will exert antineoplasticity (Swami et al. 2012). Indeed, a Vitamin D_3-supplemented diet (5000 IU/kg) caused significant tumor shrinkage (>50%) in mice bearing MCF-7 human breast cancer xenografts, which was equivalent to administered calcitriol (0.025, 0.05, or 0.1 μg/mouse, three times a week) (Swami et al. 2012). Moreover, both treatments equivalently reduced PC3 prostate cancer xenograft growth - albeit lesser than the MCF-7 tumors. Calcitriol at 0.05 μg and 0.1 μg increased serum calcium whereas there was no change in serum calcium following Vitamin D_3 administration, revealing its higher safety. Importantly, the Vitamin D_3 did not alter 1α-hydroxylase mRNA in the renal tissue, but increased it in the tumors (Swami et al. 2012). Even more noteworthy, both the vitamin D_3 and calcitriol were equipotent in reducing estrogen and other protumorigenic inflammatory and growth

Table 3 Vitamin D induces trophic factors in glioblastoma

Trophic Factors Findings	Source
Calcitriol induces IL-6 and VEGF, which are inflammatory tumor-promoting cytokine and angiogenic growth factor, respectively	Baudet et al. 1996a
Vitamin D_3 induces chemoresistance in GBM cells via reducing p53 and bax and increasing bmi1	Maleklou et al. 2016
Calcitriol stimulates NGF production	Neveu et al. 1994b
Calcitriol stimulates NT-3 production	Neveu et al. 1994a
Calcitriol stimulates GDNF production	Naveilhan et al. 1996b

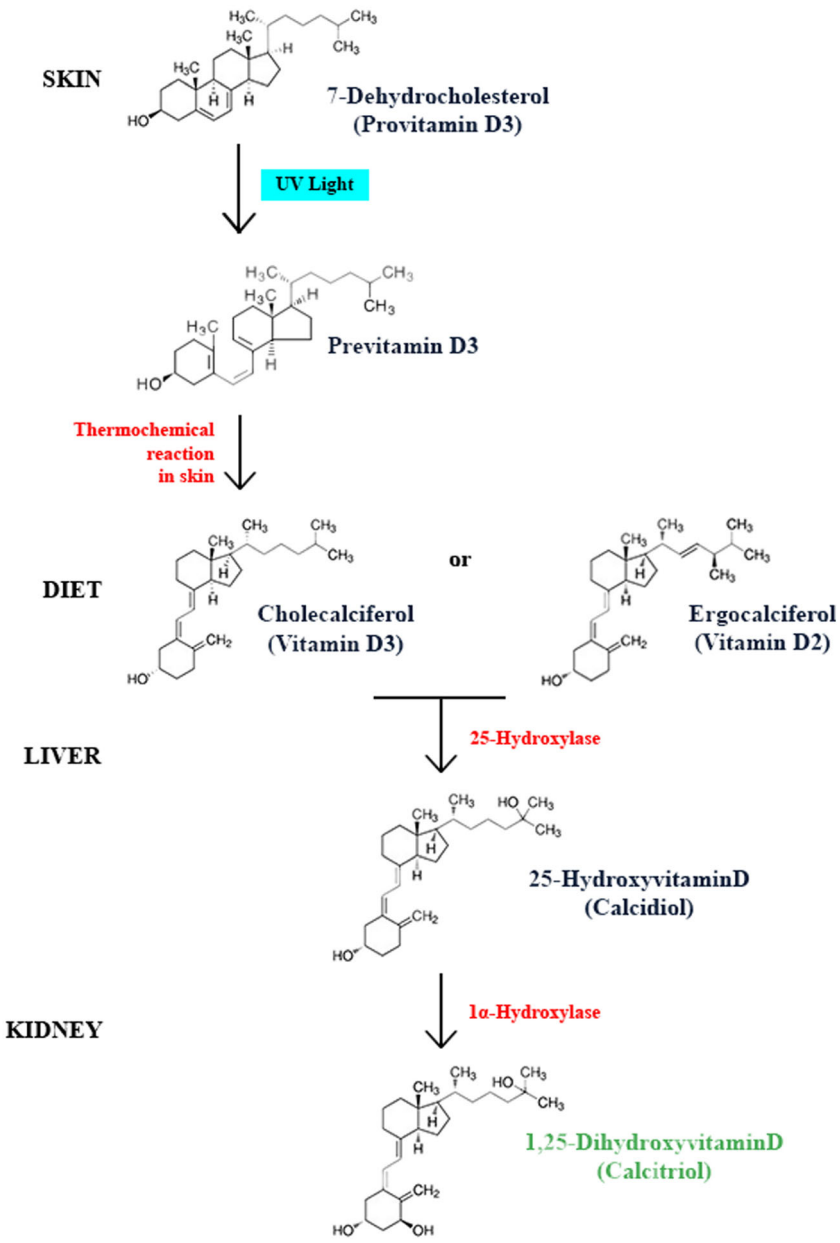

Fig. 1 Synthesis pathways of Vitamin D_2 derivatives. Vitamins D2 and D3 ingested with food and/or generated in the skin are successively hydroxylated in the liver and kidneys to the active form, calcitriol

factor synthesis and signaling pathways (Swami et al. 2012). Altogether, Vitamin D (in cholecalciferol form) can be harnessed as a safe and potent anticancer drug at high levels without causing hypercalcemia-associated toxicity.

Epidemiological studies regarding Association of Vitamin D with glioma risk or mortality

Season of birth, neonatal vitamin D levels and glioblastoma

Studies have shown that Vitamin D levels fluctuate with exposure to sunlight. In the northern hemisphere, Vitamin D level is highest in the summer months and tends to enhance for latitudes nearer the equator (Efird 2010). Infant cord blood serum concentrations of Vitamin D closely correlate with Vitamin D levels in the maternal serum (Efird 2010). In a case-control analysis conducted in adult humans, Brenner et al. demonstrated the correlations between the month of birth and the risk of cerebral neoplasia using patients enrolled at health centers in Boston, Phoenix and Pittsburgh, Pennsylvania who were diagnosed with glioma ($n = 489$), meningioma ($n = 197$), or controls ($n = 799$; subjects hospitalized for nonneoplastic diseases which were matched to cases by ethnicity, age, and gender) (Brenner et al. 2004). This study revealed a connection between the month of birth and the incidence of glial tumors and meningioma; tumor incidence

peaked in January and February and was lowest in July and August suggesting winter birth may enhance risk of brain tumor development (Brenner et al. 2004). Further, Mainio et al. investigated whether winter birth increased the risk of cerebral neoplasia in subjects from northern Finland (Mainio et al. 2006). The study group included 101 patients with a primary cerebral neoplasm. When winter births were compared with births in other seasons, a 1.39-fold (95% CI 1.01–1.77) greater incidence of primary cerebral neoplasia was found in patients with winter births in comparison to the general population ($p = 0.026$) (Mainio et al. 2006). Koch et al. investigated the date of birth in 501 patients diagnosed with gliomas in a Cancer Center in Bavaria (Koch et al. 2006). Their statistical model revealed that the incidence of glioma diagnosis exerted statistically significant fluctuations based on birth month, with the highest rate in January (Koch et al. 2006).

Bhatti et al. investigated a cohort of 247 childhood brain tumor cases using data from cancer registries in Washington State (<15 years at diagnosis) and selected for year of birth-, gender-, and race-matched 247 controls for comparison (Bhatti et al. 2015). Dried blood spots at the neonatal period were used to estimate levels of vitamin D_3 [25-(OH)D_3] in circulation. In general analysis, no significant associations were revealed. However, when the data was stratified in accordance with median birth weight (3458 g), the risk of childhood brain tumors was increased for children with higher birth weight and higher 25-(OH)D_3 levels (Bhatti et al. 2015). When compared with the lowest quartile (2.8–7.7 ng/mL), odds ratios (OR) for the 2nd (7.7- < 11.0 ng/mL), 3rd (11.0- < 14.7 ng/mL), and 4th (14.7–37.0) quartiles of 25-(OH)D_3 were 1.7, 2.4, and 2.6, respectively; also suggesting a dose-dependent relationship. Conversely, a protective effect was revealed in children with lower birth rate: ORs for the 2nd, 3rd, and 4th quartiles were 0.9, 0.7, and 0.6, respectively (Bhatti et al. 2015).The authors have concluded that the correlations of neonatal Vitamin D levels with childhood brain tumors may be associated with birth weight and circulating insulin-like growth factor 1 (IGF-1), which causes the duality of Vitamin D actions.

Season of brain tumor surgery and mortality In humans, the endogenous Vitamin D_3 synthesis is induced by dermal UV radiation, which profoundly fluctuates by season in northern Europe (Hakko et al. 2009). Vitamin D_3 synthesis and the absolute serum 25-OH-vitamin D_3 level increased by 50% in the summer and autumn compared to the non-sunny days in winter months. In northern Finland (from 64°N to 70°N), the seasonal fluctuation in UV radiation is especially prominent because of its far northern location (Hakko et al. 2009). In northern Finland, the February to March interval follows the four darkest months in the year, when the mean time period of sunshine is only few hours per day, or only polar night in Lapland. Hakko et al. analyzed 101 surgically treated patients diagnosed with a primary brain tumor living in northern Finland (from 64°N to 70°N) (Hakko et al. 2009). The dates of surgical tumor removal and mean hours of sunshine were determined by bimonthly intervals. A significant increase in deaths was found in patients who underwent surgery during the February to March interval; 40% of these patients harbored grade III–IV gliomas (Hakko et al. 2009).

Prediagnostic Calcidiol levels and risk of glioma Zigmont et al. performed a case–control analysis on specimens obtained from a blood bank in Norway (Zigmont et al. 2015). Blood donors who did not harbor glial tumors ($n = 1112$) were matched to blood donors with a diagnosis of glioma ($n = 592$) according to age and gender. They determined calcidiol levels to assess availability of vitamin D, since calcidiol has a half-life of 3 weeks, while calcitriol's half-life is only 24 h (Zigmont et al. 2015). For each control quintile of Vitamin D_2, seasonally adjusted odds ratios (ORs) were calculated via conditional logistic regression. In men, higher levels of calcidiol (>66 nmol/L) were inversely correlated to high grade gliomas from ≥2 years before diagnosis (OR = 0.59) to ≥15 years before diagnosis (OR = 0.61) suggesting that the observed association is not linked to preclinical disease (Zigmont et al. 2015).

Usage of vitamin D supplements and mortality in glioblastoma Mulpur et al. investigated the correlations of complementary treatments with mortality in 470 patients suffering GBM (Mulpur et al. 2015). According to the use of supplements, proportional hazard regressions were used to determine hazard ratios (HRs) for GBM associated mortality following multivariate adjustment for the extent of tumor resection (ESR), age, and Karnofsky Performance Score (KPS). Seventy-seven percent of the cohort reported usage of complementary medicine. Mortality correlations were not revealed with the complementary use of omega-3 fatty acids or multivitamins (Mulpur et al. 2015). Patients consuming Vitamin D as a separate supplement (containing more Vitamin D levels than in a multivitamin ADD DOSE) had lower mortality compared to nonusers (Mulpur et al. 2015).

Cell culture studies on the effects of vitamin D and analogues on glioma cells

Several members of the STRS (androgen, estrogen, glucocorticoid, and progesterone receptors) are present in astrocytomas and glioblastomas (Magrassi et al. 1992). Based on PCR assay results, Magrassi et al. were the first to demonstrate that glioblastomas express VDR significantly higher STRS than both low and high grade astrocytomas (Magrassi et al. 1992). Naveilhan et al. were the first to demonstrate that Vitamin D

may exert antitumor activity on glioma cells (Naveilhan et al. 1994). Calcitriol was applied to rat (C6) or human (GHD glioma cells) and assessed with MTT analysis for their sensitivity to calcitriol (Naveilhan et al. 1994). Cytotoxic effects of calcitriol were demonstrated at concentrations of 10 nM (Naveilhan et al. 1994). A 3-days interval was witnessed between the treatment onset and antiproliferative effects. The cytotoxic effect of calcitrol occurred following a single 24-h exposure indicating that a continuous administration was not necessary to cause a cytotoxic effect (Naveilhan et al. 1994). Calcitriol also controlled the expression of its own receptors in C6 rat glioma cells (Naveilhan et al. 1994).

In C6 glioma cells, calcitriol-induced cell death was dependent on expression of *c-myc*, *TP53*, and *gadd45* genes (Baudet et al. 1996a). The proto-oncogene *c-myc* triggers programmed cell death when its expression coincided with a stimulus for growth arrest, such as deprivation from nutrients (eg. lack of serum) (Baudet et al. 1996a). The activation of *c-myc* expression could also account for the enhanced expression of *p53* in C6.9 cells exposed to calcitriol, as *c-myc* could transactivate the *p53* promoter (Baudet et al. 1996a) robustly trigger apoptosis following irreversible DNA damage. *p53* is a transcriptional activator which triggers the activation of genes such as *gadd45* (Growth Arrest and DNA Damage-inducible 45) (Baudet et al. 1996a). GADD45 proteins act as tumor suppressors in response to various stimuli via inducing apoptosis (Tamura et al. 2012). Defects in the GADD45 signaling cascade were related to the induction and promotion of carcinogenesis; GADD45 expression is an essential step for mediating anticancer activity of multiple chemotherapeutics (Tamura et al. 2012). Tumoricidal efficacy of calcitriol can be blocked with forskolin, a drug which enhances intracellular cAMP levels, or, with genistein, is a pan-inhibitor of tyrosine kinases (Baudet et al. 1996a). However, despite DNA fragmentation in calcitriol-exposed cells, the C6.9 cells demonstrated the classical morphological signs of apoptosis (Baudet et al. 1996a). Calcitriol also stimulated genes encoding interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF); hence, the authors have speculated that calcitriol may also negatively influence prognosis of glioma in vivo through enhancing peritumoral edema (Baudet et al. 1996a).

Since the potent hypercalcemic effects of calcitriol may impair its clinical applicability in glial tumors at high dosages, Baudet et al. investigated whether several Vitamin D_3 analogues, which are less calcemic than calcitriol, could also inhibit GBM growth in vitro (Baudet et al. 1996b). Indeed, several Vitamin D_3 analogues were able to induce cell death in rat glioma cells (C6.9). The Vitamin D_3 analogue KH 1060 was the most potent agent in triggering cell death, while MC 1288 and CB 1093 were equipotent to calcitriol (Baudet et al. 1996b). EB 1089, a compound currently employed for the treatment of psoriasis, exerted only a weak effect on C6.9 cells (Baudet et al. 1996b). The effective doses were around 1 nM for calcitriol and 0.1 nM for KH 1060. Cell death-inducing efficacies exerted by calcitriol and its analogues produced several features of apoptosis, such as DNA fragmentation and induction of the *c-myc* protooncogene (Baudet et al. 1996b).

Baudet et al. analyzed differential screening of a rat brain cDNA library with probes derived from mock and calcitriol-exposed cells and found gene expression in C6.9 glioblastoma cells during calcitriol induced cytotoxicity (Baudet et al. 1998). Using this methodology, 61 differentially expressed cDNAs were defined. Among these, the genes encoding for Peripheral Myelin Protein (PMP22/gas3), osteonectin/SPARC, and Dynein Heavy Chain/MAP1C were decreased by more than a factor of five (Baudet et al. 1998). The downregulation of the *PMP22/gas3* gene enhanced the entrance of cells into $S + G_2/M$ phases, which was previously demonstrated to occur during the course of calcitriol induced cytotoxicity. Decrease of the *Dynein Heavy Chain/MAP1C* gene activity could be directly connected to execution of apoptosis (Baudet et al. 1998). The *Dynein Heavy Chain/MAP1C* gene codes the heavy chain of dynein and is responsible for the intracellular transport of endosomes, lysosomes, and Golgi apparatus components of microtubules (Baudet et al. 1998). Dynein regulates mitotic spindle formation and loss of its light chain causes robust apoptosis in Drosophila. Osteonectin (also called as Secreted Protein Acidic and Rich in Cysteine (SPARC)) was initially defined in bone, and later in brain (Baudet et al. 1998). Osteonectin controls matrix deposition and cell proliferation by abrogating cellular interactions with growth factors including PDGF and bFGF (Baudet et al. 1998). Hence, the downregulation of this molecule may be an attempt by tumor cells to reach growth factors for alleviating calcitriol induced death program. *S100β* and *Aldolase C* genes were reduced by factors of 3.5 and 1.5, respectively (Baudet et al. 1998). A noteworthy feature of these genes is that their corresponding proteins exert mutual interactions. Aldolase enzymes are involved in glycolysis; Aldolase C is the isoform specific to brain. Given the high glycolytic activity demand in tumor cells, reduction of Aldolase C may specifically hinder energetic pathways of brain tumors. A perturbation of the microtubular cytoskeleton causes a specific decrease in the S100 mRNA in C6 glioma cells (Baudet et al. 1998). S100 protein is a low molecular weight, Ca^{2+}-binding protein, and a decrease of S100β mRNA could hamper calcium-buffering efficacy of the cells (Baudet et al. 1998).

Among the upregulated genes, *Cysteine-rich protein (CRP)* gene, which codes a protein harboring a double zinc finger-like motif, was increased 7.1-fold (Baudet et al. 1998). The expression of this gene is modified during brain development. When expressed within the adherent fibroblasts, CRP family members bind specifically to the actin cytoskeleton and interact with the cytoskeletal proteins zyxin and α-actinin. Further, the *MGP* gene demonstrated a 3.8 –fold increase. This gene

encodes a Vitamin K-dependent Matrix Gla (gamma carboxyglutamic acid) Protein and is the substrate for the γ-carboxylase enzyme which catalyzes the conversion of glutamic acid to a γ-carboxyglutamic acid (Baudet et al. 1998). MGP mRNA exists in diverse tissues, including the brain, and its expression is upregulated in rat prostate following castration, which induces apoptotic death of androgen-dependent prostate cells. One of the detected cDNA clones encodes the β-tubulin (Baudet et al. 1998). This 4.3-fold increase of β-tubulin can be ascribed to the increased expression of TCP-1-δ since this latter protein is a member of cytoplasmic chaperonins necessary for the proper folding of tubulin and β-actin (Baudet et al. 1998). Relative β-tubulin immunofluorescence in CCRF-CEM human T cell leukemia cells is upregulated in apoptotic cells following exposure to a cytotoxic drug. Further, in *S. cerevisae*, a supraphysiological enhancement in the β-tubulin to α-tubulin ratio leads to the disassembly of microtubules and cytotoxicity (Baudet et al. 1998). The detection of *Mortalin* (*mitochondrial heat shock-70 protein* (*mtHsp70*)) was among the upregulated genes that could be directly ascribed to cellular senescence and death (Baudet et al. 1998). Fifteen of the increased cDNAs coded for ribosomal proteins indicating a likely contribution of the translational machinery in the apoptotic program induced by calcitriol (Baudet et al. 1998). Supporting this assumption, this induction of genes encoding ribosomal proteins was not demonstrated in benign astrocytes exposed to calcitriol. Normally, these cells respond to calcitriol by the stimulation of the expression of various genes including VDR, but do not undergo apoptosis (Baudet et al. 1998).

Below, we will suggest that cholecalciferol, which weakly binds to VDR, can trigger cell death in glioma cells via activating sphingomyelinase and enhancing ceramide levels. Therefore, VDR may not be essential in mediating tumoricidal efficacy of Vitamin D analogues. Conversely, other studies have reported contradictory data. Davoust et al. established a calcitriol resistant C6 glioma subclone in which VDR expression did not occur at the mRNA or protein levels (Davoust et al. 1998). Of note, the transfection of this subclone with VDR cDNA induced vulnerability to calcitriol, which occurred simultaneously with a robust increase of *c-myc* RNA expression (Davoust et al. 1998). However, one of the subclones with significant VDR expression following transfection failed to respond to calcitriol, suggesting that VDR is necessary, but may not be sufficient for calcitriol's tumoricidal activity. The authors proposed that any future clinical attempt to employ Vitamin D analogues in treatment of glial tumors should consider the tumoral expression of VDR. Alvarez-Dolado et al. demonstrated that calcitriol inhibited expression of Tenascin-C in C6 glioma cells in a dose-dependent manner (Alvarez-Dolado et al. 1999). Tenascin-C is an extracellular tissue matrix protein with high expression in normal glial cells during development, but has greatly reduced expression in adult brain. Glioma cells profoundly reactivate this protein, which provokes tumor growth, invasion, and angiogenesis; the authors have proposed that one of the antitumoral mechanisms of calcitriol in glioma cells may be the downregulation of Tenascin-C (Alvarez-Dolado et al. 1999).

Likely role of epigenetic regulation in vitamin D induced cell death in glioblastoma

In mammalian DNA, 2–7% of cytosine nucleotides exist as 5-methylcytosine. Methylation patterns, which mostly occur in the 5'-CG-3′ dinucleotide, are cell type specific and conserved (Canova et al. 1998). Cytosine methylation results in epigenetic suppression of genes involved in DNA repair, maintenance of chromosomal structure, genomic imprinting, and cell senescence (Canova et al. 1998). In addition to housekeeping genes, viral DNA sequences are highly methylated in cells with latent infection, whereas cells producing viri harbor unmethylated viral DNA. Cancer cells and viruses such as adenovirus protein E1B-19 K, Epstein–Barr virus protein BHFR1, baculovirus gene product *p35*, cowpox protein CrmA, and herpesvirus 8 protein KSbcl-2 encode receptor proteins with activities that inhibit apoptotic responses in the cells (Canova et al. 1998). Inaccuracy in the DNA methylation (both improperly high and low DNA methylation) may promote tumor formation (Canova et al. 1998). Calcitriol induced cell death of C6.9 cells was blocked by the DNA demethylating drugs 5-azacytidine (5-AzaC) and 5-aza-2′-deoxycytidine exposure (Canova et al. 1998). Furthermore, this effect was demonstrated through increased cell proliferation following removal of 5-AzaC and apoptotic internucleosomal DNA degradation was inhibited following treatment with 5-AzaC (Canova et al. 1998). On the other hand, c-*myc* gene expression was not affected which demonstrated that 5-AzaC did not fully block C6.9 cell calcitriol response. These results indicated that calcitriol induced apoptosis in glioma cells may require hypermethylation/epigenetic silencing of certain genes, presumably anti-apoptotic oncogenes, endogenous viral sequences, or genes regulating cell differentiation (Canova et al. 1998).

Vitamin D precursor cholecalciferol triggers ceramide pathway related cell death in glioblastoma cells

The name "ceramide" describes molecules with a long chain base (e.g. sphingosine) and amide-linked fatty acid with differing lengths (often between 16 to 24 carbons in mammals) (Magrassi et al. 1998). An elevation in cellular ceramide triggers the apoptotic pathway and can be stimulated by different stress conditions in cells such as cytokine TNF-α, Fas,

anthracyclines, and radiation (Magrassi et al. 1998). Differences in ceramide levels may act via dysregulation of the balance between ceramide and other sphingolipids (e.g. sphingosine-1-phosphate) exerting opposite actions on different MAPKs (mitogen activated protein kinases). Two biochemical mechanisms could lead an increase in intracellular ceramide: i) sphingomyelinase-mediated hydrolysis of the membrane phospholipid to produce phoshophocholine and ceramide, ii) activation of the cytosolic ceramide synthase leading to production of ceramide de novo (Magrassi et al. 1998).

Cholecalciferol, a relatively week VDR ligand, stimulates cell death in HU197 human glioblastoma cells and primary cultures obtained from a recurring human glioblastoma indicating that the actions of Vitamin D derivatives on glioma cells may be partially independent from VDR activation (Magrassi et al. 1998). Magrassi et al. assessed the actions of cholecalciferol on sphingomyelin ceramide and ganglioside GD3 levels in Hu197 cells (Magrassi et al. 1998). A prominent enhancement in ceramide levels and proportional decline in sphingomyelin occurred following 6 h of cholecalciferol treatment. Further, the increase in ceramide levels was followed by massive cell death. Ceramide III levels following treatment with sphingomyelinase were specifically enhanced while other ceramide levels declined or remained unchanged. Exposure to ceramides (N-acetylsphingosine or ceramide from bovine brain) or bacterial sphingomyelinase also triggered cytotoxicity in similar cell types (Magrassi et al. 1998).

Association between Tumoral expression of VDR and survival in glioblastoma and role of VDR in glioblastoma invasion in vitro

Salomón et al. analyzed immunohistochemical expression of VDR in human glioma cells to determine the association between the expression of VDR and clinico-pathological features (Salomón et al. 2014). They further investigated the actions of genetic and pharmacologic modification of VDR on invasion and survival of glioma cells. They found that VDR expression in glioma cells was significantly increased in comparison to VDR expression in benign brain tissues, and was associated with higher survival rates in glioblastoma patients (Salomón et al. 2014). Of note, the glioma samples used in this study were obtained from patients who were not treated with temozolomide between 1993 and 2003 (Salomón et al. 2014). Hence, the change in treatment regime following the temozolomide era would not have affected the obtained data. However, the authors have also noted that the correlation between VDR expression and survival might not be relevant to patients being treated with temozolomide (Salomón et al. 2014). The authors demonstrated that suppression of VDR expression in T98G cells significantly enhanced cell survival, while calcitriol-exposure stimulated VDR mRNA and protein levels, and decreased cell survival. In parallel with a role in arresting the cell cycle, calcitriol boosted levels of p21, p27, p57, and decreased cyclin D1expression (Salomón et al. 2014). Cyclin-dependent kinase (CDK) inhibitor p27 was expressed in the nucleus following calcitriol exposure indicating that calcitriol stimulated p27 activation. Furthermore, T98G cells with VDR expression through a specific silencing shRNA demonstrated increased invasion potential (Salomón et al. 2014). Conversely, calcitriol exposure caused a decline in the invasive potential of T98G cells, a process that could be blocked by silencing VDR activity. This invasion blockage by calcitriol and VDR could also be ascribed to intranuclear localization of p27, as intracytoplasmic p27 stimulates glioma cell invasion (Salomón et al. 2014).

Polymorphisms in the VDR and in genes encoding vitamin D-metabolizing enzymes and their association with glioma risk

In a case-control study, Anic et al. evaluated the association between single nucleotide polymorphisms (SNPs) in the genes involved in the Vitamin D signaling cascade, and glioma risk and prognosis in 622 glioma cases and 628 healthy controls (Anic et al. 2012). Subjects were genotyped for 7 SNPs in the *VDR* gene, and 8 SNPs in the *NADSYN1*, *GC*, *CYP24A1*, *CYP2R1*, and *C10ORF88* genes which were found to correlate with serum Vitamin D concentrations in genome-wide association studies (Anic et al. 2012). Risk of astrocytic tumors was associated with the following SNPs: rs2228570 (*Fok1, VDR*); rs731236 (*Taq1, VDR*); rs3829251 (NADSYN1); and rs10741657 (CYP2R1) (Anic et al. 2012). Survival associations were observed with SNPs rs1544410 (*Bsm1, VDR*) and rs6013897 (*CYP24A1*). rs2228570 (*Fok1*) was found to reduce glioma risk, while rs1544410 (*Bsm1*) was associated with worse survival (Anic et al. 2012). The VDR *Fok1* SNP is functionally relevant; the variant f(T) allele in *Fok1* produces a VDR protein that is three amino acids longer than the wild-type F(C) allele. Anic et al. observed a lowered glioma risk associated with the *Fok1* variant. Further, a relatively recent meta-analysis also demonstrated the protective role of *Fok1* variant in glioma (Xu et al. 2014). The influence of commonly studied VDR polymorphisms including *Taq1* (rs731236), *Apa1*(rs7975232), and *Bsm1* (rs1544410) is not well known, yet some clues suggest functional importance (Anic et al. 2012). RFLPs *Bsm1* and *Taq1* have high linkage disequilibrium in the 3′-untranslated region of the VDR and are among the SNPs most frequently investigated in relation to malignancies. *Taq1* and *Bsm1* do not influence the VDR protein structure, yet they are strongly linked to a poly(A) microsatellite repeat in the 3′-untranslated

region that may be associated with the VDR mRNA stability (Anic et al. 2012). The *CYP2R1* gene encodes a cytochrome P450 enzyme that hydroxylates Vitamin D to the active form of Vitamin D and its SNP rs10741657 is associated with higher vitamin D levels. Anic et al. found that the rs10741657 SNP reduced glioma risk (Anic et al. 2012).

Vitamin D potentiation of Temozolomide efficacy in glioma cells in vitro and in vivo

Temozolomide is an alkylating agent employed in the standard treatment of GBM, and acts via methylation of purine bases in the DNA (O6-guanine; N7-guanine and N3-adenine) to induce autophagic cell death (Bak et al. 2016). Autophagic Beclin-1 protein and mRNA levels are lower in the cytoplasm of GBM cells compared to lower-grade astrocytomas and healthy brain tissue. Further, analysis of glioma tumor specimens revealed that high levels of Beclin-1 in glioma cell cytoplasm was positively associated with improved survival (Bak et al. 2016). Since calcitriol exerts autophagic activities, Bak et al. investigated the antitumor effect of calcitriol combined with temozolomide in GBM (Bak et al. 2016). Temozolomide and calcitriol exerted synergistic antitumor effects in cell viability, colony formation, and wound healing assays in C6 glioma cells in vitro. Ultrastructural appearance of autophagosomes enhanced the number and size of microtubule-associated protein-1 light chain 3 (LC3) puncta, and enhanced conversion of LC3-I to LC3-II. This suggests robust activation of autophagy in temozolomide and calcitriol co-treated C6 cells (Bak et al. 2016). However, the rates of apoptosis did not differ between cells exposed to temozolomide and calcitriol and those exposed to temozolomide alone. 3-methyladenine, an autophagy inhibitor, significantly hindered the antitumor effect of temozolomide and calcitriol cotreatment, which suggested that calcitriol sensitization to temozolomide occurred via autophagic pathways (Bak et al. 2016). Lastly, and most importantly, temozolomide and calcitriol co-treatment significantly blocked progression of tumor growth and enhanced survival in glioblastoma xenograft models in comparison to temozolomide treatment alone (Bak et al. 2016).

Clinical trial with calcitriol in glioblastoma

Trouillas et al. conducted a phase II trial on 11 patients (10 glioblastoma and 1 anaplastic astrocytoma) treated with surgery or biopsy, radiotherapy (64 Gy), chemotherapy with teniposide-lomustine or fotemustine, and alfacalcidol (1-hydroxycholecalciferol) at the daily dose of 0.04 microg/kg. MRI took place every 6 months (Trouillas et al. 2001). Three out of 11 patients (27%, 2 GBM and 1 astrocytoma grade III) demonstrated progressive shrinkage of the radiological lesion, with shrinkage of the gadolinium-enhanced area (Trouillas et al. 2001). These patients also had complete clinical remission for 7, 5, and 4 years, respectively. Although these patients had normal or subnormal calcemia, cessation of the alfacalcidol was not required. Alfacalcidol was considered to be safe and acted in synergy with standart treatments, leading to progressive and durable tumor regression in 20% of malignant gliomas (Trouillas et al. 2001).

Vitamin D metabolism in glioblastoma and dose dependent dual effects of calcitriol on human glioblastoma growth in vitro

Diesel et al. treated C6 rat glioma, established human glioblastoma cell lines TX3868 and TX3095, and other primary glioblastoma cells with calcitriol and evaluated *CYP27B1* (described as 1α-hydroxylase above; converts calcidiol to calcitriol), 1α,25-dihydroxyvitamin D3–24-hydroxylase (CYP24, catabolizes calcitriol), and *VDR* expression (Diesel et al. 2005). Sixteen splice variants of *CYP27B1* in glioblastoma were defined with varying expression between GBM and healthy tissues. Preliminary data was obtained for the enzymatic action of CYP27B1in GBM cells, but not for the functional activities of the splice variants (Diesel et al. 2005). The expression of the *CYP27B*1 mRNA was enhanced in the majority of cultured GBM cell lines compared to the corresponding tumor biopsies (Diesel et al. 2005). The authors suggested that selective pressure for increased *CYP27B1* expression in GBM cell cultures may have played a role in CYP27B1 activity for malignant GBM growth. In contrast to data obtained from many previous studies on rat C6 glioma cell lines, they found that calcitriol at low doses (1 nM) stimulated GBM cell growth, while at high doses (1 μM) blocked GBM cell growth in vitro (Diesel et al. 2005). They further demonstrated the absence of significant VDR expression in GBM cell lines, and concluded that VDR-independent pathways may mediate actions of Vitamin D analogues. Most of the GBM cell cultures did not express CYP24 at significant levels (Diesel et al. 2005). Treatment with calcitriol induced higher expression of both CYP27B1and CYP24 but did not influence VDR expression (Diesel et al. 2005). Overall, their findings demonstrated that while GBM cell lines metabolize calcidiol, low doses of calcitriol may propagate tumor growth, and some of Vitamin D actions may be independent of VDR.

Vitamin D stimulation of stem cell populations and Chemoresistance in glioblastoma cell cultures

Maleklou et al. demonstrated that nanoparticle encapsulated Vitamin D3 (VD_3NP) in combination with doxorubicin and epirubicin significantly enhanced chemotherapy resistance and cancer stem cell (CSC) properties in C6 glioma cell lines (Maleklou et al. 2016). However, the VD3NP-docetaxel combination sensitized C6 cells to ionizing irradiation. Although the targeted delivery of Vitamin D_3 alone inhibited C6 glioma cells, it also exerted potential harmful effects by lowering chemosensitivity and enhancing CSC features (Maleklou et al. 2016). Combining Vitamin D_3 with doxorubicin reduced *p53* and *bax* expression and increased *bmi1* expression. The proapoptotic and anticancer actions of wild-type *p53* and *bax* are well established. *Bmi1* is an oncogene that encodes a zing finger protein belonging to the Polycomb Group Complex 1 (PRC1), and its aberrant expression is detected in numerous cancers (www.ncbi.nlm.nih.gov/gene/648). PRC1 regulates chromatin remodeling and is an important epigenetic regulator of various genes controlling embryonic development and renewal of somatic stem cells.

Vitamin D regulation of Neurotrophins and Neurotrophin receptors in glioma cells

Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), and Neurotrophin-4 (NT-4) are the four members of the neurotrophin family with specific roles in the development and functions of the nervous system (Neveu et al. 1994a). Neurotrophins are predominantly expressed in neural cells, but are also expressed in primary astrocytic cell cultures (Neveu et al. 1994a). Neurotrophins bind to two different classes of membrane receptors: the tyrosine kinase receptors (Trk) and the low-affinity neurotrophin receptor ($P75^{NTR}$) (Naveilhan et al. 1996a, 1996b). Members of the Trk family function as signal transducers and regulate the biological function of neurotrophins. Their affinity to neurotrophins is high ($K_d = 10^{-11}$ M) and the specificity of their neurotrophic effects is ascribed to selective interactions between various neurotrophins and Trk members (Naveilhan et al. 1996a, 1996b). TrkA is an NGF receptor, TrkB is a BDNF receptor, and NT-4/5, and TrkC are receptors for NT-3 (Naveilhan et al. 1996a, 1996b). Additionally, *TrkB* and *TrkC* genes undergo alternative splicing; the *TrkB* gene, in addition to the full-length form (f-TrkB), encodes two other receptor forms without the kinase domain (t-TrkB) (Naveilhan et al. 1996a, 1996b). For TrkC, in addition to the full-length isoform (f-TrkC), there are four truncated isoforms lacking the kinase domain (t-TrkC) and three isoforms containing either 14, 25, or 39 amino acid insertions in the tyrosine kinase domain (ki-TrkC) (Naveilhan et al. 1996a, 1996b). In contrast to Trk receptors, the low-affinity neurotrophin receptor $P75^{NTR}$ possesses similar affinity for all neurotrophins ($K_d = 10^{-9}$ M). $P75^{NTR}$ is a 75 kDa glycoprotein that functions as a ligand-presenting protein to enhance the levels of local neurotrophin control over the Trk receptors ligand specificity, and to regulate the biological responses to neurotrophins (Naveilhan et al. 1996a, 1996b). However, $P75^{NTR}$ is also structurally similar to the tumor necrosis factor receptor (TNFR) and induces the ceramide pathway (Naveilhan et al. 1996a, 1996b). Finally, neurotrophin receptors are expressed in both neural and glial cells, and their expression is controlled during ontogenesis and after neural injury.

Neveu et al. investigated the effects of calcitriol on NGF synthesis in primary astrocytic cultures obtained from brain of neonatal rats (Neveu et al. 1994b). NGF stimulates proliferation or differentiation of basal forebrain cholinergic neurons, and VDR expression was found to be lower in Alzheimer's Disease brains (Neveu et al. 1994b). Calcitriol stimulated synthesis of *NGF* mRNA in a dose-dependent manner with a peak effect at 100 nM, which persisted for a minimum 48 h (Neveu et al. 1994b). Calcitriol also enhanced levels of *VDR* mRNA in primary astrocytic cells and C6 glioma cells. Treatment of primary glial cells with 10 nM calcitriol stimulated a 2-fold enhancement of the NGF protein secretion following 3 days of treatment (Neveu et al. 1994b). Moreover, a 5-fold enhancement occurred 3 days following a second exposure to calcitriol.

Similarly, pretreatment with lower doses of calcitriol (such as 1 or 10 nM) augmented the cellular responses to a 24-h 10 nM calcitriol treatment. Hence, exposure duration regulates the synthesis of NGF, likely through an increase in *VDR* expression (Neveu et al. 1994b). The specificity of calcitriol was demonstrated by the absence of NFG synthesis following 100 nM treatment with the Vitamin D_3 metabolite, $24,25\text{-}(OH)_2D_3$ (Neveu et al. 1994b). In this setting, astrocytes showed the most robust response to calcitriol treatment. The removal of microglia or oligodendroglia did not affect the time interval or extent of the cellular response to calcitriol (Neveu et al. 1994b). Further, calcitriol did not affect NGF synthesis in meningeal fibroblasts, but stimulated *NGF* mRNA levels in C6 glioma cells (Neveu et al. 1994b).

Neveu et al. also investigated the effects of calcitriol on NT-3, NT-4, and BDNF levels in primary astrocytic cultures (Neveu et al. 1994a). They found that a 24-h calcitriol exposure increased NGF, but not BDNF levels in astrocytes. This is in contrast to the findings in neurons, in which the regulation of BDNF and NGF is mostly similar (Neveu et al. 1994a). Calcitriol also enhanced levels of NT-3 while decreasing levels of NT-4 (Neveu et al. 1994a). NT-3 is widely expressed in early CNS development, but in the adult brain, high levels only exist in the hippocampus and cerebellum (Neveu et al. 1994a). NT-3 is primarily expressed in neurons, but some data

suggests it is also expressed in glia. Regulation of NT-3 neuronal expression is different from that of NGF and BDNF. Both *NGF* and *BDNF* levels increase in response to a wide range of excitatory stimuli, while neuronal *NT-3* mRNA levels are not generally affected by neurotransmitters and decrease following seizures (Neveu et al. 1994a). TrkA is the signal transducing receptor in adult brain and is mainly restricted to basal forebrain cholinergic neurons (Neveu et al. 1994a). Therefore, calcitriol induced intracerebral stimulation of NGF may protect a small population of neurons. On the other hand, the NT-3 receptor, TrkC is widely expressed in the brain and stimulation of NT-3 levels by calcitriol may promote the survival of a larger neural population (Neveu et al. 1994a).

Both primary astrocytes and C6 glioma cells synthesize in vitro mRNAs that encode the varying forms of neurotrophin receptors, but not TrkA (Naveilhan et al. 1996a). Truncated Trk receptors are the dominant form in glial cells; both full-length TrkB and TrkC are expressed in primary glial cell cultures, while only the full length TrkB is expressed in C6 cells (Naveilhan et al. 1996a). Of note, calcitriol did not modify the synthesis of full length or truncated forms of *TrkB* or *TrkC* receptor mRNA in primary glial cultures or in C6 glioma cells (Naveilhan et al. 1996a). Conversely, low levels of calcitriol enhanced low-affinity neurotrophin receptor ($P75^{NTR}$) mRNA expression and protein levels in C6 glioma cells, but not in primary astrocytic cultures, even at concentration as high as 1 μM (Naveilhan et al. 1996a).

Given that the basal level of $P75^{NTR}$ mRNA in glia is 50-fold lower than in C6 glioma cells, it is presumed that a low basal level of $P75^{NTR}$ in glia hindered any upregulation by calcitriol (Naveilhan et al. 1996a).

It should be noted that *VDR* mRNA upregulation in primary glia required higher concentrations of hormone (10^{-9} M) in comparison to C6 glioma cells (10^{-11} M) (Naveilhan et al. 1996a). Since long-term exposure to calcitriol triggers cytotoxicity in C6 glioma cells, but not in primary glia, a likely involvement of $P75^{NTR}$ in calcitriol's specific tumoricidal activity was suggested (Naveilhan et al. 1996a). Indeed, previous studies have shown that a high synthesis of $P75^{NTR}$ in the absence of its ligand could trigger apoptosis. In addition, $P75^{NTR}$ exerts partial homology with TNFR to induce apoptosis via activation of the ceramide pathway. Given that the binding of NGF to $P75^{NTR}$ also stimulates the ceramide pathway, simultaneous overexpression of $P75^{NTR}$ and NGF with calcitriol treatment may trigger apoptosis in glioma cells via an autocrine loop (Naveilhan et al. 1996a). Putative VDRE in the $P75^{NTR}$ promoter was studied by transecting plasmids, including sequences from $P75^{NTR}$ promoter fused to a cat reporter gene. A region between 610 and 860 base pairs upstream from the translation start codon responded to calcitriol (Naveilhan et al. 1996a). In vivo experiments demonstrated that calcitriol exposure in 15-day-old and adult rats lowered the $P75^{NTR}$ mRNA expression in the spinal cord without an effect in dorsal root ganglion neurons or sciatic nerve neurons. These data demonstrated a specific regulatory role of calcitriol on expression of $P75^{NTR}$ in vivo (Naveilhan et al. 1996a).

Calcitriol stimulates NGF production in glioma. But how does NGF act on glial tumors?

As suggested above, some authors proposed that simultaneous stimulation of $P75^{NTR}$ and NGF with calcitriol treatment may trigger apoptosis in glioma cells via the ceramide pathway (Naveilhan et al. 1996a). NGF treatment in rats implanted with an anaplastic glioma (F-98) decreased tumor growth rate and prolonged survival (Vinores and Koestner 1980). NGF also reduced tumor cell proliferation and enhanced F-98 glioma cell differentiation (Vinores and Koestner 1980). Hansen et al. showed an inverse correlation of TrkA expression in higher grade human gliomas, and when they transfected human TrkA cDNA into the human glioblastoma cell line G55, they observed cell cycle arrest and enhanced autophagy (Hansen et al. 2007). Despite these data, other studies consistently demonstrated the opposite effect, and proposed a tumor propagating role of NGF in gliomas. However, these studies are based on cell culture and biopsy specimens and no in vivo animal models have been tested. Singer et al. observed enhanced proliferation in the human glioma cell lines U251, U87, and U373 when they added NGF into the serum free medium (Singer et al. 1999). Brown et al. further showed that NGF triggered proliferation of glioma cells via α9β1 integrin, a factor not expressed by benign astrocytes, in correlation with increasing tumor grade (Brown et al. 2008). Chinese researchers also showed that nuclear presence of NGF in human glial tumors correlates with tumor grade (Li et al. 2011). Berghoff et al. demonstrated a pro-invasive role for NGF in glioma cells via two distinct pathways; γ-secretase, and Cadherin-11 in a γ-secretase-independent manner (Berghoff et al. 2015). Ahn et al. demonstrated that $P75^{NTR}$ stimulated human glioma cell invasion in vitro using PDLIM1 as a signaling adaptor (Ahn et al. 2016). Lastly, Zhou et al. revealed that $P75^{NTR}$ could block p53 activity by inducing proteolysis via an MDM2-ubiquitin cascade and by direct binding to its DNA binding domain (Zhou et al. 2016). Conversely, ablation of $P75^{NTR}$ stimulated p53, apoptosis, and lowered clonogenicity and survival of cancer cells (Zhou et al. 2016).

Calcitriol stimulates NT-3 in glioma. Do NT-3 and its receptor TrkC have any role in gliomas?

Above we have suggested calcitriol stimulated glioma secretion of NT-3, which is widely expressed in early CNS

development, but mostly limited to the hippocampus and cerebellum in adult brain (Neveu et al. 1994a). Lawn et al. demonstrated that specific activation of TrkC by its ligand NT3 promoted survival of cells capable of initiating glial tumor growth via stimulation of ERK and Akt, and that this process can be reversed with genetic or pharmacological suppression of TrkC or its downstream targets, ERK and Akt (Lawn et al. 2015). Decreased proliferation of glial tumor-initiating cells through EGFR inhibitors could be induced by TrkC signaling, and the neurotrophin cascade is sufficient for sustained proliferation of glial tumor-initiating cells as tumor spheres in the absence of the growth factors EGF and FGF (Lawn et al. 2015). Lastly, Jawhari et al. demonstrated robust expression of NT-3 and TrkC in GBM specimens, and that NT-3/TrkC signaling enhances in vitro survival of GBM cells in a hypoxic environment (Jawhari et al. 2017).

Calcitriol robustly stimulates GDNF production in C6 glioma. GDNF as a likely promoter of glioma progression

Glial cell line-derived neurotrophic factor (GDNF) is not a neurotrophin, yet it exerts prominent therapeutic potentials for neurodegenerative diseases (Naveilhan et al. 1996b). Treatment of C6 cells with 100 nM calcitriol for 48 h induced an 18.5-fold enhancement in GDNF mRNA expression. Further, calcitriol was effective at levels as low as 0.1 nM (Naveilhan et al. 1996b). Wiesenhofer et al. assessed and compared expression of GDNF in fibroblasts, healthy rat and human brain tissues, C6 glioma cells, and human glioma tissues (Wiesenhofer et al. 2000a). C6 cells exerted the highest synthesis of GDNF, whereas 3 T3 fibroblasts lacked expression of GDNF. Immunohistochemistry demonstrated robust GDNF- and GDNF receptor-α1-expressing cells in human glial tumors, which was also confirmed with RT-PCR mRNA. Glial tumors, including the most undifferentiated GBM, expressed GDNF levels at concentrations up to five times that of healthy brain (Wiesenhofer et al. 2000a). Blockage of GDNF- and GDNF receptor-α1 expression with antisense nucleotides inhibited C6 glioma cell proliferation (Wiesenhofer et al. 2000b). In human glial tumors, GFRα1b is the dominant spliced receptor variant, while RET9 is the dominant coreceptor of GDNF (Ng et al. 2009). Exposure of GBM cells to GDNF, but not to the alternative ligand neurturin, increased proliferation and provided resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) via Akt stimulation and JNK inhibition (Ng et al. 2009). Very recently, Sun et al. isolated neuropilin-1 (NRP1) as a GDNF receptor in C6 glioma cells. When analyzed human glioma tissues, it was found that NRP1 was an independent risk factor for recurrence and shortened survival in GBM patients (Sun et al. 2017). Expression of high *NRP1* mRNA levels correlated with shorter overall and disease-free survival (Sun et al. 2017).

Overall, calcitriol induced stimulation of GDNF and neurotrophins, NGF and NT-3, may adversely affect glioma cell growth, despite contrary in vitro and in vivo studies. It should be noted that even cytotoxic chemotherapeutic agents and radiation treatment could simultaneously stimulate pro- and anti-apoptotic signals in malignant cells. Here, the important outcome is the net effect of antitumor activity, which is likely attenuated with simultaneously activated protective/proliferative signals. Very likely, a similar mechanism accounts for the antineoplasticity of calcitriol; its major effect would be antitumoral although it stimulates NGF, NT-3, and GDNF, which may alleviate its anticancer potency. Therefore, agents that act synergistically with VDR signaling and boost tumoricidal activity of calcitriol may increase the net antitumoral potency.

The possible risky effect of vitamin D in glioblastoma is mostly experimental and cell-culture based, yet a careful approach is needed

In this manuscript, we discussed the potential propagating effects of Vitamin D on glioma cell growth. However, it is also important to note that the majority of epidemiological human studies demonstrated a beneficial effect of Vitamin D on glioblastoma risk and survival. This is also corroborated with findings that showed a correlation of VDR expression with enhanced survival in GBM patients (Salomón et al. 2014). Above, we suggested that calcitriol enhanced the stem cell population in glioma cell cultures however, as the authors themselves have also admitted (Maleklou et al. 2016) that this effect was seen with almost every antitumor agent, as cancer cells that survive a noxious event generally exert stem cell features. Moreover, in vivo data obtained from rat studies showed synergism between calcitriol and temozolomide (Bak et al. 2016). Pilot clinical studies also showed potential efficacy of Vitamin D treatment for GBM. Still, a careful approach and well-planned strategies are needed to avoid any negative effects of Vitamin D, although they could potentiate the efficacy and benefits of this molecule. In this aspect, we strongly believe that combining Vitamin D, temozolomide, and ATRA (All-Trans Retinoic Acid) would be a logical strategy to minimize risks and maximize benefits of Vitamin D in GBM management. Below, we will provide data to strengthen our proposal.

Potential synergistic and antagonistic actions of vitamin D and vitamin a derivatives in anticancer activity. Any surrogate marker to predict risks versus benefits?

As suggested above, VDR controls gene translation via binding to specific DNA-response sequences of target genes following heterodimerization with the retinoid X receptor (RXR) (retinoids are analogues of Vitamin A) and the recruitment of various nuclear receptor coactivator proteins, including steroid receptor coactivator family members (Garcion et al. 2002). VDR heterodimerizations with the nuclear receptors for thyroid hormone, ATRA, and Smad3 (a SMAD protein family-associate transmitting the signal for transforming growth factor β (TGF-β)) were also demonstrated (Garcion et al. 2002). To the best of our knowledge, no epidemiological studies have investigated the mutual interactions between Vitamin D and Vitamin A in modifying risk of glial tumors. However, studies have shown that these two group of molecules may have synergistic or antagonistic functions in modifying risk of cancer. For instance, in colorectal cancer, the dose-response analysis of the interaction between circulating calcidiol concentration and level of dietary retinol intake revealed that higher calcidiol levels were more prominent at lower intakes of retinol (Jenab et al. 2010). Similarly, Field et al. observed a reduction in the protective effect of vitamin D on overall survival in melanoma patients harboring high vitamin A levels (Field et al. 2013). On the other hand, Cheng et al. demonstrated that Vitamin A synergized with Vitamin D in preventing pulmonary cancer among smokers (Cheng et al. 2014). Similar studies showed anticancer synergisms between Vitamin A and D in prostate cancer cells, which was accompanied by an elevation in *Bax* expression and apoptotic cell death (Sha et al. 2013).

These conflicting epidemiological studies on Vitamin A and D interactions in modifying cancer risk are not easily explicable. Nonetheless, some clues are provided by cell culture studies that focused on the receptors of these two diverse vitamins/hormones. AML is characterized by an accumulation of neoplastic immature myeloid precursors and is currently treated with ATRA, which makes it a desirable model (Marchwicka et al. 2016). Studies have suggested synergistic antineoplastic effects of ATRA and Vitamin D in AML. However, the effect of combination treatment varies in different AML cell lines due to ATRA induced upregulation or downregulation of VDR transcription among AML cell lines (Marchwicka et al. 2016). The transcriptional regulation pathway of VDR in response to ATRA has not been fully illuminated. Some studies have shown that the retinoic acid receptor α (RARα) is responsible for regulating VDR transcription in AML cells. In cells with a high basal level of RARα protein and in the absence of a RARα agonist, the VDR gene is transcriptionally repressed, while the down-regulation of RARα leads to increased VDR expression (Marchwicka et al. 2016).

These associations are complex and may be cell specific (Gocek et al. 2012). Generally, calcitriol stimulates monocyte-like differentiation of AML cells while ATRA induces granulocyte-like cells. However, AML constitutes heterogeneous blood dyscrasias characterized by a block at various phases of hematopoietic differentiation and a differential response to therapies with calcitriol or ATRA. For instance, the AML cell line KG-1 is resistant to calcitriol-induced monocytic differentiation, while sensitive to ATRA-induced granulocytic differentiation; these cells harbor very low level of VDR protein that is upregulated by ATRA. On the other hand, in the AML cell line HL60, *VDR* mRNA is downregulated by ATRA (Gocek et al. 2012). Even more intriguing, differential species-specific interactions between Vitamin D and A receptors indicate that further scientific experiments are warranted. Indeed, Janik et al. revealed that the regulation of VDR expression in humans differs from mice (Janik et al. 2017). VDR and RAR receptors are present and transcriptionally active in blood cells of both species, especially at early phases of haematopoiesis, while induction with different ligands is species-specific. In early haemotopoiesis, ATRA, but not calcitriol, upregulates the expression of VDR in human blood cells, while in murine blood cells calcitriol, but not ATRA, upregulates VDR expression (Janik et al. 2017).

Magrassi et al. analyzed the effects of calcidiol and calcitriol on two human glioblastoma cell lines in the absence and presence of all trans-retinoic acid (ATRA) (Magrassi et al. 1995). Both Vitamin D analogues significantly reduced human glial tumor cell growth at levels over 5 μM. When the HU 70 cell line was exposed to calcitriol combined with 1 μM of ATRA, significant inhibition was witnessed, even after addition of calcitriol in the nanomolar range, which mainly occurred via enhanced cell death (Magrassi et al. 1995). Here, we suggest that tumoricidal synergism between ATRA and calcitriol may have also occurred via boosting synthesis of neurosteroids, particularly progesterone. Both neurons and astrocytes can produce steroids de novo from cholesterol; steroids produced within the central or peripheral nervous systems have been termed “neurosteroids” (Yagishita et al. 2012). The mitochondrial cytochrome P450scc (CYP11A1) is an enzyme that cleaves the cholesterol side chain responsible for catalyzing the de novo production of pregnenolone and 3β-hydroxysteroid dehydrogenase (HSD3B1), the enzyme responsible for synthesis of progesterone from pregnenolone (Yagishita et al. 2012). Yagishita et al. demonstrated that calcitriol enhanced *CYP11A1* and *HSD3B1* gene expression and progesterone synthesis in human glioma cells, which also occurred following exposure to calcipotriol, another VDR-agonist (Yagishita et al. 2012). Additionally, calcitriol increased ATRA-induced *CYP11A1* gene expression and progesterone production (Yagishita et al. 2012). As we (Altinoz

et al. 2001; Altinoz et al. 2007; Altinoz et al. 2018) and others (Atif et al. 2015a; Atif et al. 2015b) have demonstrated, progesterone efficacy reduced glial tumor growth, and therefore, the synergism between ATRA and calcitriol may have also occurred via neurosteroidogenesis. On the other hand, as suggested above, Vitamin D and Vitamin A receptors can be differentially regulated by their agonists in different cell lines of the same malignancy (i.e. AML); a similar phenomenon may also occur in glioblastoma cells. To avoid any risks of combining ATRA and Vitamin D in GBM management, primary monolayer (and 3-dimensional spheroid) cell cultures obtained from patient's tumor tissues were exposed to ATRA and/or Vitamin D. The responses of growth reduction and receptor expression will then be analyzed in order to predict a clinically meaningful benefit of a differentiation therapy.

Clinical studies with Temozolomide and retinoic acid combination in glioblastoma

In 2003, Jaeckle reported their Phase II trial which assessed the efficacy of combined treatment with 13-*cis* Retinoic Acid and temozolomide in recurrent glioma (Jaeckle et al. 2003). Overall survival was 46% (36% to 57%) at 52 weeks and 21% (13 to 31%) at 104 weeks. Of the 84 evaluable patients, there were two (3%) complete and eight (12%) partial responses (complete plus partial response, 15%) (Jaeckle et al. 2003). Temozolomide and 13-*cis* Retinoic Acid were active, surpassing their 20% thresholds for progression free survival (Jaeckle et al. 2003). Another Phase II trial performed at the University of California at San Francisco found no survival advantage of temozolomide and 13-*cis* Retinoic Acid combination in comparison to historical trials using radiotherapy either with adjuvant nitrosourea, with temozolomide alone, or with the combination of temozolomide and thalidomide (Butowski et al. 2005). Sirachainan et al. investigated the potential of 13-*cis* Retinoic Acid and temozolomide combination with radiotherapy in children with pons glioma (Sirachainan et al. 2008). The prognosis of diffuse pons glioma is grave with a median survival of 9 months with radiotherapy. Their treatment protocol included simultaneous radiotherapy (55.8–59.4 Gy) with temozolomide (75 mg/m^2/day) and *cis*-retinoic acid (Sirachainan et al. 2008). Ten of the 12 patients responded after completion of simultaneous radiotherapy. Seven patients responded partially, four exerted disease stabilization, and one progressed. Progression-free survival at one-year was 42% and one-year overall survival was about 58%, which was an encouraging result (Sirachainan et al. 2008). Clarke et al. randomly assigned 85 adult patients with a new GBM diagnosis to radiotherapy with simultaneous daily temozolomide, followed by six cycles of either dose-dense or metronomic temozolomide (Clarke et al. 2009). If a patient did not respond to six cycles of temozolomide either clinically or radiographically, therapy was switched to single-agent 13-*cis*-retinoic acid 100 mg/m^2 daily during days 1 to 21 of a 28-day cycle. The 1-year survival was 80% for the dose-dense and 69% for the metronomic arm, while median overall survival was 17.1 months (14.0 to 28.1 months) and 15.1 months (12.3 to 18.9 months), respectively (Clarke et al. 2009).

Grauer et al. published results of a prospective phase-II pilot study which determined the potential of adjuvant temozolomide and 13-*cis* Retinoic Acid combination in patients with recently diagnosed anaplastic gliomas following total or subtotal surgical resection (Grauer et al. 2011). Adult patients ($n = 32$) were enrolled in the study and treated with a median number of 10 temozolomide and 13-*cis* Retinoic Acid cycles (range 1–26). Temozolomide was given at 200 mg/m^2 on days 1 through 5 of 28-day cycles. 13-*cis* Retinoic Acid was administered at a single dose of 60 mg/m^2 daily on days 1–21 and repeated following completion of a 28-day cycle (Grauer et al. 2011). Escalations of dosages in steps of 10 mg/m^2/cycle up to 100 mg/m^2 were employed if no serious side effects were witnessed during the previous cycle. In patients with grade 3–4 toxicity, a dose tapering was employed in steps of 10 mg/m^2/cycle. The majority of patients had good prognostic features including young age, total surgical resection, oligodendroglial histology, co-deletion of 1p/19q, methylation of the O6-methylguanine-DNA methyltransferase (MGMT) gene, and isocitrate dehydrogenase 1 (IDH1) mutation (Grauer et al. 2011). The median progression free survival was 38 months (22.2–53.4). The 6-, 12- and 24-months progression free survival rates were 84.4, 75, and 42.4% (Grauer et al. 2011). The authors concluded that 13-*cis* Retinoic Acid and temozolomide combination did not yield a survival advantage when they compared the temozolomide-treated group within a previous randomized NOA-04 phase-III trial (Grauer et al. 2011). Overall, adding retinoic acid treatment to adjuvant temozolomide had only modest activity in high grade glial tumors.

Rationale for calcitriol+ATRA+Temozolomide strategy

Here, we will try to justify our proposal that a triple combination of calcitriol, ATRA, and temozolomide for adjuvant treatment of GBM would exert further efficacy. Above, we suggested that ATRA and calcitriol exerted robust synergism in suppressing glioma growth (Magrassi et al. 1995) and calcitriol increased ATRA-induced *CYP11A1* gene expression and progesterone production (Yagishita et al. 2012), which may be of benefit in glioblastoma treatment. There are additional studies that support our proposal. For example, Wang et al. demonstrated that pretreating human breast cancer cells with calcitriol and ATRA prior to treatment with paclitaxel or

doxorubicin lowered the ED_{50} for blockage of clonogenic growth or for apoptosis by up to 2 logs for paclitaxel and up to 1 log for doxorubicin (Wang et al. 2000). They also demonstrated that both calcitriol and ATRA augmented paclitaxel induced phosphorylation of Bcl-2 (Wang et al. 2000). c-Jun NH2-terminal kinase–mediated multiple-site Bcl-2 phosphorylation was shown to block its protective activity in paclitaxel-induced apoptosis (Yamamoto et al. 1999); hence both ATRA and calcitriol-mediated augmentation of chemotherapy-associated Bcl-2 phosphorylation may be a specific chemosensitizing mechanism.

Bcl-2 was first defined as an oncogene in B cell follicular lymphomas and is a key regulator of apoptosis often harnessed for intrinsic resistance to cell death in many malignancies, including lymphoma, pulmonary carcinoma, and GBM (Kouri et al. 2012). *Bcl-2* blocks the intrinsic mitochondria-dependent apoptotic pathway, which is often induced by antineoplastic agents. Paradoxical results regarding expression of *Bcl-2* and tumor grade in gliomas have demonstrated that low-grade gliomas were found to express higher levels of Bcl-2 protein (Kouri et al. 2012). On the other hand, in a mouse model of oligodendroglioma, coexpression of PDGF-β and Bcl-2 promoted tumor progression to anaplastic disease (Kouri et al. 2012). Bcl-2 also exerts proinvasive activities in glioma cells via enhanced activity of furin, which proteolytically activates invasive metalloproteinases (MMP) and TGF-*β*. Lastly, miR-153, a negative microRNA regulator of *Bcl-2*, triggers cell death in GBM (Kouri et al. 2012). Therefore, *Bcl-2* suppressive phosphorylating efficacies elicited both by ATRA and calcitriol may lower the apoptotic threshold of glioblastoma cells.

In squamous cell carcinoma, both ATRA and calcitriol alone induced apoptosis and decreased survivin levels, which synergistically potentiated the effects of the chemotherapeutic 5-Fluorouracil (5-FU), a DNA synthesis inhibitor chemotherapeutic like temozolomide (Satake et al. 2003). Survivin belongs to the family of "inhibitor of apoptosis (IAP) proteins" that block caspase activity and prevent apoptosis. This protein is highly produced in most malignancies and is associated with worse prognosis (Jaiswal et al. 2015). Survivin has consistently been associated with high tumor grades, lower survival, and enhanced recurrence. Very recently, it was also shown that nuclear expression of survivin is a poor prognostic factor in GBM patients (Saito et al. 2017). As both calcitriol and ATRA are potential repressors of survivin and induce chemosensitization to 5-FU, it would be logical to hypothesize that they may also exert synergistical benefits in chemosensitization to GBM. Lastly, both ATRA and calcitriol synergize with Epidermal Growth Factor Receptor (EGFR)-inhibitors to induce differentiation, senescence, and apoptosis in leukemia cells (Lainey et al. 2013), suggesting that both agents may target similar tyrosine kinase pathways involved in cellular growth and resistance against cell death.

In 2012, a Czech study group reported efficacy of COMBAT regime, which includes Vitamin D_3, retinoic acid (isotretinoin 100 mg/m^2), celecoxib, and fenofibrate in addition to low dose metronomic temozolomide and etoposide in management of 74 children with advanced refractory malignancies (Zapletalova et al. 2012). Median response time was 6 months and the 2-years overall survival was about 43% (range 1.3–70 months). At the time of the report, 24 patients were alive; 6 (8%) with disease progression, 7 (9%) with partial, and 11 (15%) with complete responses. The treatment was well tolerated and exerted clinical efficacy (Zapletalova et al. 2012).

Both ATRA and vitamin D may cause Pseudotumor Cerebri. The rationale for simultaneous acetazolamide application to avoid a significant side effect of CAT protocol

Pseudotumor cerebri (or idiopathic intracranial hypertension) is a disease characterized by increased intracranial pressure with a mostly unknown etiology. Symptoms include headache (sometimes severe) and vision problems, including complete vision loss. Several theories were proposed to explain the pathogenetic mechanisms of the pseudotumor cerebri; i) an excess of cerebrospinal fluid (CSF) production, ii) increased volume of intracranial blood or brain tissue, iii) obstruction of the veins that drain blood from the brain, and iv) a combination of these etiologies. Both ATRA and Vitamin D may induce intracranial hypertension (Coombs et al. 2016; Zaki et al. 2013). Here, we propose that adding acetazolamide to CAT protocol (Calcitriol-ATRA-Temozolomide) may provide dual benefits including lowering intracranial pressure and exerting direct antitumoral features. Acetazolamide is a sulfonamide carbonic anhydrase inhibitor that mediates reabsorption of bicarbonate, sodium, and chloride in the renal proximal tubule. By blocking this enzyme, bicarbonate, sodium, and chloride ions are excreted, along with excess water causing a reduction in blood, intracranial, and intraocular pressure. Peculiarly and very importantly, acetozolamide was also found to increase antineoplastic potency of temozolomide in glioblastoma cell lines (Das et al. 2008) and to block hypoxia adaptation of glioblastoma cells by inhibiting overexpression of carbonic anhydrase (Said et al. 2013). Overall, adding acetazolamide to the proposed CAT protocol would be a very logical approach for both reducing the likely side effects and for augmenting treatment efficacy.

Conclusions and future perspectives

Despite the small number of experimental studies suggesting that Vitamin D analogues may stimulate growth or drug resistant stem cell population, the majority of human epidemiological, clinical, and pathological data suggest that adding Vitamin D to conventional treatment of high-grade glial tumors would be beneficial. These data can be summarized as: i) Association of low sun light exposure with enhanced glioma risk or worse survival in glioma, ii) Lowered risk of glioma in association with high prediagnostic Vitamin D levels, iii) Enhanced survival of glioma patients who consume Vitamin D supplements, iv) Better survival of glioblastoma patients whose tumors express Vitamin D receptor, and v) Results of a pilot Phase-II clinical investigation showing significant survival benefit in glioma patients (about 20%) treated with Vitamin D in addition to conventional treatments. Nonetheless, Vitamin D stimulation of NGF, $p75^{NTR}$, NT-3, GDNF, VEGF, and IL-6 may limit its efficacy to block glioma cell growth. However, it should be noted that many conventional treatment strategies including chemotherapeutic agents and radiotherapy also induce pro-survival or even proliferative signals in cancer, leading to the hypothesis of drug-resistant cancer stem cells. Hence, drug cocktails that hinder innapropriate dual signals may be of great benefit. The Vitamin A derivative, ATRA, robustly potentiates antitumor effects of calcitriol in glioma cell cultures and both agents suppress the activity of antiapoptotic molecules *Bcl-2* and Survivin. Therefore, we hypothesized that a triple regimen including calcitriol, ATRA, and temozolomide would exert more potent survival benefit in glioblastoma. Given the poor prognosis associated with GBM and all of these agents being commercially available and currently employed in clinic, clinical trials with a triple combination therapy would be plausible.

Author contributions İ.E. and M.A.A. developed the hypothesis and evaluated final draft of the manuscript. A.O., A.O and J.L.P. reviewed the manuscript and made relevant criticisms. M.A.A. performed literature analysis and made relevant changes according to the advises of İ.E. and A.O.

Compliance with ethical standards

Conflict of interest None.

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