

The effect of combined treatment with sodium phenylbutyrate and cisplatin, erlotinib, or gefitinib on resistant NSCLC cells

Maha S Al-Keilani¹
Karem H Alzoubi¹
Saied A Jaradat^{2,3}

¹Department of Clinical Pharmacy, College of Pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan; ²Department of Biotechnology and Genetic Engineering, Jordan University of Science and Technology, Irbid 22110, Jordan; ³Princess Haya Center for Biotechnology, Jordan University of Science and Technology, Irbid 22110, Jordan

Background: Chemotherapy resistance is the main cause of the marginal clinical benefit of platinum-based chemotherapy and tyrosine kinase inhibitors in advanced non-small-cell lung cancer (NSCLC). Thus, the identification of new therapeutic agents that can enhance the sensitivity of these drugs is of clinical importance. Histone deacetylase inhibitors (HDACIs) are emerging as new promising agents with strong antiproliferative effects against different types of cancers. This study investigates the synergistic potential of sodium phenylbutyrate (NaPB) added on top of standard chemotherapy used against NSCLC.

Objective: The objective of this study was to evaluate the ability of NaPB to overcome the resistance of NSCLC cell lines to cisplatin, gefitinib, and erlotinib.

Methods: MTT cell proliferation assay was used to measure the anticancer effects of cisplatin, erlotinib, or gefitinib alone or combined with various concentrations of NaPB against A549, Calu1, and H1650 NSCLC cell lines. Synergism was estimated by measuring synergy value (R), which is equal to the ratio of IC_{50} of each primary drug alone divided by combination IC_{50} s. Student's t -test analysis was used to evaluate the potential differences between IC_{50} values. ANOVA followed by Tukey's post hoc was used to evaluate the potential differences among monotherapy and combination treatment groups. Analyses were performed using R 3.3.2 software. P -value <0.05 was considered to be statistically significant.

Results: NaPB was shown to inhibit the growth of A549, Calu1, and H1650 cell lines in a dose-dependent manner (IC_{50} 10, 8.53, and 4.53 mM, respectively). Furthermore, the addition of NaPB along with cisplatin, erlotinib, or gefitinib to A549, Calu1, and H1650 cell lines resulted in a synergistic antiproliferative effect against the three NSCLC cell lines ($R>1.6$, P -value <0.05), thus suggesting that NaPB can potentiate the effect of cisplatin, erlotinib, and gefitinib on A549, Calu1, and H1650 cell lines.

Conclusion: Current results suggest a potential role of NaPB as a sensitizing agent in NSCLC.

Keywords: NSCLC, resistance, histone deacetylase inhibitor, sodium phenylbutyrate, synergism, sensitizing chemotherapy

Introduction

Lung cancer is a health concern worldwide since it is one of the most common cancers accounting for 17% of cancer cases in men and 7% of cases in women. It is also the leading cause of cancer-related mortality in both sexes being responsible for about 19% of deaths.¹ The non-small-cell lung cell (NSCLC) accounts for the majority of lung cancer cases;² however, about three quarters of the patients with NSCLC are diagnosed at late stages (IIIB and IV).³ Platinum-based chemotherapy is considered the main-

Correspondence: Maha S Al-Keilani
Department of Clinical Pharmacy,
College of Pharmacy, Jordan University of
Science and Technology, PO Box 3030,
Irbid 22110, Jordan
Tel +962 2720 1000
Email mskeilani@just.edu.jo

stay of therapy for patients with advanced, metastatic, and recurrent NSCLC.^{4,5} The marginal clinical benefit associated with the use of platinum-based chemotherapy and targeted molecular therapy including the epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs), gefitinib and erlotinib, in advanced NSCLC is mainly due to resistance, either intrinsic or acquired.^{4,6} Thus, identification of new therapeutic agents that can be used as sensitizing agents for the treatment of NSCLC is unequivocal.

Histone deacetylase inhibitors (HDACIs) are under investigation as promising future chemotherapeutic agents for the treatment of various types of cancers. Examples are vorinostat, romidepsin, panobinostat, and belinostat that have been approved by the US Food and Drug Administration for the treatment of hematological cancers.^{7,8}

The HDACIs showed anticancer effects through the inhibition of tumor-cell growth, induction of cellular apoptosis, and differentiation in malignant cells.⁹ Their recruitment in the anticancer regimen enhanced the cytotoxicity of several anticancer agents.^{10,11} Sodium phenylbutyrate (NaPB), the salt of a short-chain fatty acid, is another HDACI and was the first of its class to be encountered.^{12,13} Several studies revealed the antineoplastic potential of this agent in different types of cancers including lung cancer using both *in vitro* and *in vivo* studies.^{14–17} Unfortunately, *in vivo* studies and clinical trials failed to reveal beneficial anticancer effect of NaPB when used as monotherapy and suggested that it is better for NaPB to be used as part of combination chemotherapies.¹⁸ To the best of our knowledge, there are no *in vitro* studies that focus on the synergistic potential of NaPB in combination with standard chemotherapy for the treatment of NSCLC. Thus, in this study, the ability of NaPB to sensitize the NSCLC cell lines to cisplatin, erlotinib, and gefitinib and overcome their resistance against these agents was investigated.

Materials and methods

Cell culture and drug treatment

Human NSCLC, A549 (Kirsten Rat Sarcoma Virus [KRAS]-mutant; G12S, EGFR-wild-type), Calu1 (KRAS-mutant; G12C, EGFR-wild-type), and H1650 (KRAS-wild-type, EGFR-mutant; a deletion in exon 19 of the EGFR gene delE746A750) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The A549 cell line was cultured in DMEM (Euroclone S.P.A, Pero, MI, Italy), and Calu1 and H1650 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI; Euroclone). Culture media were supplemented with 10% fetal bovine serum (Euroclone) and 1% penicillin/streptomycin

(100 IU/mL penicillin and 100 µg/mL streptomycin; Euroclone). Cell lines were maintained in a humidified incubator of 95% air and 5% CO₂ atmosphere at 37°C.

Cisplatin, erlotinib, gefitinib, and NaPB were purchased from Combi-Blocks (San Diego, CA, USA). Cisplatin, erlotinib, and gefitinib were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 100, 10, and 10 mM, respectively. NaPB was dissolved in nuclease-free water at a concentration of 2 M. All drug stocks were stored in dark-colored bottles at –20°C as stock solutions. The drugs were diluted with culture media to the required concentrations immediately before use. Before treatment with the drugs, the medium was removed when cells were adherent and about 80% confluent.

MTT assay

The cells were seeded in 96-well plates at a density of 5×10^3 in 200 µL of medium per well, and the cells were incubated and allowed to attach overnight. After that media were replaced with fresh ones containing various concentrations of drugs; NaPB (0–32 mM), cisplatin (0–96 µM), erlotinib (0–32 µM), or gefitinib (0–32 µM) alone or in combination with three different concentrations of NaPB (IC_{50} , $0.5 \times IC_{50}$, $2 \times IC_{50}$). Cells grown in medium alone (for treatment with NaPB only) or containing an equivalent amount of DMSO (for other treatment conditions) served as control.

After incubation for 72 hours at 37°C, 20 µL of MTT dye was added to the treated cells (5 mg/mL in PBS) and then incubated for 3 hours at 37°C. After that, the MTT dye was discarded, and the formazan product was dissolved by adding 100 µL of DMSO to each well, followed by shaking for 5 minutes. The spectrophotometric absorbance of the plates was measured using an ELISA plate reader at 570 nm with a reference wavelength of 690 nm. Cell viability was calculated as follows: absorbance of the experimental group/absorbance of the control group. The IC_{50} value was defined as the concentration that inhibited cell proliferation by 50% when compared to that of the controls. IC_{50} values were calculated using Compusyn software 1.0 (Composyn Inc., Paramus, NJ, USA). All measurements were done in triplicates, and the values shown in the figure and tables represent the mean value of the three wells.

Synergism analyses

The three NSCLC cell lines were treated with cisplatin, erlotinib, or gefitinib alone or in combination with NaPB at fixed concentrations that corresponded to the IC_{50} , $0.5 \times IC_{50}$, and $2 \times IC_{50}$ in each cell line. The degree of interaction between NaPB and cisplatin, erlotinib, or gefitinib was calculated

through the synergy values (R) as previously described by Martinez-Marignac et al.¹⁹ The R was measured by dividing the value of IC_{50} of each primary drug alone (cisplatin, erlotinib, or gefitinib) divided by IC_{50} value for the combination therapy. R values >1.6 , $=1$, and <1 indicated synergism, additive effect, and antagonism, respectively.

Statistical analyses

Student's t -test analysis was used to evaluate the potential differences between IC_{50} values. ANOVA followed by Tukey's post hoc test was used to evaluate the potential differences among monotherapy and combination treatment groups. Analyses were performed using R 3.3.2 software. P -value <0.05 was considered to be statistically significant.

Results

NaPB inhibited the growth of NSCLC cells

Three NSCLC cell lines A549, Calu1, and H1650 were utilized. Initially, the effects of NaPB on the proliferation of the three cell lines were investigated, using the MTT assay performed after 72 hours of treatment. The NaPB inhibited the growth of the three cell lines in a dose-dependent manner. The IC_{50} values were 10, 8.5, and 4.5 mM in A549, Calu1, and H1650, respectively (Table 1).

Synergistic growth inhibition by NaPB in combination with cisplatin, erlotinib, and gefitinib

For the combined treatment, the three NSCLC cell lines were treated with a series of concentrations of cisplatin (0–96 μ M), erlotinib (0–32 μ M), or gefitinib (0–32 μ M) in the presence or absence of three concentrations of NaPB that corresponded to $0.5 \times IC_{50}$, IC_{50} , and $2 \times IC_{50}$.

A549 and Calu1 cell lines containing activating *KRAS* mutations causing resistance to gefitinib and erlotinib were exposed to 5, 10, and 20 mM and 4, 8, and 16 mM of NaPB, respectively. While H1650 cell lines containing an activating

EGFR deletion and are resistant to erlotinib and gefitinib were exposed to 2, 4, and 8 mM of NaPB. The growth inhibitory effect was measured in the three cell lines by MTT assays. As shown in Figure 1, current results revealed that NaPB enhanced the anticancer effects of the three tested chemotherapeutic agents in a dose-dependent manner. Moreover, the addition of NaPB to A549, Calu1, and H1650 along with cisplatin, erlotinib, or gefitinib resulted in synergistic antiproliferative effect against the three NSCLC cell lines (Table 2; $R > 1.6$, P -value < 0.05), thus suggesting that NaPB can potentiate the effect of cisplatin, erlotinib, and gefitinib on A549, Calu1, and H1650. The degree of synergism was the highest in A549 cell line.

Discussion

Platinum-based regimens and EGFR-TKIs remain the major chemotherapy for NSCLC, but resistance to these agents has negatively affected the overall survival of patients. Resistance to anticancer therapy can occur through different mechanisms such as impaired permeability, DNA mutations, metabolic changes, DNA damage repair, and epigenetic alterations.¹¹ Epigenetic mechanisms are critical for normal genetic development of the mammalian cells, and cancer is believed to develop due to the accumulation of genetic and epigenetic aberrations through a multistep process,¹⁰ creating a heterogeneous disease at both cellular and molecular levels.²⁰ Thus, epigenetic aberrations play a major role in cancer development and progression and anticancer therapy resistance.¹¹

There is a continued interest in the inclusion of relatively safe drugs such as HDACIs to the conventional chemotherapy. There are also several lines of evidence that support the effectiveness of HDACIs in enhancing the anticancer activity of cisplatin and TKIs. In ovarian cancer cell lines, overexpression of HDAC isoforms resulted in resistance to cisplatin,²¹ and combination of cisplatin with panobinostat, a HDACI, enhanced ovarian cells sensitivity.²² Synergistic anticancer effect was also evident when cisplatin was combined with belinostat in NSCLC cells.^{22,23} In patients with NSCLC, HDAC class I and II overexpression served as a negative prognostic biomarker.^{24,25} On the other hand, combining trichostatin A with erlotinib was associated with the stimulation of NSCLC cell apoptosis.²⁶

The current study was performed in an attempt to identify the synergistic potential of the HDACI NaPB when combined with the conventional chemotherapy in NSCLC utilizing cell lines that have genetic mutations making them resistant to therapy. *KRAS* and *EGFR* are two oncogenes that are among the genes that are frequently mutated in lung cancer

Table 1 IC_{50} values

	NSCLC cell line		
	A549	Calu1	H1650
NaPB (mM)	9.94	8.53	4.53
Cisplatin (μ M)	416.43	240.89	628.38
Erlotinib (μ M)	15.86	41.36	3.18
Gefitinib (μ M)	6	11.93	8.79

Note: IC_{50} is the inhibitory concentration of 50% viability.

Abbreviations: NaPB, sodium phenylbutyrate; NSCLC, non-small-cell lung cancer.

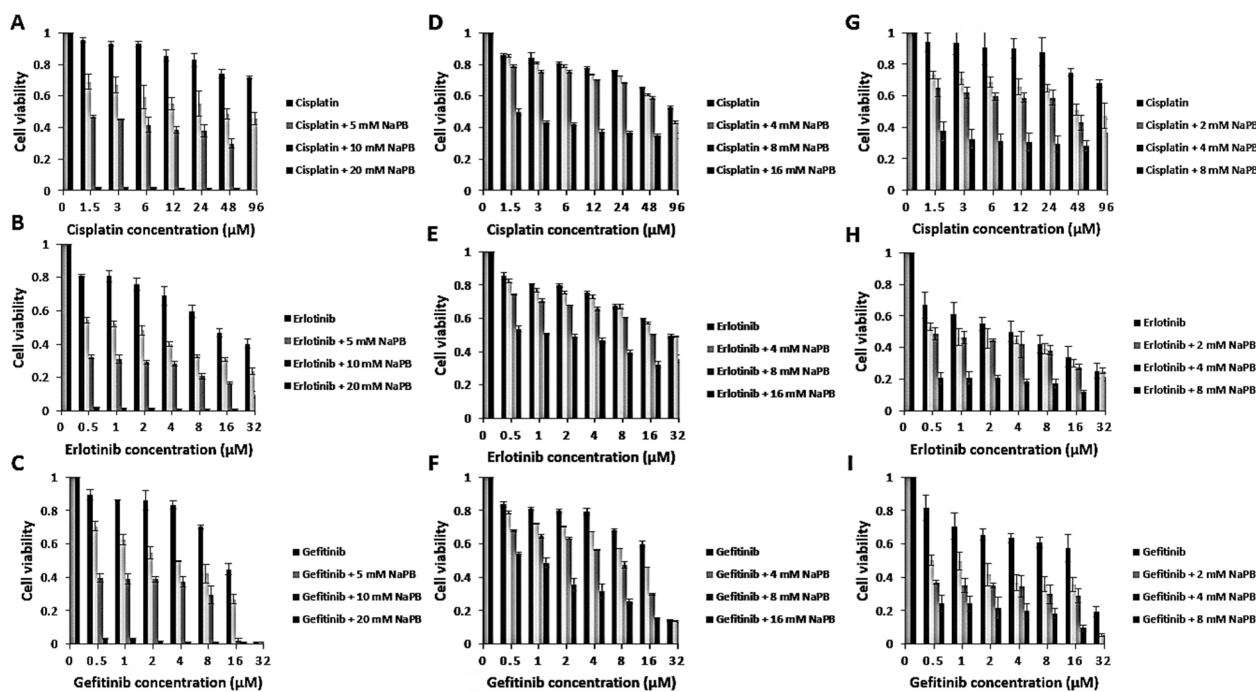


Figure 1 NaPB combined with cisplatin, erlotinib, or gefitinib showed potential synergism in A549 (A–C), Calu1 (D–F), and H1650 (G–I) NSCLC cell lines.

Notes: Cell lines were incubated for 72 hours with a series of concentrations of cisplatin, erlotinib, or gefitinib alone or in combination with NaPB at three fixed concentrations corresponding to $0.5 \times IC_{50}$, IC_{50} , $2 \times IC_{50}$ in each cell line. Cells grown in medium containing an equivalent amount of DMSO served as control. After treatment, cell proliferation was measured using MTT assay. Data are expressed as mean \pm SD of triplicates. ANOVA followed by Tukey's post hoc test was used to evaluate the potential differences among monotherapy and combination treatment groups. P -value < 0.05 was considered to be statistically significant.

Abbreviations: DMSO, dimethyl sulfoxide; NaPB, sodium phenylbutyrate; NSCLC, non-small-cell lung cancer.

resulting in treatment resistance mainly to the EGFR-TKIs erlotinib and gefitinib.^{27,28} Studies on chemotherapy-resistant NSCLC cell lines have shown a synergistic potential of the HDACis, panobinostat and romidepsin when combined with erlotinib, and vorinostat when combined with gefitinib and erlotinib.^{29–31} Thus, HDACis hold a big promise in overcoming resistance to erlotinib and gefitinib in NSCLC cell lines harboring mutations in *KRAS* or *EGFR* genes.

NaPB was more effective in inhibiting H1650 cell proliferation than Calu1, consistent with the results of the previous studies that showed a cell-type-specific anticancer effect of NaPB.¹⁸ Current results also revealed that the resistance of NSCLC cells to cisplatin, erlotinib, and gefitinib can be overcome via combination of these agents with NaPB. Moreover, the current study has demonstrated that NaPB inhibited the growth of NSCLC cell lines in a dose-dependent manner and that it synergized with the three drugs in inhibition of tumor cell growth as evidenced by the synergy values (R).

This synergistic effect can be explained by several mechanisms, as NaPB inhibits class I and IIa HDAC enzymes

resulting in reprogramming of gene expression and post-translational modifications.^{18,32} A gene expression microarray analysis revealed that sodium butyrate, a derivative of NaPB, downregulated 100 cell cycle-associated genes in NSCLC cell lines.³³ As a consequence, NaPB therapy is associated with the inhibition of cellular proliferation, metastasis, and angiogenesis, induction of cell cycle arrest and apoptosis.

Conclusion

Current results indicate that the combination of NaPB with the selected anticancer agents in NSCLC is a successful strategy that may reduce the risk of resistance. This supports the use of NaPB as sensitizing chemotherapy in NSCLC rather than a monotherapy. Future work is suggested to focus on testing the tolerability and efficacy of such combinations in preclinical models.

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Table 2 Combination IC₅₀* and synergy values (R) in A549, Calu1, and HI 650 NSCLC cell lines

A549	IC ₅₀ * (µM)	P-value**	R	Calu1	IC ₅₀ * (µM)	P-value**	R	HI 650	IC ₅₀ * (µM)	P-value**	R
Cisplatin											
Plus 5 mM NaPB	40.97	<0.00	10.17	Plus 4 mM NaPB	109.96	0.008	2.19	Plus 2 mM NaPB	93.83	<0.00	6.7
Plus 10 mM NaPB	1.04	<0.00	400.58	Plus 8 mM NaPB	70.39	0.001	3.42	Plus 4 mM NaPB	25.39	<0.00	24.75
Plus 20 mM NaPB	7.30E-19	<0.00	5.7E+20	Plus 16 mM NaPB	0.95	<0.00	254.67	Plus 8 mM NaPB	0.02	<0.00	4.145
Erlotinib											
Plus 5 mM NaPB	1.15	0.0006	13.76	Plus 4 mM NaPB	39.63	0.004	1.044	Plus 2 mM NaPB	0.94	0.017	3.37
Plus 10 mM NaPB	0.11	<0.00	148.32	Plus 8 mM NaPB	14.43	<0.00	2.87	Plus 4 mM NaPB	0.71	0.007	4.46
Plus 20 mM NaPB	7.90E-12	<0.00	2.01E+12	Plus 16 mM NaPB	1.36	<0.00	30.47	Plus 8 mM NaPB	0.002	0.001	1,655.3
Gefitinib											
Plus 5 mM NaPB	2.19	0.002	2.74	Plus 4 mM NaPB	6.5	0.002	1.84	Plus 2 mM NaPB	1.02	0.0005	8.61
Plus 10 mM NaPB	0.83	0.001	7.2	Plus 8 mM NaPB	3.41	0.002	3.5	Plus 4 mM NaPB	0.32	0.0009	27.14
Plus 20 mM NaPB	4.98E-06	0.002	1,203,422	Plus 16 mM NaPB	0.77	0.001	15.40	Plus 8 mM NaPB	0.03	0.001	313.74

Notes: *IC₅₀, inhibitory concentration of 50% viability; **IC₅₀, values of monotherapy and combination were compared using the Student's t-test; R, is the synergy value.
Abbreviations: NaPB, sodium phenylbutyrate; NSCLC, non-small-cell lung cancer.

Author contributions

All authors contributed toward data analysis, drafting, and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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