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Dexamethasone Alone and in Combination with Desipramine, Phenytoin, Valproic Acid or Levetiracetam Interferes with 5-ALA-Mediated PpIX Production and Cellular Retention of PpIX in Glioblastoma Cells

Johnathan E. Lawrence
Upper Michigan Brain Tumor Center, Northern Michigan University, jolawren@nmu.edu

Christopher J. Steele
College of Human Medicine, Michigan State University

Richard A. Rovin
Aurora Neuroscience Innovation Institute, Aurora Health Care

Robert J. Belton Jr
Northern Michigan University

Robert J. Winn
Northern Michigan University

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LABORATORY INVESTIGATION

Dexamethasone alone and in combination with desipramine, phenytoin, valproic acid or levetiracetam interferes with 5-ALA-mediated PpIX production and cellular retention in glioblastoma cells

Johnathan E. Lawrence[1] · Christopher J. Steele[2] · Richard A. Rovin[3] · Robert J. Belton Jr.[1] · Robert J. Winn[1]

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Abstract Extent of resection of glioblastoma (GBM) correlates with overall survival. Fluorescence-guided resection (FGR) using 5-aminolevulinic acid (5-ALA) can improve the extent of resection. Unfortunately not all patients given 5-ALA accumulate sufficient quantities of protoporphyrin IX (PpIX) for successful FGR. In this study, we investigated the effects of dexamethasone, desipramine, phenytoin, valproic acid, and levetiracetam on the production and accumulation of PpIX in U87MG cells. All of these drugs, except levetiracetam, reduce the total amount of PpIX produced by GBM cells ($p < 0.05$). When dexamethasone is mixed with another drug (desipramine, phenytoin, valproic acid or levetiracetam) the amount of PpIX produced is further decreased ($p < 0.01$). However, when cells are analyzed for PpIX cellular retention, dexamethasone accumulated significantly more PpIX than the vehicle control ($p < 0.05$). Cellular retention of PpIX was not different from controls in cells treated with dexamethasone plus desipramine, valproic acid or levetiracetam, but was significantly less for dexamethasone plus phenytoin ($p < 0.01$). These data suggest that medications given before and during surgery may interfere with PpIX accumulation in malignant cells. At this time, levetiracetam appears to be the best medication in its class (anticonvulsants) for patients undergoing 5-ALA-mediated FGR.

Keywords Glioma · 5-Aminolevulinic acid · Protoporphryn IX · Fluorescence-guided surgery · Photodynamic therapy

Introduction

Patients with glioblastoma multiforme (GBM) have a less than 5 % 5-year survival rate [1]. The extent of resection is the most important treatment-related variable for improving prognosis [2]. Extent of resection can be limited by the difficulty of discriminating between tumor and adjacent white matter. An emerging tool to achieve maximal safe resection and thereby increase overall survival is the use of 5-aminolevulinic acid (5-ALA) for fluorescence-guided resection (FGR). 5-ALA is selectively taken up by neoplastic cells and metabolized to protoporphyrin IX (PpIX). PpIX fluorescence under light of 400-410 nm allows neurosurgeons to distinguish neoplastic from non-neoplastic tissue. Unfortunately, not all patients given 5-ALA before surgery accumulate sufficient PpIX in their malignant cells to make the use of FGR. This may in part be due to medications administered in the perioperative period.

5-ALA is a precursor compound for porphyrin production in the heme biosynthesis pathway [3]. Stummer and colleagues showed that rat C6 glioma cells metabolize 5-ALA and accumulate excess PpIX in vitro and in vivo permitting fluorescent detection [4]. Subsequent clinical trials confirmed the effectiveness of 5-ALA for FGR with better resection rates and improved overall survival [5–8]. Volumetric analysis showed that PpIX fluorescence extends beyond the enhancing tumor margin on MRI [9].

✉ Robert J. Winn
rwinn@nmu.edu

[1] Upper Michigan Brain Tumor Center, Northern Michigan University, 1401 Presque Isle Ave, Marquette, MI 49855, USA

[2] College of Human Medicine, Michigan State University, 965 Fee Rd, Room A-110, Lansing, MI 48824, USA

[3] Aurora Neuroscience Innovation Institute, Aurora Health Care, 2801 W Kinnickinnic River Pkwy, Milwaukee, WI 53215, USA

5-ALA administration may not only assist in resection, it may also be used as a diagnostic tool. In one study, 100 % of fluorescent positive biopsies were later confirmed by tissue staining as malignant or high-grade brain neoplasms [10]. Further, urine analysis of PpIX in GBM patients may provide a non-invasive diagnostic tool for the disease. Utsuki et al. analyzed urine as a predictive model for intraoperative fluorescence and found that 100 % of glioblastoma, 50 % of benign astrocytoma and 50 % of metastatic brain tumor patients exhibited PpIX fluorescent urine 2 h after 5-ALA administration [11].

Prescreening for PpIX accumulation in the urine may help determine which patients will respond to 5-ALA during surgery [11], but it does not explain why some patients do not accumulate adequate PpIX for detection during surgery. Removal of the 5-ALA/PpIX compounds from the cell and/or ferrochelatase activity may result in poor PpIX accumulation. The ATP-binding cassette transporter ABCG2 shuttles porphyrins out of cells, and blocking ABCG2 function results in porphyrin accumulation [12]. ABGC2 transporters are also known to transport anticancer drugs out of malignant cells and are overexpressed and correlated with tumor grade in glioma [13]. In addition, the silencing of ferrochelatase production in glioma cells lines using siRNA augments PpIX accumulation and fluorescence [14]. It has also been hypothesized that medications may interfere with the cells ability to metabolize 5-ALA and/or accumulate PpIX [15].

Anticonvulsants are commonly used in the management of seizures in brain tumor patients. In 2012, Hefti and coworkers, reported that phenytoin, but not levetiracetam, interferes with metabolism of 5-ALA and the production of PpIX in glioma cells [15]. Additionally, there are other classes of drugs used prior and during surgery that may interfere with 5-ALA metabolism and/or PpIX accumulation. Here we expand on the previous work with phenytoin and levetiracetam to investigate the effects of dexamethasone (corticosteroid), desipramine (antidepressant), and valproic acid (anticonvulsant) on the production and accumulation of PpIX in GBM cells using an in vitro model system to measure relative PpIX fluorescence [16].

Methods

Cell lines

U-87 MG cells (ATCC, Manassas, VA, USA) were previously stably transfected with the yellow fluorescent protein (YFP) expression vector pEYFP-C1 (YFP-U87) [16]. YFP-U87 Cells were cultured in standard conditions with Eagle's Minimum Essential Medium (EMEM) (Lonza, Portsmouth, NH, USA), 10 % fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 0.2 mg/mL G418 (Gold Biotechnology, St. Louis, MO, USA) and 1 % penicillin/streptomycin/amphotericin B (PSA) (Lonza, Portsmouth, NH, USA).

Cell number to YFP fluorescence correlation

YFP-U87s were re-suspended in phenol red-free Dulbecco's Modified Eagle Medium supplemented with L-glutamine (Lonza, Portsmouth, NH, USA) and 10 % FBS. Cells were serially diluted and plated in black-bottom microtiter plates (Greiner Bio-One, Monroe, NC, USA). Four replications per plate were used for each of four separate assays. The cells were allowed to adhere to the plates for 4 h. A Modulus Microplate Reader (Promega BioSystems, Sunnyvale, CA, USA) measured YFP fluorescence due to its constitutive production within the YFP-U87 cells, using a standard 525 nm excitation/580–640 nm emission filter. Relative light units (RLU) from these YFP fluorescence recordings were plotted against cell number to determine the correlation between YFP production and cell number.

Treatments

Dexamethasone, desipramine hydrochloride, 5,5-diphenylhydantoin (phenytoin), valproic acid sodium salt, and levetiracetam (Sigma, St Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) (Amresco, Solon, OH, USA) before diluting in phenol red-containing EMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin/amphotericin B. Two sets of treatment media were created: one with a single treatment, and another with the single treatment plus the addition of a therapeutic concentration of dexamethasone. All drugs for administration in cell culture were within therapeutic plasma concentrations as referenced on the brand drug product sheets or cited in the literature when not reported on the product sheet: ~25–160 ng/mL dexamethasone in first 6 h after dosing [17], 50–300 ng/mL desipramine (Norpramin product sheet), 10–20 μg/mL phenytoin (Dilatin product sheet), 50–125 μg/mL valproic acid (Stavzor product sheet), and the effective levetiracetam dose of 11 μg/mL [18]. The final concentrations used were 100 ng/mL dexamethasone, 300 ng/mL Desipramine, 20 μg/mL phenytoin, 50 μg/uL valproic acid, 11 μg/mL levetiracetam (Table 1). Control media was also prepared with either 0.1 % DMSO, for comparison to single treatment groups, or 0.2 % DMSO, for comparison with single treatment with the addition of a therapeutic concentration of dexamethasone in order to control for treatment vehicle effects.

Table 1 Medication treatment groups and difference in PpIX production/retention relative to appropriate DMSO positive control

Treatments	Drug class	Drug concentration	DMSO (%)	5-ALA	Production (%)	*p* value	Retention (%)	*p* value
Dexamethasone	Corticosteroid	100 ng/mL	0.1	Yes	−44 ± 4	<0.01	30 ± 12	<0.05
Desipramine	Antidepressant	300 ng/mL	0.1	Yes	−12 ± 6	<0.01	−11 ± 8	=0.055
Valproic Acid	Anticonvulsant	50 μg/mL	0.1	Yes	−25 ± 4	<0.01	−28 ± 5	<0.01
Phenytoin	Anticonvulsant	20 μg/mL	0.1	Yes	−31 ± 5	<0.01	−40 ± 10	<0.01
Levetiracetam	Anticonvulsant	11 μg/mL	0.1	Yes	7 ± 7	>0.05	6 ± 10	>0.05
0.1 % (−) Control	–	–	0.1	No	−86 ± 6	<0.01	−120 ± 8	<0.01
0.0 % (+) Control	–	–	–	Yes	23 ± 8	<0.01	4 ± 8	>0.05
0.1 % (+) Control	–	–	0.1	Yes	–	–	–	–
2X Dex	Corticosteroid	200 ng/mL	0.2	Yes	−46 ± 4	<0.01	26 ± 9	<0.05
Des + Dex	AD + C	300 + 100 ng/mL	0.2	Yes	−36 ± 7	<0.01	14 ± 9	>0.05
Val + Dex	AC + C	50 μg/mL + 100 ng/mL	0.2	Yes	−33 ± 3	<0.01	−9 ± 7	>0.05
Phe + Dex	AC + C	20 μg/mL + 100 ng/mL	0.2	Yes	−55 ± 3	<0.01	−43 ± 8	<0.01
Lev + Dex	AC + C	11 μg/mL + 100 ng/mL	0.2	Yes	−35 ± 4	<0.01	−3 ± 11	>0.05
0.2 % (−) Control	–	–	0.2	No	−87 ± 6	<0.01	−124 ± 10	<0.01
0.0 % (+) Control	–	–	–	Yes	9 ± 7	>0.05	23 ± 10	<0.01
0.2 % (+) Control	–	–	0.2	Yes	–	–	–	–

Dex dexamethasone, *Des* desipramine, *Val* valproic acid, *Lev* levetiracetam, *Phe* phenytoin, *AD* antidepressant, *AC* anticonvulsant, *C* corticosteroid

Production = % Increase (+ Value) or Decrease (− Value) in Total PpIX in Cells + Media Compared to Appropriate DMSO Control

Retention = % Increase (+ Value) or Decrease (− Value) in PpIX Found Only Within Cells (Media Removed)

The negative controls include either 0.1 or 0.2 % DMSO without 5-ALA

The positive controls include either 0.1 or 0.2 % DMSO with 2 mM 5-ALA

All % changes are normalized to either 0.1 % DMSO controls for single drug treatments or 0.2 % DMSO controls for dual drug treatments

Values are mean ± standard error

Treatment of YFP-U87s

Each treatment group was represented with at least six replicates per assay and completed in triplicate. YFP-U87s were plated at a density of 15,000 cells per well in black-bottom, 96-well microplates (Greiner Bio-One, Monroe, NC, USA). Cells were allowed to adhere to the plate overnight, then fresh media containing the treatments or DMSO vehicle was added to the respective treatment groups to create therapeutic concentrations of each medication within the wells. Treatment groups (Table 1) were dexamethasone, desipramine, phenytoin, valproic acid, levetiracetam, 0.1 % DMSO (positive control), 2X dexamethasone, desipramine + dexamethasone, phenytoin + dexamethasone, valproic acid + dexamethasone, levetiracetam + dexamethasone, 0.2 % DMSO (positive control) and negative control (no treatment, no 5-ALA). Positive controls without DMSO (5-ALA only) were also tested. Treatment containing media was replaced with fresh treatment media at 24 and 48 h consistent with previous studies in the literature [15].

5-Aminolevulinic acid treatment

After 72 h of drug treatment, all treatment groups were washed with EMEM without phenol red supplemented with 4 mM L-glutamine (Gibco, LifeTechnologies, Grand Island, NY, USA) and 10 % FBS. After washing, 100 μL of EMEM without phenol red containing 2 mM 5-ALA (Acros Organics, Thermo Fisher Scientific, Pittsburg, PA, USA), 4 mM L-glutamine, 10 % FBS, 30 μg/mL insulin (Sigma-Aldrich, St. Louis, MO, USA), 0.1 % DMSO, and 2 % v/v of 0.1 mM EDTA (Thermo Fisher Scientific, Pittsburg, PA, USA) was added to each well. All groups, except the negative controls, then received 5-ALA treatment media and incubated in standard cell culture conditions for 4 h protected from light.

PpIX production and accumulation

PpIX and YFP fluorescence was measured using a Modulus Microplate Reader with a 525 nm excitation/580-640 nm emission filter to detect YFP fluorescence and a 405 nm

excitation/580–640 nm emission filter (Promega Corporation, Madison, WI, USA) to detect PpIX fluorescence. PpIX and YFP fluorescence was measured in the same plate in sequential fashion in order to analyze the total production of PpIX after 4 h. Briefly, after measuring total fluorescence in the well, the media was aspirated from all wells and fluorescence was again measured to determine the amount of PpIX retained within the cells. Treatment group data were normalized by subtracting the average relative light unit (RLU) value of the non-5-ALA treated wells from the 5-ALA treated wells. PpIX total production per well (readings with media) and retention by cells (readings after aspiration) were then calculated by determining a PpIX/YFP quotient (dividing PpIX RLU by YFP RLU). The PpIX/YFP quotient provides a relative value for the PpIX production per cell mass in the well. The treatment PpIX/YFP quotients were compared to the 5-ALA plus DMSO treated groups to detect statistical differences. A Paired-Samples *T* test was performed to determine significant differences between respective treatment groups and the appropriate DMSO containing vehicle control.

Results

YFP-U87 standard curve

The YFP-U87 standard curve had a strong positive Pearson correlation coefficient (r = 0.987) indicating a significant linear relationship between YFP RLU and cell number (Fig. 1).

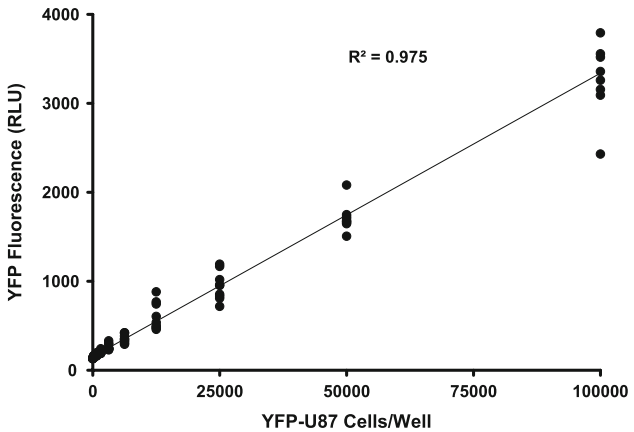

Fig. 1 Yellow fluorescent protein (YFP) light emission correlates with cell number. Relative light units (RLU) were plotted against the number of YFP-U87 cells 4 h following plating in a 96-well microplate. Analysis was completed using raw data from nine experiments. The Pearson correlation coefficient was 0.987 ($r^2 = 0.975$) and confirms a strong, direct relationship between cells plated and YFP fluorescence as shown previously [16]

Effects of medication treatment on the total production of PpIX

PpIX production quotients for total fluorescence were compared for each treatment combination. All treatments, except levetiracetam, experienced a significant decrease ($p < 0.05$) in the total PpIX production quotient compared to the 0.1 % DMSO control group, as shown in Fig. 2a. The greatest reduction of PpIX produced was found in the dexamethasone and phenytoin groups, with approximately

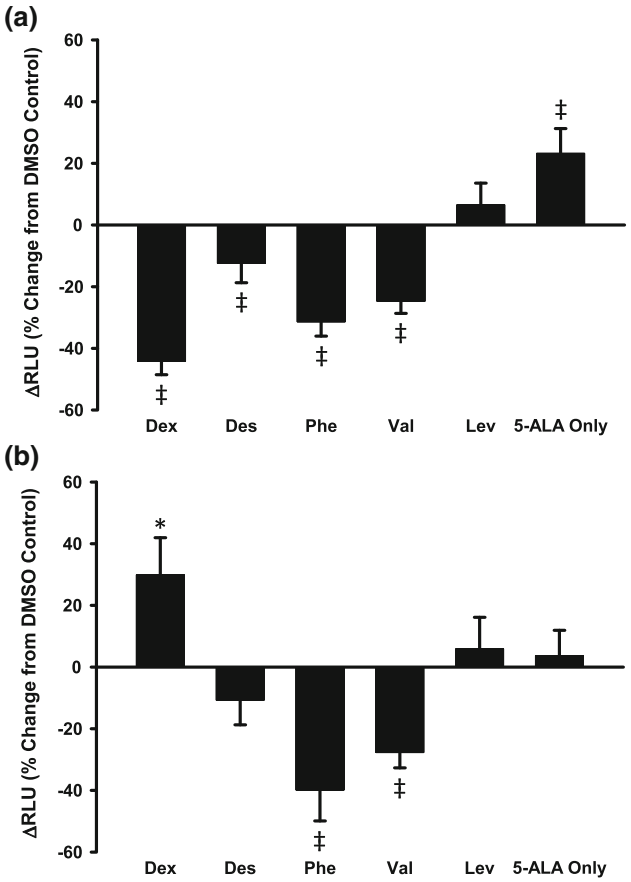

Fig. 2 Single medication treatment of cells reduces PpIX production or retention in cells. Relative PpIX fluorescence (PpIX fluorescence per cell) is shown as a percent difference of relative light units (ΔRLU) between treatment groups and the 0.1 % DMSO positive control. Relative PpIX fluorescence was determine by calculating a PpIX RLU/YFP RLU quotient. YFP-U87 cells were treated with a single medication for 72 h before application of 2 mM 5-ALA and plates were read 4 h later. **a** The relative total production of PpIX (readings of cells + media in wells) was significantly less in all groups compared to the 0.1 % DMSO control, except levetiracetam. **b** The relative retention of PpIX in cells was determined by removing the media prior to measuring fluorescence. Values are presented as the percent difference from the 0.1 % DMSO positive control. While dexamethasone treated cells produced significantly less PpIX compared to controls, the dexamethasone reduces the efflux of the PpIX from the cells. *Dex* dexamethasone, *Des* desipramine, *Phe* phenytoin, *Val* valproic acid, *Lev* levetiracetam, $^{*}p < 0.05$, $^{\ddagger}p < 0.01$

44 and 31 % reduction, respectively. Because brain tumor patients are often given these drugs in combination with dexamethasone, YFP-U87 cells were also treated with dexamethasone plus one of the other medications. The reduction of PpIX in response to these medication combinations was additive. Desipramine, which resulted in 12 % reduction of PpIX, further reduced PpIX production to 36 % in the presence of dexamethasone. Likewise, phenytoin, which resulted in 31 % reduction in PpIX, further reduced PpIX production to 55 % in the presence of dexamethasone. While levetiracetam alone did not reduce PpIX production, the addition of dexamethasone significantly reduced PpIX production (Fig. 3a).

Effects of treatments on cellular retention of PpIX

PpIX retention quotients for intraellular fluorescence were compared for each treatment combination. Dexamethasone treated cells, which produced lower total levels of PpIX, was the only treatment group that retained more PpIX within the cells ($p < 0.05$; Fig. 2b). Compared to controls, the amount of intracellular PpIX after phenytoin and valproic acid treatment was significantly less ($p < 0.05$) while unchanged following desipramine and levetiracetam treatments. In the presence of dexamethasone, PpIX retention varied depending on the treatment combination (Fig. 3b). The addition of dexamethosone to desipramine, valproic acid and levetiracetam treatments had no effect on intracellular PpIX compared to controls. However, when added to phenytoin, dexamethasone intracellular PpIX remained significantly lower compared to controls ($p < 0.05$).

Discussion

Fluorescence guided resection of brain tumors is an important addition to the surgical management of brain tumors. Yet, not all tumors display adequate PpIX fluorescence after 5-ALA administration. We tested several medications commonly prescribed to brain tumor patients, alone or in combination, to determine if they might alter metabolism of 5-ALA and/or retention of PpIX in GBM cells. The novel findings of our study include: (1) dexamethasone, desipramine, and valproic acid appear to inhibit production of PpIX, (2) the addition of dexamethasone to the other drugs tested further reduced PpIX production, and (3) although dexamethasone reduced the amount of PpIX produced more than any other drug tested, it promotes the retention of PpIX within cells.

These findings were derived using the YFP/PpIX fluorescence quotient model [16]. It is important to note that these data were collected using the cell line, YFP-U87 cells. As demonstrated previously by Hefti et al. [15], the

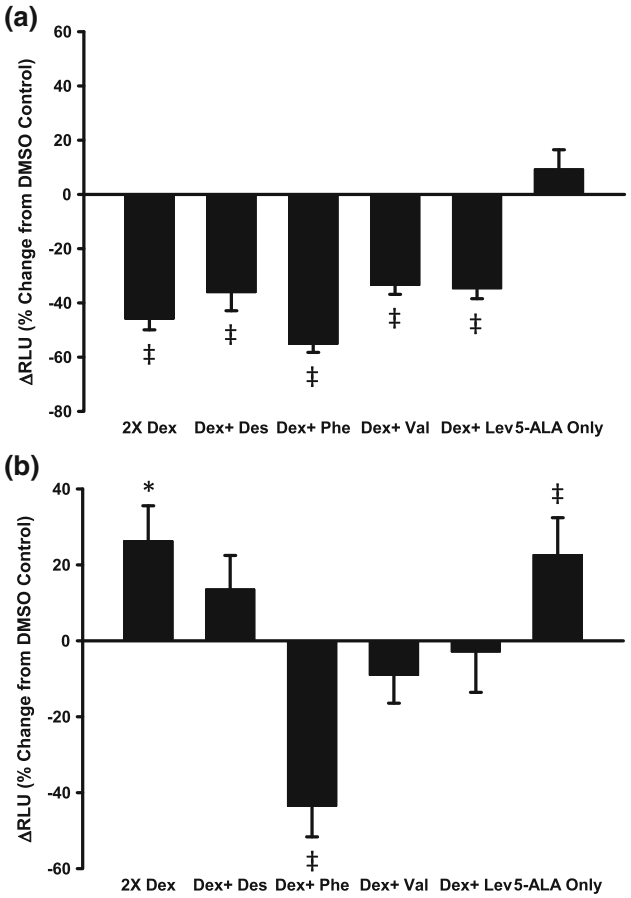

Fig. 3 Dual medication treatment of cells further reduces PpIX production or retention in cells. Relative PpIX fluorescence (PpIX fluorescence per cell) is plotted as the percent difference of relative light units (ΔRLU) between treatment groups and the 0.2 % DMSO positive control. YFP-U87 cells were treated with both medications for 72 h before application of 2 mM 5-ALA and plates were read 4 h later. **a** After 4 h, the total relative production of PpIX was determined by taking readings with cells and media in the wells. There was a reduction in the production quotients (PpIX RLU/YFP RLU) for all dual drug treatments, with several combinations (Des + Dex; Phe + Dex; Lev + Dex) appearing to have a strong synergistic PpIX reduction. **b** The relative retention of PpIX was unchanged in cells treated with two drugs (drug + Dex) versus single drug treatments. Dex = Dexamethasone, Des = Desipramine, Phe = Phenytoin, Val = Valproic Acid, Lev = Levetiracetam, $^{*}p < 0.05$, $^{\ddagger}p < 0.01$

responses of U-87MG cell line to levetiracetam or phenytoin were indistinguishable from the responses seen in another GBM cell line (U373 MG) or primary GBM cultures in both cell growth and PpIX production [15]. While the conclusions obtained in the present study are consistent with the Hefti et al. study, it will be important to follow up this study with additional cell lines and primary cultures, as the cellular heterogeneity within glioma tumors may result in different responses to drug treatment.

Because of glioma's infiltrative properties, tumor margins are not clearly defined and make gross total resection

difficult. The advantage of good intraoperative imaging and/or visualization of the tumor is evident. Research and case studies continue to report that 5-ALA improves total resection rates and consequently progression free survival [19, 20]. However, the difficulty of getting FDA approval in the United States has generated interest in alternatives to 5-ALA FGR like intraoperative MRI [21], conjugated chlorotoxin FGR [22] and fluorescein sodium FGR [23]. While these other resection modalities may improve total resection over white light, it is yet to be determined if these modalities are better at achieving gross total resection than 5-ALA [24, 25]. Interestingly, these alternative resection aids may improve resection in an additive manner when used in combination with 5-ALA as seen with intraoperative MRI + 5-ALA [19, 26].

The use of 5-ALA FGR can improve resection of glioma and ultimately progression-free survival, but its use may also be expanded to destroy residual tumor cells after resection or to treat inoperable lesions using intraoperative or stereotactic photodynamic therapy [27]. Photodynamic therapy with 5-ALA is based upon the toxicity of PpIX when excited by red light (630–635 nm) in combination with oxygen [28]. This 5-ALA photodynamic therapy releases singlet oxygen and causes apoptosis and necrosis [28]. Although photodynamic therapy is not well established for treatment of brain tumors, 5-ALA's potential use in this manner is intriguing. Stummer et al. (2008) reported a long-term response in a "non-resectable, distant recurrence of glioblastoma multiforme" using 5-ALA photodynamic therapy [27].

Unfortunately, not all tumors treated with 5-ALA generate adequate PpIX needed for FGR and photodynamic therapy. Here we show that the medications used to manage brain tumor patients may interfere with the cell's ability to metabolize 5-ALA and accumulate intracellular PpIX. Malignant glioblastoma cells (YFP-U87s) readily convert 5-ALA to PpIX when they are not treated with medication. All medications except levetiracetam lowered total PpIX production. A noteworthy observation with the dexamethasone treatment was that it acted as a strong inhibitor of PpIX production, while promoting retention of the PpIX within the cells. We also confirmed the previous report that phenytoin, but not levetiracetam, interferes with metabolism of 5-ALA and the production of PpIX in glioma cells [15].

These data suggest that levetiracetam may be a better choice for 5-ALA FGR when a preoperative anticonvulsant is needed as it does not appear to decrease the production and intracellular retention of PpIX as do phenytoin and valproic acid. The antidepressant desipramine does not have a significant affect upon PpIX accumulation in cells even though it does reduce total PpIX production similarly to the drugs dexamethasone, phenytoin and valproic acid. Further studies on additional antidepressants are warranted to determine what drugs may offer the best outcome for 5-ALA FGR. Lastly, dexamethasone, a common corticosteroid given to brain tumor patients to manage intracranial pressure and vasogenic edema, may significantly reduce the total amount of 5-ALA that is converted to PpIX. Dexamethasone's use in patients undergoing 5-ALA FGR should be studied clinically to determine if it is truly disrupting the tumor from fluorescing adequately for FGR. The observation that cells treated with dexamethasone were able to retain PpIX better than others will be followed up in future work to determine the mechanism behind the increase in retention. The authors acknowledge that drug interactions alone may not be the primary reason behind a lack of fluorescence in patients undergoing 5-ALA FGR. Intrinsic cell pathways and the molecular events within different cells may also alter PpIX production and retention as shown in a recent study indicated where the mutation status of IDH1 alone could significantly alter 5-ALA mediated fluorescence [29].

In summary, we report that dexamethasone, desipramine, phenytoin and valproic acid, but not levetiracetam reduce PpIX production in GBM cells. We confirmed that levetiracetam may possibly be a better choice for seizure management in patients undergoing 5-ALA FGR. However, if the patient is also on dexamethasone, the benefits of using levetiracetam may be offset. It seems evident that medications are having an effect on PpIX production and retention. Future work is warranted to determine the best medications and combination of these drugs to achieve the desired management of the patient's symptoms, but also to achieve the highest amount of tumor fluorescence. Optimizing 5-ALA FGR may translate into 5-ALA photodynamic therapy that effectively kills residual tumor cells after surgery or to photokill inoperable tumors via stereotactic photodynamic therapy.

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