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# Enhanced effects by 4-phenylbutyrate in combination with RTK inhibitors on proliferation in brain tumor cell models

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ABSTRACT

We have investigated *in vitro* effects of anticancer therapy with the histone deacetylase inhibitor (HDACi) 4-phenylbutyrate (4-PB) combined with receptor tyrosine kinase inhibitors (RTKi) gefitinib or vandetanib on the survival of glioblastoma (U343MGa) and medulloblastoma (D324Med) cells. In comparison with individual effects of these drugs, combined treatment with gefitinib/4-PB or vandetanib/4-PB resulted in enhanced cell killing and reduced clonogenic survival in both cell lines. Our results suggest that combined treatment using HDACi and RTKi may beneficially affect the outcome of cancer therapy.



## 1. Introduction

Glioma is the most frequent primary brain tumor in adults. Patients with the most aggressive type of glioma, glioblastoma multiforme, have a poor prognosis, where only about 12% of patients survive 5 years after diagnosis [1]. In contrast, for medulloblastoma, the most common form of pediatric brain tumor, up to 40–70% of patients survive 5 years after diagnosis with present treatment strategies [2,3]. However, the treatment for medulloblastoma, which includes surgery, chemotherapy and radiation, has frequent, severe and long term side effects. For the reasons outlined above, both glioblastoma and medulloblastoma urgently prompt for new therapeutic strategies.

Growth factors bind to extracellular domains of transmembrane receptors, thereby triggering intracellular signals which eventually lead to alterations in the transcription machineries through epigenetic mechanisms. Members of the receptor tyrosine kinase (RTK) family, such as the epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR), have all been associated with the development and progression of glioblastoma and medulloblastoma [4–6], and the downstream members of the MAPK signaling pathway, ERK1/2 and Akt, have been reported to be aberrantly expressed and activated in many cancers [7–9].

Histone deacetylase inhibitors (HDACi) are potent inducers of cell differentiation and apoptosis. They act by shifting the acetylation/deacetylation equilibrium of core histones in favor of acetylation. In addition, HDACi treatment results in DNA demethylation [10,11]. HDACi can by themselves induce apoptosis, cell cycle arrest and differentiation. They can also potentiate the effects of other drugs. The HDACi 4-phenylbutyrate (4-PB) is an aromatic fatty acid with anti-neoplastic activity that has, e.g. been shown to reduce hepatoma xenograft tumor growth [12] and furthermore, has been tested in cancer therapy [13–15]. It was also evaluated in medulloblastoma and glioblastoma cell lines, where increased acetylation of histones was followed by differentiation effects [16]. HDACi have also been shown to inhibit medulloblastoma cell growth, induce cell death [17] and enhance the effects of ionizing radiation [18]. The advantage of HDACi partly lies in its preferential induction of cancer cell death, while normal cells are relatively insensitive to the drugs [14,19].

The potential of combination of small molecules that modulate RTKs and HDAC activity for the treatment of glioblastoma and medulloblastoma should be better investigated. The EGFR inhibitor

*Abbreviations:* 4-PB, 4-phenylbutyrate; HDAC, histone deacetylase; RTK, receptor tyrosine kinase; HDACi, histone deacetylase inhibitor; RTKi, receptor tyrosine kinase inhibitor.

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gefitinib (Iressa) and the EGFR/VEGFR-2 antagonist vandetanib (Zactima) have previously been tested in treatment of glioblastoma [20,21]. Gefitinib but not vandetanib has already shown successful results in medulloblastoma cells [22].

We have previously shown that 4-PB and another HDACi, Trichostatin A, induced differentiation and apoptosis in the U343MGa cell line [23]. Here, we are showing that 4-PB, when given in combination with the RTK activity modulators gefitinib and vandetanib, potentiates their effects in glioblastoma (U343MGa) and medulloblastoma (D324Med) cells by inhibiting cell proliferation and inducing loss of clonogenic survival.

## 2. Materials and methods

### 2.1. Cell lines and culture conditions

The human glioblastoma multiforme cell line U343MGa and the medulloblastoma cell line D324Med (DAOY) were obtained from Dr. Monica Nistér. Cells were cultured in DMEM (U343MGa) or MEM (D324Med) medium, supplemented with 10% fetal calf serum (Gibco), penicillin (100 units/ml) and streptomycin (100 μg/ml; Gibco). Cells were plated at a density of 1500 cells/cm$^2$, and cultured in flasks with regular medium replacement every fourth day, in 5% $CO_2$ at 37 °C.

### 2.2. Drugs and treatments

Stock solutions of 50 mM gefitinib (Iressa) and 40 mM vandetanib (Zactima) were prepared in dimethyl sulfoxide (DMSO) and diluted in the cell culture media to final concentrations. Final concentration of DMSO was 0.02%. Final drug concentrations in the cell culturing medium were kept in a range to avoid excessive cell loss [15,24–26]. Gefitinib was given at 5 and 10 μM (D324Med) or 10 and 20 μM (U343MGa) and vandetanib was given at 500 nM and 1 μM (D324Med) or 4 and 8 μM (U343MGa). Stock solution of 4-PB (2 M) was prepared in phosphate buffered saline (PBS). The final concentration of 4-PB was 2 mM. During prolonged treatment (2–5 days) the cell culturing medium with the treatment agent was replaced every day. Control cells received 0.02% DMSO final concentration.

### 2.3. Cell viability assessment

Cell viability was assessed using the cell proliferation reagent WST-1 (Roche). Cells were plated in a 96-well plate (density > $1 \times 10^5$ cells/plate) in culture medium. 10 μl of WST-1 was added after 72 h of treatment with gefitinib, vandetanib or 4-PB and incubated at 37 °C for 4 h. Absorbance at 450 nm, with reference at 650 nm, was measured with a Versa max microplate reader (Molecular Devices). The results were expressed as proportion of viable cells compared to controls (100%).

### 2.4. Clonogenic assay

To determine colony formation efficiency, the U343MGa and D324Med cells were seeded in 50 mm$^2$ Cell$^+$ Petri dishes (Sarstedt) at a concentration of 150 cells/dish, and allowed to attach to the surface for 5 h before a 72 h treatment with 2 mM 4-PB and gefitinib (10 and 20 μM for U343MGa; 5 and 10 μM for D324Med); and vandetanib (4 and 8 μM for U343MGa; 0.5 and 1 μM for D324Med). After 12 days of incubation in drug-free medium, the cell cultures were rinsed with phosphate-buffered saline, fixed in formaldehyde, and stained with Giemsa according to standard protocol (Gibco BRL). Colonies (>75 cells) with 50% plate efficiency (PE) were counted manually in a stereo microscope using a colony counter. For each treatment combination, the surviving fraction was calculated as the ratio of the mean PE of treated cells over the PE of untreated control cells.

### 2.5. Statistical analysis

Data are shown as mean values and +/− standard deviations. $p$ values were calculated using ANOVA (to compare three or more treated arms) and Student's $t$-test (to compare two treatment arms).

## 3. Results

### 3.1. Reduced cell viability after combined treatments

In order to investigate cell killing effects after combining 4-PB with either gefitinib or vandetanib, we evaluated the cell viability using the WST-1 assay on U343MGa glioblastoma and D324Med medulloblastoma cells. We observed that treatments with 4-PB, gefitinib or vandetanib alone resulted in no or moderate response (Fig. 1). In contrast, substantially enhanced cell killing was observed in both cell lines when gefitinib was combined with 4-PB. When compared to both controls and to 4-PB treated cells, the combination treatments with 4-PB together with gefitinib caused highly significant reduction in cell viability, ($p = 0.0001$) in both cell lines, although 4-PB alone had no significant effect. Similarly, combination treatments with 4-PB/vandetanib resulted in significantly enhanced cell killing of both cell lines, compared to both controls and to 4-PB treated cells ($p = 0.0001$). Vandetanib/4-PB compared with vandetanib alone did not show any difference in the U343MGa cells ($p = 0.0929$), while the D324Med cells were significantly affected ($p = 0.0002$).

### 3.2. 4-PB potentiates the effects of gefitinib and vandetanib on glioblastoma and medulloblastoma cell colony formation

Clonogenic assays were performed to investigate the effects of combined treatment on colony formation. Two concentrations of the RTKIs were used with a fixed 4-PB concentration of 2 mM. For U343MGa cells (Fig. 2), gefitinib was used at 10 and 20 μM, and vandetanib at 4 and 10 μM, respectively. In the U343MGa cells, 4-PB treatment caused minor change in colony formation compared to control cells. For gefitinib, both concentrations as mono-treatment significantly affected colony formation negatively. The lower concentration (10 μM) resulted in a reduction with ($p = 0.007$) and 20 μM gefitinib was substantially more effective ($p = 0.0002$) with complete eradication of colony formation. By using the lower concentration of gefitinib it was possible to identify an enhancement by combining with 4-PB compared to gefitinib alone ($p = 0.05$). Vantetanib was also tested at two concentrations (4 and 10 μM) in the U343MGa cells. Here, the effect by mono-therapy was significant at both concentrations. Compared to control cells, 4 μM vandetanib gave an approximately 40% ($p = 0.008$), and 10 μM a 75% ($p = 0.0004$) reduction in colony formation, respectively (Fig. 2). A significant additional effect over mono-therapy was evident by addition of 4-PB, at both vandetanib concentrations.

The D324Med cell line displayed similar response to the treatments as the U343MGa cells except that this cell line is more sensitive 4-PB mono-therapy (Fig. 3). The two tested concentrations of the RTKIs were 5 and 10 μM for gefitinib, and 500 nM and 1 μM for vandetanib. The effect on colony formation by mono-treatment was similar for both drugs at the respective concentrations displaying 40–50% reduction in colony formation at the lower concentrations with $p = 0.0008$ for gefitinib and

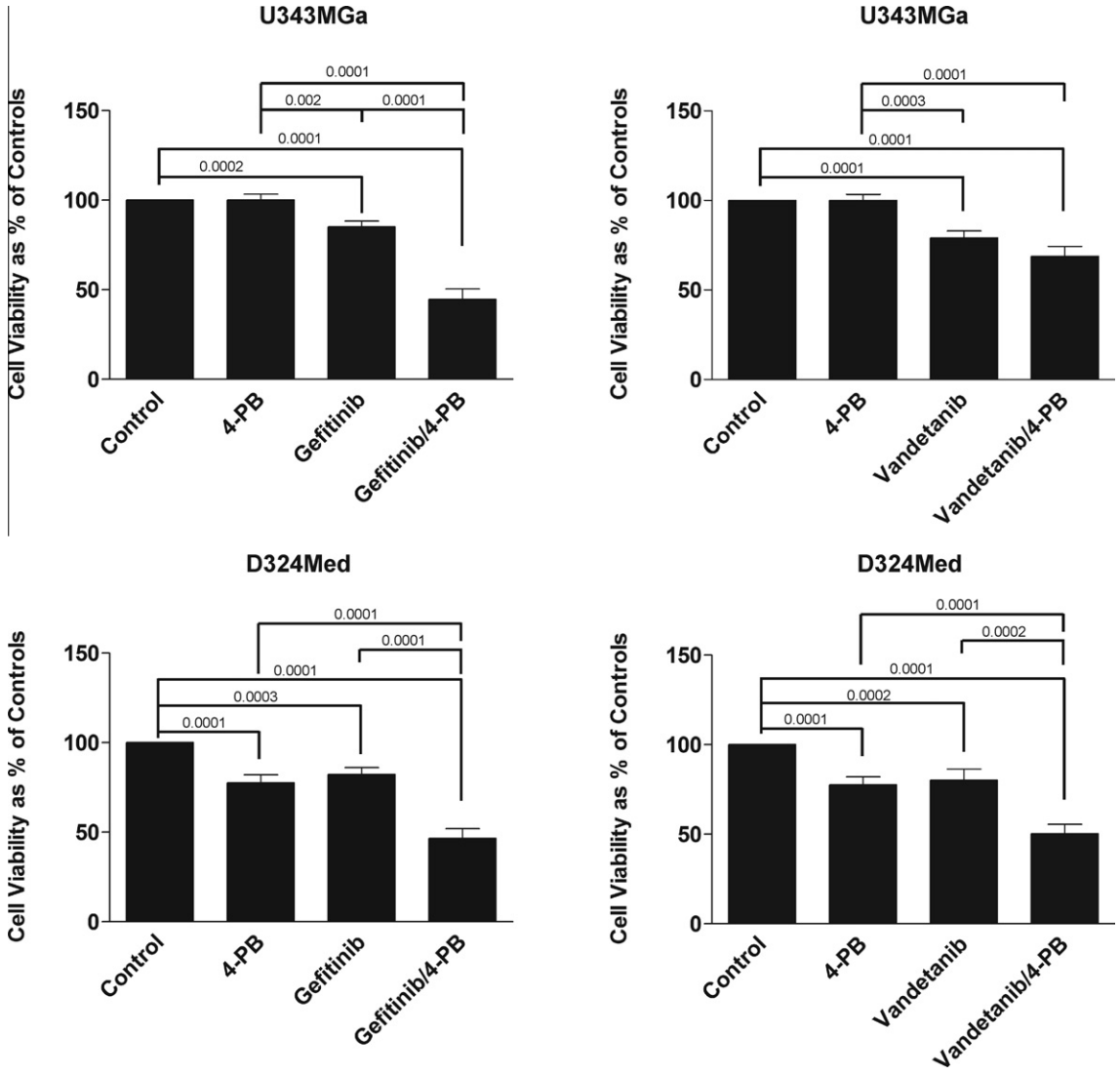


**Fig. 1.** Results from cell viability assays of glioblastoma U343MGa and medulloblastoma D324Med cells after 72 h treatment with the HDAC inhibitor 4-PB and a RTK modulator (gefitinib or vandetanib). U343MGa cells were treated with 2 mM 4-PB, 10 μM gefitinib, or 4 μM vandetanib. D324Med cells were treated with 2 mM 4-PB, 5 μM gefitinib, or 500 nM vandetanib. Cell survival was determined using WST-1 assay and is presented in relation to untreated control cells (100%). Each experiment point was performed in five replicates. The experiments were repeated six times with similar results. Bars represent standard error. Statistically significant changes are indicated as *p*-values.

$p = 0.0001$) for vandetanib. The combination with 4-PB resulted in additional effect to approximately 75–80% reduction in colony formation (Fig. 3).

At the higher respective RTKi concentrations, additional significant colony reduction was evident when comparing gefitinib/4-PB vs gefitinib alone ($p = 0.01$), while vandetanib/4-PB did not cause any additional significant effect over vandetanib alone.

## 4. Discussion

There is an urgent need for new treatment paradigms for glioblastoma to achieve better efficacy, and for medulloblastoma to enhance therapy and avoid the current side effects and long term morbidity of current therapeutic protocols. To achieve this, efficient cancer therapeutics will require the reactivation or inhibition of multiple targets by the use of different drug combinations. The ideal situation will be to find drugs that when combined, will allow lower individual doses but yet result in enhanced cell killing with less expected side effects.

Aberrant expression of HDACs has been shown to often correlate with neoplastic transformation and drug resistance [27–29]. Therefore, the possibility to revert the effects caused by overexpression of HDACs with HDACi has resulted in potentiated effects when combined with RTKi's, such as gefitinib and imatinib [30,31]. The combined cell killing effects of the HDACi 4-PB and the RTK activity modulators gefitinib (EGFR inhibitor), and vandetanib (EGFR/VEGFR-2 antagonist) were thus investigated in two brain tumor models, the glioblastoma U343MGa and medulloblastoma D324Med cell lines. By combining 4-PB with gefitinib or vandetanib we have shown an effect in cell viability, as measured by the WST-1 test, as an apparent 4-PB potentiation of the cell killing of both gefitinib and vandetanib in the D324Med cell line. However, although 4-PB combined with gefitinib resulted in enhanced cell killing also in the U343MGa cells, the effect was substantially less when combined with vandetanib. The basis for the differences in sensitivity between the cell types likely stems from cell type-specific surface receptors and differently affected intracellular signaling pathways downstream of EGFR and VEGFR in the case of glioblastoma, and the PDGFR, SHH, and Wnt in the

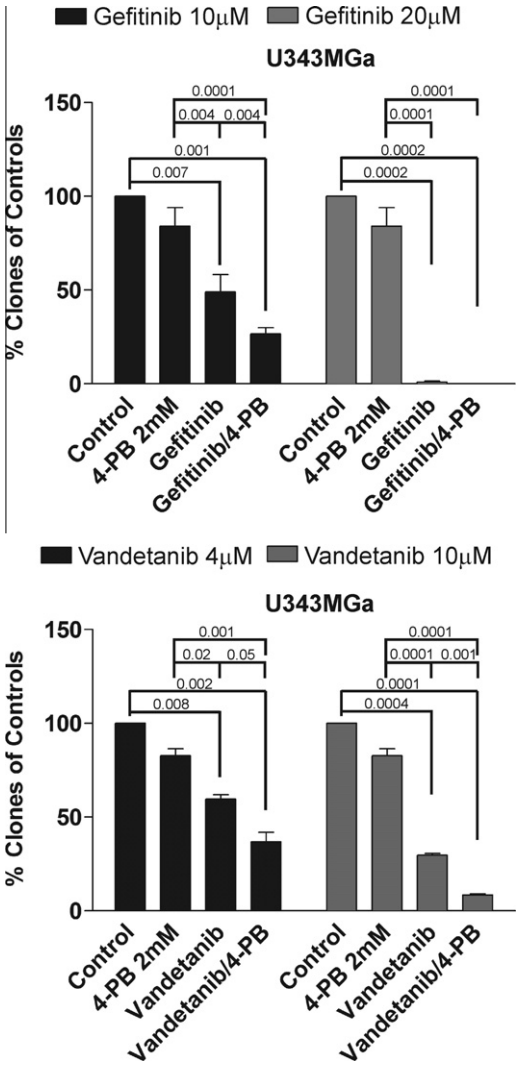


**Fig. 2.** Colony formation efficiency determined by clonogenic assays of the U343MGa cells. Cells were treated for 72 h with 2 mM 4-PB, 10 μM/20 μM gefitinib, or 4 μM 10 μM vandetanib, alone or in combination and assayed after 12 days in drug free medium. The experiments were repeated three times. Bars represent standard error. Statistically significant changes are indicated as *p*-values.

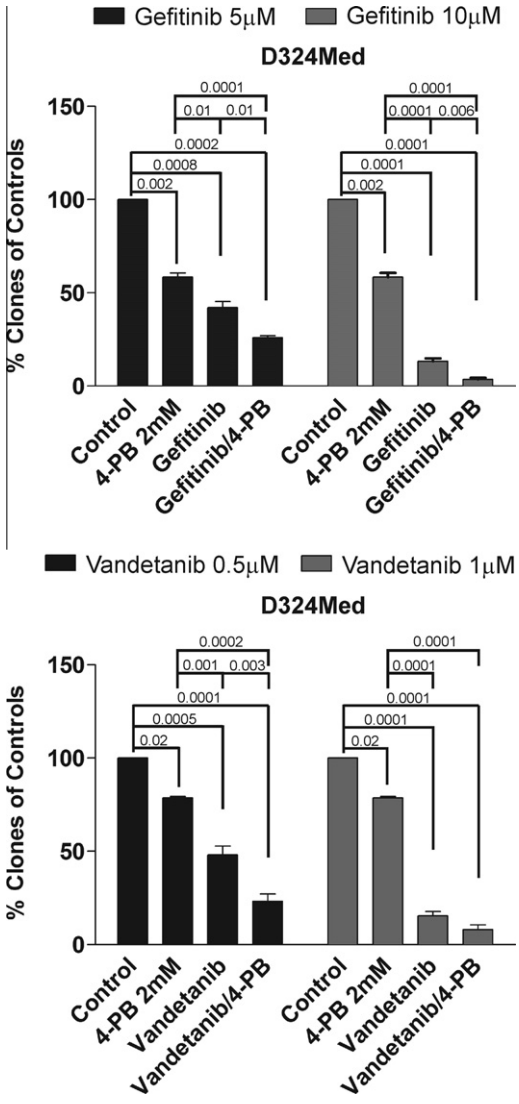


**Fig. 3.** Colony formation assays of the D324Med cells following treatments. Cells were treated for 72 h with 2 mM 4-PB, 5 μM/10 μM gefitinib, or 500 nM/1 μM vandetanib, alone or in combination and assayed after12 days culturing without medium. The experiments were repeated three times. Bars represent standard error. Statistically significant changes are indicated as *p*-values.

case of medulloblastoma [32,33]. Importantly, aberrant constitutive activation of downstream members of the cell signaling pathways, which may not be inhibited by the drugs, will keep the cell proliferation machineries activated.

4-PB inhibits class 1 HDACs, and members of this family are involved in transcriptional repression through interaction with Rb [13,34]. Therefore, treatment with 4-PB might lead to Rb mediated activation of gene transcription of subsets of genes, which eventually lead to cell cycle arrest. In addition, HDACs inhibit the activity of the tumor suppressor protein p53 by deacetylation. Thus, its increased acetylation caused by HDACi may result in induction of apoptosis [11,34].

The observed reduction in cell viability prompted us to investigate by clonogenic assays the effects of combined treatment on the ability of the surviving cells to proliferate. This assessment involved a temporal treatment followed by a recovery phase. We could show that gefitinib and vandetanib in combination with 4-PB potentiated the inhibition of colony formation in both cell lines. These results suggest that combination with 4-PB could be a promising therapy for glioblastoma and medulloblastoma. Its beneficial effect for reducing the consequences of long term treatment and possible side effects should be tested in xenograft models.

HDACi can also by themselves induce apoptosis, cell cycle arrest, and differentiation, and can potentiate the effects of other drugs. This study suggests that HDACi may be considered to be included in the treatment of glioblastoma and medulloblastoma in combination with modern cell surface receptor inhibitors/modulators.

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