Research Paper

Valproic Acid Reduces Tumor Cell Survival and Proliferation with Inhibitors of Downstream Molecules of Epidermal Growth Factor Receptor Pathway

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Abstract

Objectives: To evaluate the beneficial effect of treating tumor cells with valproic acid (VPA) in combination with inhibitors of various downstream molecules of epidermal growth factor receptor pathway for limiting tumor cell growth. **Materials and Methods:** Cytotoxic effect of VPA was tested with various combinations of inhibitors of PI3K-AKT, ERK1/2, Jun N-terminal kinases (JNK) as well as p38 kinases in A375 cells using methyl-thiazol-tetrazolium assay, clonogenic assay, and caspase assay. Antiproliferative effect of the combination was tested with ethynyl-2′-deoxyuridine incorporation assay. **Results:** Among the combinations tested, a combination of VPA with PI3K-AKT inhibitor showed enhanced tumor cell death and reduced tumor cell proliferation compared to the combination of VPA with ERK1/2, JNK, and p38 inhibitors at lower doses. **Conclusions:** Combination of VPA with PI3K-AKT inhibitor at lower concentration reduced tumor cell growth.

Keywords: Cancer, histone deacetylase inhibitors, Ly294002, valproic acid

Introduction

Valproic acid (VPA) is a histone deacetylase inhibitor (HDACi) with a branched short chain fatty acid, widely used as a generic low-cost antiepileptic and mood stabilizer. It has been in medical use since 1962 and has a good long-term safety profile in the therapeutic doses comparable to other HDACi, with limited toxicities.[1-3] Because of this, it has been considered as a good candidate for anticancer therapy. Recently, its anticancer activity is being evaluated in different cancer models by several groups either as a monotherapy or in combination with chemotherapeutic agents both *in vitro* and *in vivo*. Although as a monotherapy agent VPA failed to show good response, in combination with other chemotherapeutic agents, it demonstrated good therapeutic efficacy both in cell culture and animal studies. Because of this, multiple translational clinical trials were undertaken to find the effect of adding VPA in treating multiple types of solid tumors in combination with other conventional chemotherapeutic agents.[4-14] Most of the studies showed moderate therapeutic responses while none of the studies showed exciting outcomes.

Targeted therapeutics have gained much importance for treating multiple cancer types in recent years. Among the targetable molecules, epidermal growth factor receptor (EGFR) and its downstream molecules occupies a central role since this pathway plays a critical role in driving tumor cell proliferation, migration, and their survival.[15] ERK1/2, PI3K-AKT, Jun N-terminal kinases (JNK), and p38 are well-known downstream molecules of EGFR pathway. In the present study, we analyzed the antiproliferative and cell killing effect of VPA in combination with inhibitors of ERK1/2, PI3K-AKT, JNK, and p38 kinase using A375 cells.

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Materials and Methods

Cell culture and inhibitors

Human melanoma cell line A375 was purchased from the National Centre for Cell Science (Pune, Maharashtra, India), cultured in Dulbecco's modified Eagle's medium (DMEM) media (Himedia, India) supplemented with 2 mM L-glutamine 10% fetal bovine serum (Himedia, India) and 1% antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin), (Himedia, India), and maintained at 37°C in a humidified incubator of 5% CO_2. U0126 (ERK1/2 inhibitor), LY294002 (PI3K-AKT kinase inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 mitogen-activated protein kinase inhibitor) were procured from Calbiochem, USA. All these inhibitors were dissolved in dimethyl sulfoxide (DMSO) (Himedia, India), aliquoted for single time use and stored at − 20°C. VPA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMEM medium immediately before use.

Measurement of cell viability

Cell viability was measured by methyl-thiazol-tetrazolium (MTT) assay. In triplicates, cells were seeded into 96-well plates (Nunclon Cat no: 167008, Thermo Scientific); at 3×10^3 cells per well (100 µl) for 24 h. The next day, 100 µl of fresh medium was added containing different concentrations of drugs or vehicle (DMSO). VPA was used at a concentration of 0.5–6 mM; U0126 and LY294002 at 0.5–5 µM; SP600125 and SB203580 at 2.5–30 µM. For combination therapy, VPA was used with 0.5–2 mM with different concentrations of U0126, LY294002, SP600125, and SB203580 with multiple combinations. After incubation for 72 h, 100 µl MTT (3-[4, 5-dimethylthiazol-2-yl]-3, 5-diphenyltetrazolium bromide) solution (Himedia, India) was added to each well, at a final concentration of 1 mg/ml, and incubated at 37°C for 4 h. After incubation, the media/MTT solution was aspirated and Formazan crystals were dissolved in 100 µl DMSO (Himedia, India). The absorbance at 570nm was measured using a multi-mode microplate reader (FLUOstar Omega; BMG Labtech).

Clonogenic assay

A 375 cells were seeded at a density of 10^5 cells in 30 mm tissue culture dish and allowed to adhere overnight. Cells were re-fed with fresh media containing either 2 mM of VPA or 2 µM of LY294002 or combination of 2 mM VPA and 2 µM LY294002. After 24 h of incubation, cells were trypsinized and plated onto new culture dishes at the density of 500 cells/dish and kept without the drug for additional 10 days. At completion of 10 days, culture dishes were washed with 1X phosphate buffered saline (PBS) (Himedia, India), fixed with methanol: Acetone (1:1 v/v) and stained with Giemsa (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. The remaining staining solution was removed, and culture dishes were washed with distilled water and dried at room temperature. Colonies (>50 cells) were counted under stereomicroscope (Stemi DV4, Carl Zeiss, Germany).

Caspase assay

To detect the incidence of cell death, caspase assay was performed using the caspase assay kit (EMD Millipore, Cat. No 4500–0540) and quantified using the flow cytometry system (Guava EasyCyte**,** EMD Millipore, Hayward, CA) according to the manufacturers' instructions. Cells were seeded in 6-well plates (2×10^4 cells per well). Following 24 h, cells were treated with either 2 mM VPA or 2 µM LY294002 or a combination of both. Twenty-four hours after treatment cells were detached and harvested, washed in 1X PBS and suspended in 50 µL media. 10 µl of 10X FLICA reagent was added, mixed, well and incubated in dark for 1 h at 37°C and 5% CO_2. Cells were washed twice and 150 µl master mix propidium iodide was added and incubated at room temp for 5 min. Cell samples were analyzed in the Guava EasyCyte (EMD Millipore).

Ethynyl-2′-deoxyuridine incorporation assay for cell proliferation

For cell proliferation, Click-iT® ethynyl-2′-deoxyuridine (EdU) Microplate Assay (Invitrogen kit; Catalog no. C10214) was used. This assay utilizes the nucleoside analog EdU (5-ethynyl-2′-deoxyuridine) for DNA incorporation during active DNA synthesis. Cells were seeded into 96-well plates in triplicates at 3×10^3 cells per well (100 µl) for 24 h in black 96-well plates (Nunclon Delta Black Microwell; Cat no: 137,101, Thermo Scientific) and after overnight attachment, treated with inhibitor/vehicle for 24 h. Next day, 10 µM EdU was added in each well containing inhibitor/vehicle-medium and incubated for another 24 h. Following incubation the assay performed according to manufacturer's instruction. The Fluorescence (520 ex/590 em) was measured using multi-mode microplate reader (FLUOstar Omega; BMG Labtech).

Statistical analysis

Data were presented as the mean ± standard error from three independent experiments. Statistical analyses were performed by Student's *t*-test as well as one way ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Reduction in cell viability with valproic acid, U0126, LY294002, SP600125, and SB203580

In order to determine the influence of the VPA, U0126, LY294002, SP600125, and SB203580 on cell viability, A375 cells were incubated in the presence of different concentrations (0.5–6 mM) of VPA. The effect of VPA on 72 h of incubation is shown in Figure 1. VPA showed the concentration-dependent reduction in cell viability. However, more than 50% reduction in cell viability was observed only at 2 mM or higher doses. U0126 was effective even at lower doses while LY294002, SP600125, and SB203580 are effective only at higher doses [Figure 1].

Combination of valproic acid with LY294002 significantly reduces cell viability

To find a combination that could reduce tumor cell viability, 1 and 2 mM concentrations of VPA was treated with 0.5, 1 and 2 µM concentrations of U0126 and LY294002. Further, 0.5, 1,

and 2 mM concentrations of VPA were treated with 5 and 10 μM concentrations of SP600125 and SB203580. Although VPA with U0126 showed a significant reduction in cell viability, it is not different from cell viability observed with U0126 alone [Figures 1 and 2]. However, we observed a significant reduction with cell viability in VPA with LY294002 compared to the effect of these two individual drugs. Even the combination of VPA with SP600125 produced a significant reduction in cell viability although at higher concentrations [Figure 2]. No significant reduction was observed with the combination of VPA with SB203580 [Figure 2].

Reduced cell survival was evident from clonogenic assay

Clonogenic assay was performed to study the effect of VPA and LY294002 combination on survival of A375 cells. Scoring of colonies containing more than 50 cells under a stereo microscope revealed that VPA (2 mM) and LY 294002 (2 μM) individual drugs showed significant reduction ($P < 0.01$ and $P < 0.05$, respectively) in survival of A375 cells compared to untreated A375 cells. Further, the combination of these at this specified concentration further reduced ($P < 0.0001$) the survival of the A375 cells [Figure 3].

Enhanced cytotoxic effect of valproic acid with LY294002 was confirmed by caspase assay

To validate the cytotoxic effect of the combination of VPA and LY294002, we have performed caspase assay for the evaluation of caspase activation in dying A375 cells. For this, control A375 cells, cells treated with 2 mM VPA, 2 μM LY294002 and their combination was tested [Figure 3]. Lower levels of caspase activity were observed in control A375 cells. Treating

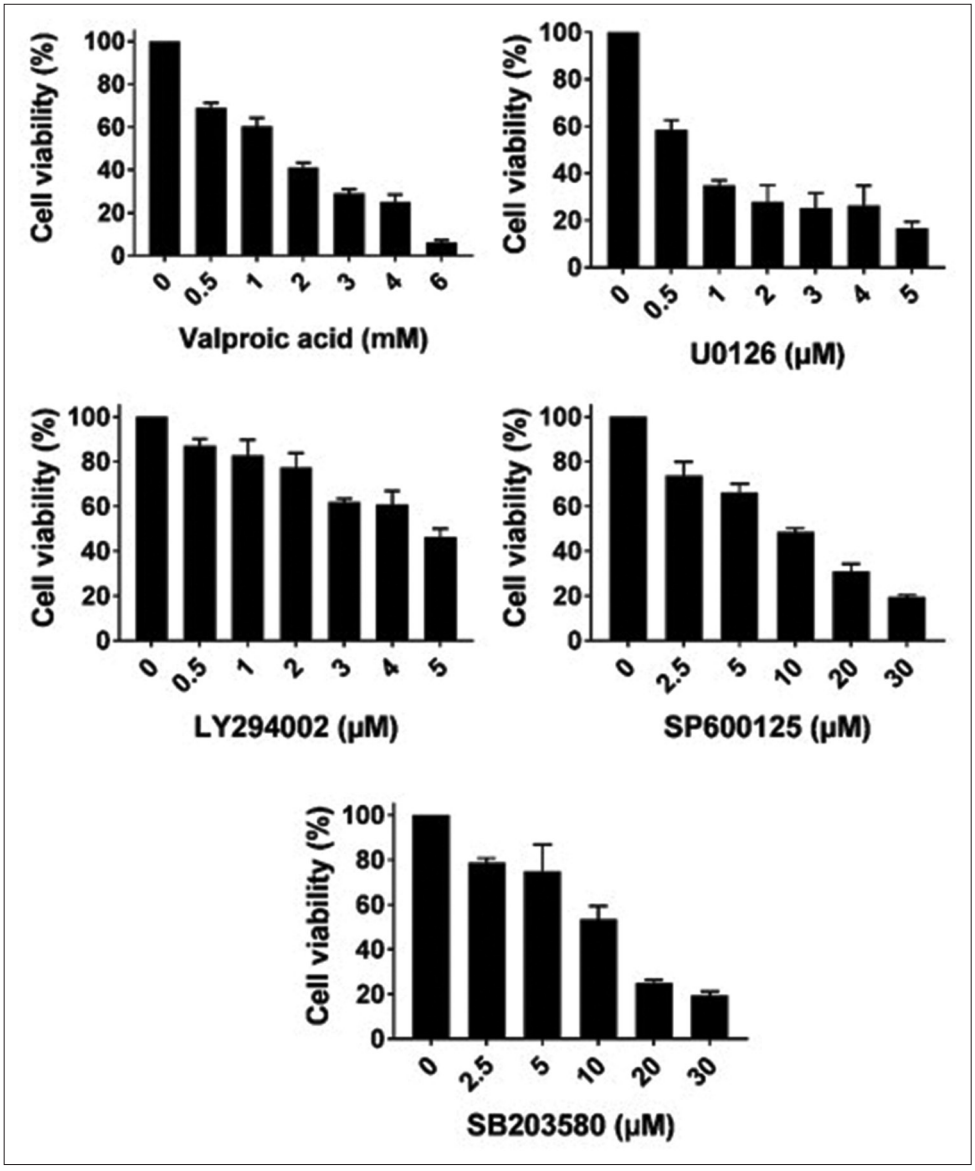

Figure 1: Cytotoxic effect of valproic acid and different inhibitors of downstream molecules of epidermal growth factor receptor pathway. Valproic acid as well as inhibitors of ERK1/2(U0126), PI3K-AKT (LY294002), Jun N-terminal kinases (SP600125) as well as p38 kinase (SB203580) were tested for their cytotoxicity on A375 cells using methyl-thiazol-tetrazolium assay

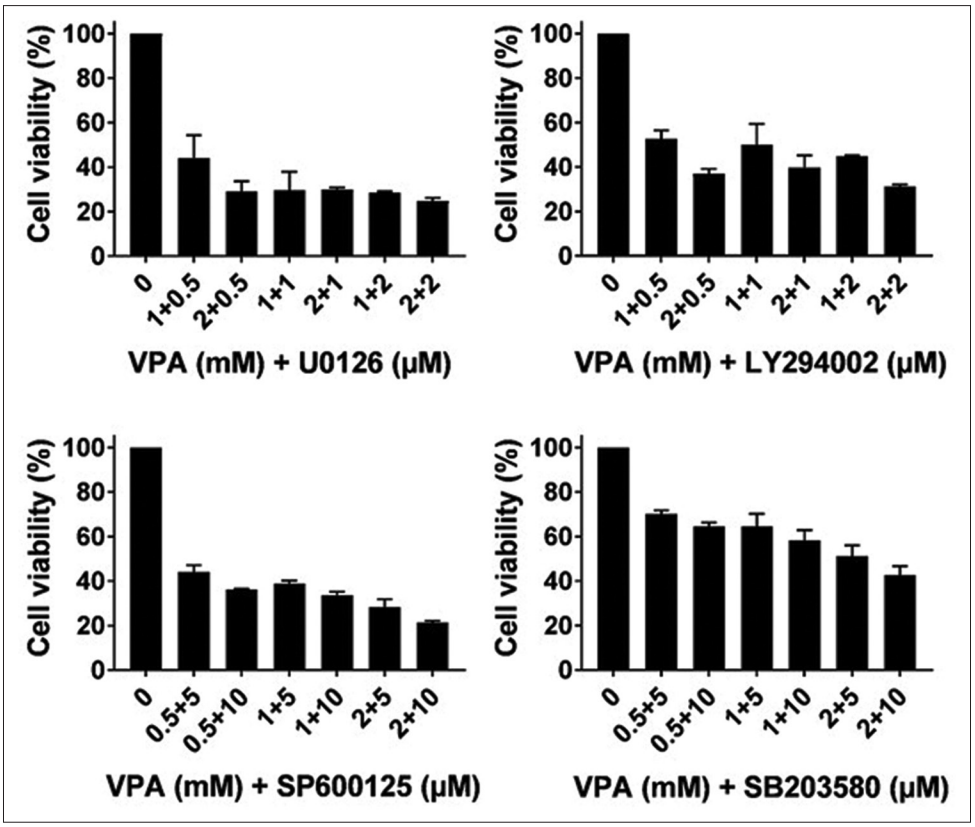

Figure 2: Cytotoxic effect of valproic acid in combination with different inhibitors of downstream molecules of epidermal growth factor receptor pathway. Cytotoxic effect of valproic acid was tested in combination with inhibitors of ERK1/2, PI3K-AKT, Jun N-terminal kinases as well as p-38 kinase at multiple concentrations on A375 cells using methyl-thiazol-tetrazolium assay

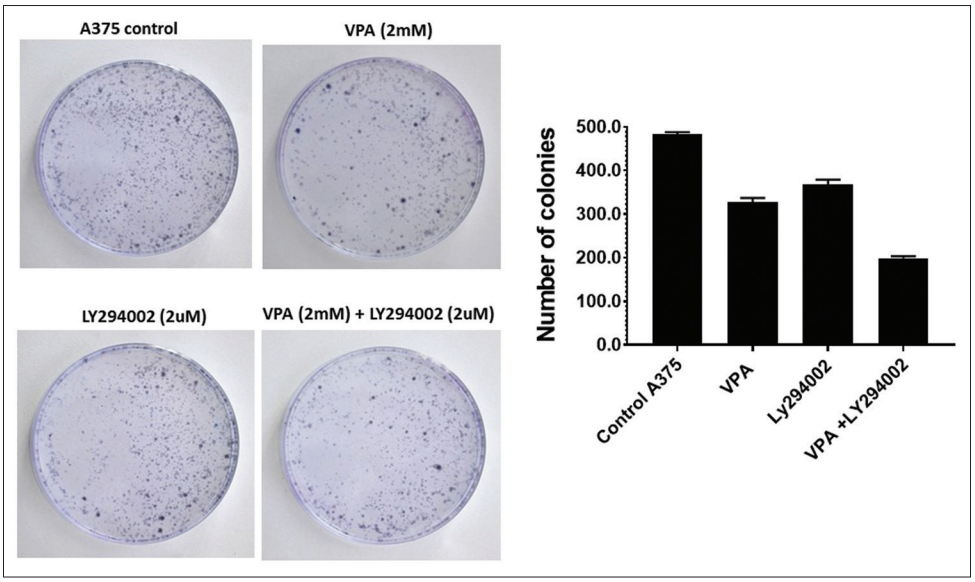

Figure 3: Clonogenic cell survival assay. A representative image of the clonogenic assay (left panels). Valproic acid (2mM) as well as LY294002 (2μM) individual drugs showed a significant reduction ($P < 0.01$ and $P < 0.05$, respectively) in survival of A375 cells compared to untreated A375 cells. Combination of valproic acid and LY294002 at the same concentrations further reduced ($P < 0.0001$) the survival of the A375 cells

cells with either VPA or LY294002 resulted in increased cell death while combined treatment further enhanced the caspase activity [Figure 4] suggesting the beneficial effects of this combined treatment.

Antiproliferative effect of combination of valproic acid with LY294002

While results from MTT assay is a measure of cell proliferation as well as cell death, to check whether the combination

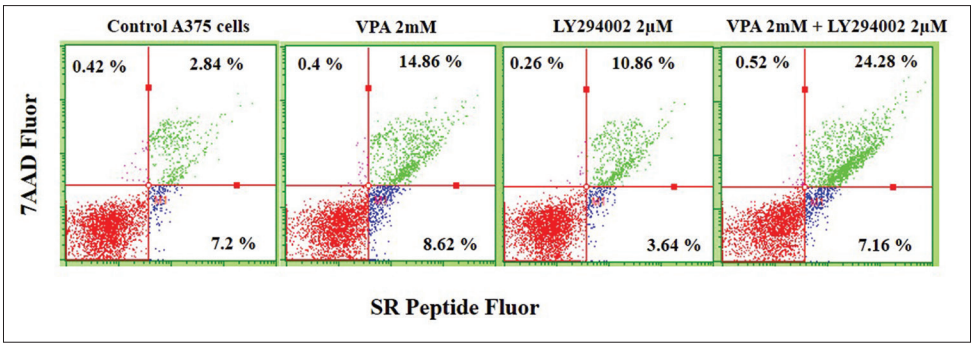

Figure 4: Cytotoxic effect of valproic acid in combination with PI3K-AKT inhibitor LY294002. Cytotoxic effect of valproic acid was tested in combination with PI3K-AKT inhibitor LY294002 on A375 cells using caspase assay. Box label: Upper left: Necrotic cells; Lower left: viable cells; Lower right: Early/mid apoptotic cells; Upper right: Late apoptotic cells

of VPA (2 mM) with LY294002 (2 μM) possess any antiproliferative effect, A375 cell proliferation was assessed by EdU incorporation assay. EdU detection is based on a click reaction-a copper-catalyzed covalent reaction between an azide and an alkyne. In this assay, the EdU contains the alkyne and Oregon Green® 488 dye contains the azide. VPA (2 mM) significantly reduced A375 cell proliferation compared to control A375 cells ($P < 0.05$) whereas LY294002 (2 μM) failed to show any antiproliferative effect at the tested concentration. However, when a combination of VPA (2 mM) was used with LY294002 (2 μM), cell proliferation was significantly reduced ($P < 0.01$) [Figure 5].

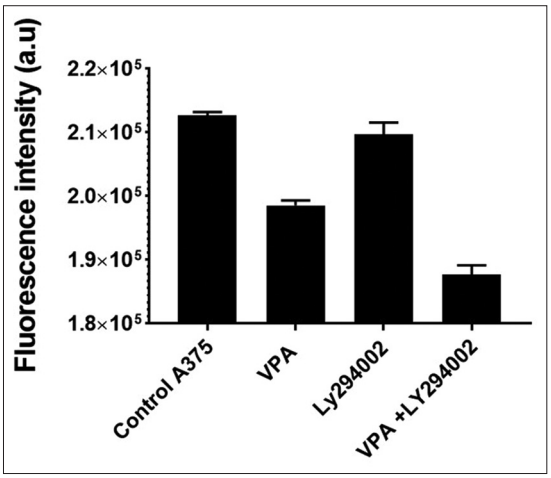

Figure 5: Evaluation of cell proliferation using ethynyl-2′-deoxyuridine incorporation assay. Anti-proliferative effect of valproic acid was tested in combination with PI3K-AKT inhibitor LY294002 on A375 cells using ethynyl-2′-deoxyuridine incorporation assay. Valproic acid (2 mM) significantly ($P < 0.05$) reduced proliferation of A375 cells while cell proliferation is unaffected with LY294002 (2μM). However, a combination of valproic acid (2 mM) and LY294002 (2μM) showed enhanced ($P < 0.01$) antiproliferative effects on A375 cells

Discussion

The strong rationale for using epigenetic agents such as VPA for treating cancer is based on the fact that epigenetic changes such as aberrant DNA methylation and histone acetylation are common in cancer.[16] Since EGFR and its downstream molecules play central roles in tumor cell survival, proliferation as well as migration, targeting them with VPA is expected to provide a clear advantage for controlling the robust growth of tumor cells. Among these downstream molecules, overexpression of ERK1/2 and PI3K-AKT were observed in multiple type of tumors whereas JNK and p-38 kinases are upregulated on induction of stress. In the present study, as monotherapy agent, VPA provided better cytotoxicity only at higher doses, similar to previous observation.[17] However, combined treatment of VPA with multiple inhibitors of EGFR downstream molecules provided varying amounts of cytotoxicity. Among the combinations, VPA with PI3K-AKT inhibitor LY294002 offered enhanced cytotoxicity compared to other combinations.

We have three observations from the present study. First of all, cell death induced by combination of VPA with ERK1/2 inhibitor is not significantly higher compared to ERK1/2 inhibition alone. Second, cell death induced by combination of VPA with PI3K-AKT inhibitor is significantly higher compared to inhibition of PI3K-AKT alone. Third, combination of VPA with JNK or p38 kinase inhibitor showed enhanced cell death only at higher concentrations. Taken together, these results indicate that combination of VPA with PI3K-AKT inhibitor is a better option if a combined treatment is required. These can be explained on the basis of differential potential of VPA in inhibiting ERK1/2 and AKT apart from its HDACi activity.[18] From our results, it appears that VPA failed to enhance ERK1/2 inhibitor-induced cell death while it enhanced PI3K-AKT inhibitor-induced cell death. This indicates that VPA mostly inhibits ERK1/2 activity apart from its HDACi activity. This is explained based on the well-known fact that tumor cells can grow when one of the PI3K-AKT or ERK1/2 pathway is active, and also ERK1/2 contribution for cell proliferation is higher while PI3K–AKT contribution for cell survival is high. When both pathways are inhibited more cytotoxicity is expected, which is seen in the combination of VPA with LY294002, confirmed through MTT assay, clonogenic assay as well as caspase assay. ERK1/2 inhibition by VPA is further confirmed from our antiproliferative assay with EdU, where

significant reduction in cell proliferation was observed with VPA and LY294002 combination whereas LY294002 at the tested concentration failed to show significant antiproliferative activity. In support of this hypothesis, an earlier study showed the up-regulation of AKT on inhibition of tumor cell growth with VPA[19] while another study[20] showed the beneficial effect of treating target of rapamycin inhibitor RAD001with VPA.

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Conflicts of interest

There are no conflicts of interest.

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