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Original Article

THE HDAC INHIBITOR SODIUM PHENYLBUTYRATE ENHANCES THE CYTOTOXICITY INDUCED BY 5-FLUOROURACIL, OXALIPLATIN, AND IRINOTECAN IN COLORECTAL CANCER CELL LINES

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ABSTRACT

Objective: The main objective of this study was to evaluate the ability of sodium phenylbutyrate (NaPB) to enhance the cytotoxicity of 5-fluorouracil, oxaliplatin, and irinotecan against colorectal cancer cell lines expressing wild-type and mutant p53.

Methods: The antiproliferative effect of NaPB alone or in combination with 5-fluorouracil, oxaliplatin, or irinotecan in HCT-116 and HT-29 colorectal cancer cell lines was investigated using the MTT cell proliferation assay. IC_{50} values were calculated using Compusyn Software 1.0 (Combosyn Inc.). Synergy values (R) were calculated using the ratio of IC_{50} of each primary drug alone divided by combination IC_{50}s. For each two pairs of experiments, student's t-test was used for analysis. In combination studies, one-way ANOVA test; Tukey post-hoc testing was performed using R 3.3.2 software. P-value<0.05 was considered significant.

Results: NaPB inhibited the growth of HCT-116 and HT-29 cell lines in a dose-dependent manner (IC_{50}s 4.7 mmol, and 10.1 mmol, respectively). HT-29 cell lines (mutant p53) were more sensitive to NaPB at low concentrations (<4 mmol). Moreover, the addition of NaPB to HCT-116 and HT-29 with 5-fluorouracil, oxaliplatin, or irinotecan synergistically induced the antiproliferative effect (R>1.6, p-value<0.05).

Conclusion: NaPB enhanced the cytotoxicity of conventional chemotherapy against colorectal cancer cell lines harboring wild-type or mutant p53. Thus NaPB is a promising potential adjuvant chemotherapy in colorectal cancer.

Keywords: Colorectal cancer, Histone deacetylase inhibitor, Sodium phenylbutyrate, Adjuvant chemotherapy

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INTRODUCTION

Colorectal cancer is the third most common cancer in both sexes and it is among the most common causes of cancer-related deaths in the United States [1]. In Jordan, colorectal cancer accounts for 10.3% and 8.9% of cancer-related deaths in males and females, respectively [2]. Surgical resection of the primary tumor and involved lymph nodes is the primary treatment for early-stage localized tumors. However, for advanced-stage metastatic tumors, adjuvant chemotherapy is the mainstay of therapy [3]. About 55% of colorectal cancer patients present with advanced stages at the time of diagnosis, and half of the patients who undergo surgery ultimately develop a metastatic disease [4-6]. Adjuvant chemotherapy consisting mainly of 5-fluorouracil, oxaliplatin, and irinotecan, has been associated with improved response rates, reduced risk of tumor recurrence and mortality, and enhanced disease-free and median survival rates [4-8]. Despite the advances in chemotherapy and targeted molecular agents, about 90% of patients with advanced metastatic colorectal cancer fail therapy, which is mainly attributed to resistance, either intrinsic or acquired [9, 10].

The high resistance and toxicity profile associated with the current chemotherapeutic agents highlight the urgent need for the identification of new adjuvant therapies. Such agents will act synergistically to sensitize tumor cells to the currently approved anticancer drugs, overcome therapy resistance, and reduce dose requirements and thus the drug-induced adverse effects. Taking advantage of potential antineoplastic agents that are currently approved and used for the treatment of disease conditions other than cancer is highly encouraged to evade the costly and exacting processes of development of novel anticancer agents. Agents targeting epigenetic mechanisms are among the future promising therapies for enhancing the response of the currently approved anticancer drugs. The proof-of-concept for that is the approval of histone deacetylase (HDAC) inhibitors (HDACIs); vorinostat, romidepsin, panobinostat, and belinostat by the US Food and Drug Administration for use in haematological cancers [11, 12].

HDACIs showed anticancer effects through the inhibition of tumour cell growth and induction of cellular apoptosis and differentiation in malignant cells [13]. Their recruitment in the anticancer regimen enhanced the cytotoxicity of several anticancer agents [14, 15]. Sodium phenylbutyrate (NaPB), the salt of a short-chain fatty acid, is a HDACI and was the first of its class to be encountered [16, 17]. Numerous studies have revealed the antineoplastic potential of this agent in different types of cancers, including colorectal cancer, *in vitro* and *in vivo* [18-21].

In this study, we were prompted to investigate the ability of NaPB to sensitize the colorectal cancer cell lines to 5-fluorouracil, oxaliplatin, and irinotecan and overcome the resistance against these agents.

MATERIALS AND METHODS

Drugs

Irinotecan was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 160 mmol, and 5-fluorouracil and oxaliplatin were purchased from Combi-Blocks (San Diego, CA, USA) and dissolved in DMSO at concentrations of 385 mmol and 378 mmol, respectively. NaPB was obtained from Combi-Blocks (San Diego, CA, USA) and dissolved in nuclease-free water at a concentration of 2 M. All drugs were stored in dark-coloured bottles at-20 °C as stock solutions.

Cell culture and drug treatment

HCT-116 and HT-29 human colorectal cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Euroclone, Italy) supplemented with 10% fetal bovine serum (Euroclone, Italy) and 1 % penicillin/

streptomycin (100 IU/ml penicillin and 100 µg/ml streptomycin, Euroclone, Italy). Both cell lines were maintained in a humidified incubator of 95% air and 5% CO2 atmosphere at 37 °C. Drug stocks were diluted to the required concentrations with culture media 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 plated in 96 well plates at a density of 5 x 10^3 in 200 µl of medium per well and the cells were incubated and allowed to attach overnight. The attached cells in the plates were treated with a series of drug concentrations; NaPB (0-32 mmol), or 5-fluorouracil (0-200 µM), oxaliplatin (0-300 µM), or irinotecan (0-160 µM) alone or in combination with three different concentrations of NaPB (IC_{50}, 0.5X IC_{50}, 2X IC_{50}). Cells grown in medium alone (for treatment with NaPB only) or containing an equivalent amount of DMSO served as control (for other treatment conditions). Cells were incubated with the drugs at the indicated concentrations for 72 h. All measurements were done in triplicate. After that, the cell proliferation assay was performed per manufacturer's protocol. Briefly, MTT dye was added to the treated cells at a final concentration of 0.5 mg/ml in PBS. After that the plates were incubated at 37 °C for 3 h, then the MTT was discarded and the formazan product was dissolved by adding 100 µl of DMSO to each well, followed by shaking for 5 min. Then the plates were read using an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm with a reference wavelength of 690 nm. Cells viability was calculated as follows: Absorbance of the experimental group/absorbance of the control group. The IC_{50} value was defined as the concentration needed for a 50% reduction in cell viability. Dose-effect analyses and IC_{50} calculations were performed using Compusyn Software 1.0 (Combosyn Inc.).

Analysis of the effects of drug combinations

The effects of different drug combinations were determined as previously described by Richard *et al.* [22]. Synergy values (R) were calculated using the ratio of IC_{50} of each primary drug alone (5-fluorouracil, oxaliplatin, or irinotecan) divided by combination IC_{50}s. R-value reflects the extent of synergism or antagonism for two drugs: R>1.6, synergism; R=1, additive effect; R<1, antagonism.

Statistical analysis

All results were expressed as the mean±standard deviation. For each two pairs of experiments, student's t-test was used for analysis. For comparison of IC_{50} values of monotherapy versus combination IC_{50}, student's t-test was performed. For analysis of the results of combination studies, One-way ANOVA test; Tukey post-hoc testing was performed using R 3.3.2 Software. Statistical significance was considered if p-value<0.05.

RESULTS

NaPB inhibited the growth of colorectal cancer cell lines

We utilized two different colorectal cancer cell lines; HCT-116 and HT-29. Initially, we investigated the effects of NaPB on the proliferation of the two cell lines, using the MTT assay performed after 72 h of treatment. As shown in fig. 1, NaPB exerted antiproliferative effects in a dose-dependent manner against the two cell lines.

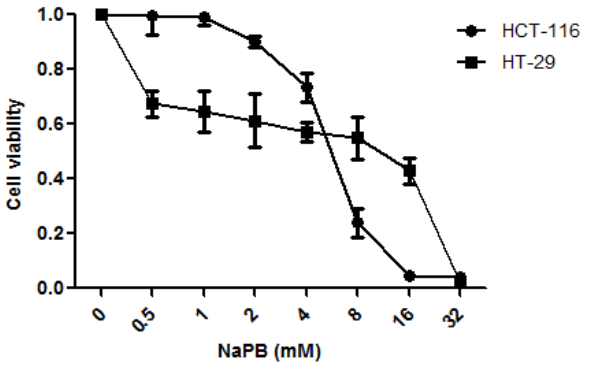

Fig. 1: Antiproliferative effect of NaPB on HCT-116 and HT-29 colorectal cancer cell lines, NaPB concentrations were ranging from 0.5-32 mmol. Attached cells were treated and maintained in the drug-containing medium for 72 h before being analyzed by MTT. Triplicate experiments were performed. Data are expressed as mean±standard deviation. NaPB inhibited the growth of HCT-116 and HT-29 cell lines in a dose-dependent manner. Student's t-test was used for analysis and statistical significance was considered if p<0.05

The IC_{50} values were about 5 mmol and 10 mmol in HCT-116 and HT-29, respectively (table 1).

Table 1: Inhibitory concentrations of 50% viability (IC_{50}s) for NaPB and chemotherapy drugs in HCT-116 and HT-29

Colorectal cancer cell lines	HCT-116	HT-29
NaPB (mM)	4.7	10.1
5-fluorouracil (µM)	2.9	200
Oxaliplatin (µM)	9.8	9.8
Irinotecan (µM)	0.4	1.7

Synergistic growth inhibition by NaPB in combination with 5-fluorouracil, oxaliplatin, and irinotecan

For the combined treatment, the two colorectal cancer cell lines were treated with varying concentrations of 5-fluorouracil (0-200 µM), oxaliplatin (0-300 µM), or irinotecan (0-160 µM) in the presence or absence of three different concentrations of NaPB that corresponded to 0.5X IC_{50}, IC_{50}, and 2X IC_{50} (2.5, 5, and 10 mmol in HCT-116; and 5, 10, and 20 mmol in HT-29). The growth inhibitory effect was measured in the two cell lines by MTT assays. As shown in fig. 2, our results revealed that at clinically achievable and non-toxic concentrations, NaPB enhanced the antiproliferative effect of the three tested anticancer agents in a dose-dependent manner.

Table 2: Combination IC_{50}s* and synergy values (R) in HCT-116 and HT-29 colorectal cancer cell lines

HCT-116	IC_{50}	p-value	R	HT-29	IC_{50}	p-value	R
5-fluorouracil				**5-fluorouracil**			
Plus 2.5 mmol NaPB	0.2	0.006	14.5	Plus 5 mmol NaPB	36	0.000	5.6
Plus 5 mmol NaPB	6.60E-10	0.029	4.39E+09	Plus 10 mmol NaPB	1.6	0.000	125
Plus 10 mmol NaPB	4.01E-07	0.026	7.23E+06	Plus 20 mmol NaPB	3.40E-10	0.000	5.87E+11
Oxaliplatin				**Oxaliplatin**			
Plus 2.5 mmol NaPB	1.7	0.022	5.8	Plus 5 mmol NaPB	1.3	0.004	7.8
Plus 5 mmol NaPB	0.96	0.019	10.2	Plus 10 mmol NaPB	0.8	0.002	12.9
Plus 10 mmol NaPB	0.0007	0.002	14000	Plus 20 mmol NaPB	9.40E-08	0.006	1.04E+08
Irinotecan				**Irinotecan**			
Plus 2.5 mmol NaPB	0.0075	0.011	53.39	Plus 5 mmol NaPB	0.3	0.017	5.7
Plus 5 mmol NaPB	1.49E-38	0.008	2.69E+37	Plus 10 mmol NaPB	0.08	0.019	21.3
Plus 10 mmol NaPB	1.11E-55	0.008	3.59E+54	Plus 20 mmol NaPB	8.70E-06	0.019	1.95E+05

*IC_{50}: inhibitory concentration of 50% viability, **IC_{50} values of monotherapy and combination were compared using the student's t-test, ***R: synergy value

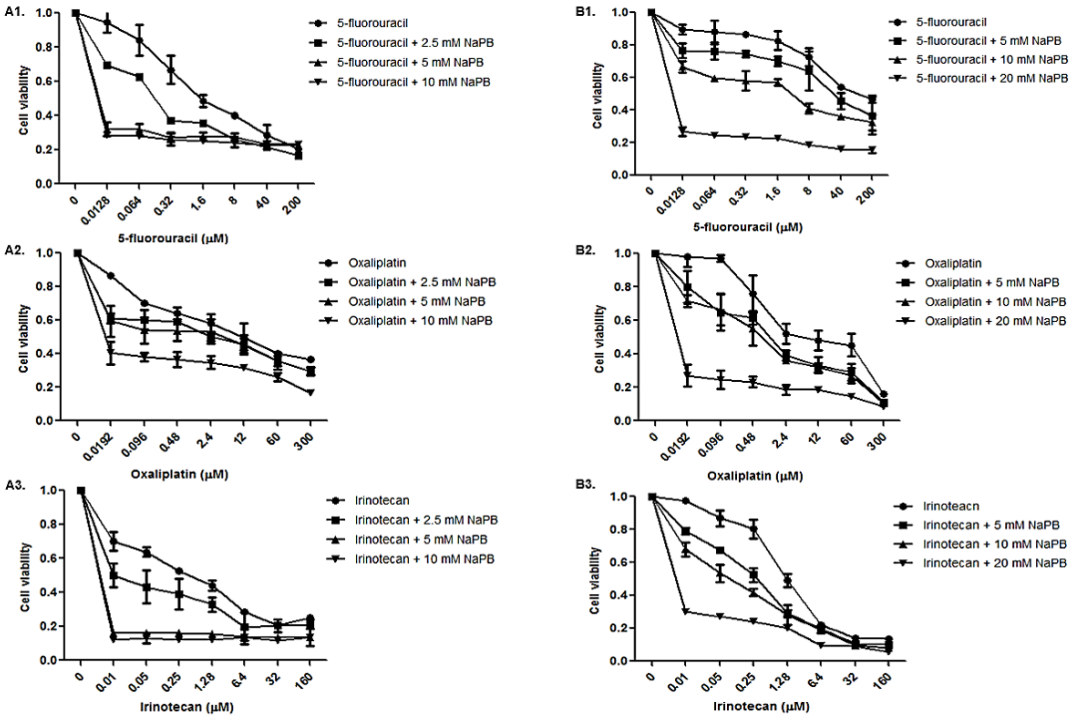

Fig. 2: Effect of treatment with 5-fluorouracil, oxaliplatin, and irinotecan, alone or combined with NaPB on cell proliferation of HCT-116 (A1-A3), and HT-29 (B1-B3) colorectal cancer cell lines, Cell viability was assessed using MTT assay. Cell viability was measured after treatment with a series of drugs' concentrations; 5-fluorouracil (0-200 µM) [A1 and B1], oxaliplatin (0-300 µM) [A2 and B2], or irinotecan (0-160 µM) [A3 and B3] alone or combined with three different concentrations of NaPB (0.5X IC_{50}, IC_{50}, 2X IC_{50}) for 72 h. Cells grown in medium containing an equivalent amount of DMSO served as control. Each treatment condition was performed in triplicate. Data are expressed as mean±standard deviation. One-way ANOVA test; Tukey post-hoc was used for analysis and statistical significance was considered if p-value<0.05

After that synergy values (R) were quantified using the ratio of IC_{50} of each primary drug alone (5-fluorouracil, oxaliplatin, and irinotecan) divided by combination IC_{50}s. NaPB showed a statistically significant synergistic effect against the two cell lines when combined with 5-fluorouracil, oxaliplatin, and irinotecan, as indicated by (R) values (p-value<0.05) (table 2).

DISCUSSION

Conventional chemotherapy containing 5-fluorouracil, oxaliplatin, and irinotecan remain the major anticancer therapy for colorectal cancer, but the 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, epigenetic alterations, and others [15]. Epigenetic mechanisms are critical for the 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 [14-23], creating a heterogeneous disease at both cellular and molecular levels [24].

Thus, epigenetic aberrations play a significant role in cancer development and progression and anticancer therapy resistance [15]. There is a high continued interest toward the inclusion of relatively safe drugs such as HDACIs in the conventional chemotherapy and there are several lines of evidence that support the effectiveness of HDACIs in enhancing the antineoplastic activity of anticancer agents in colorectal cancer. CG2 (a HDACI) enhanced the cytotoxicity of 5-fluorouracil, oxaliplatin, and irinotecan against HCT-116 colorectal cancer cell lines [25]. Furthermore, PXD101 (a HDACI) plus 5-fluorouracil or irinotecan combination showed a synergistic effect in colorectal cancer *in vitro* and *in vivo* [26, 27]. MS275 or SBHA (HDACIs) combined with oxaliplatin showed high synergistic activity against colorectal cancer cells [28].

The current study was performed in an attempt to identify the synergistic potential of the HDACI NaPB when combined with 5-fluorouracil, oxaliplatin, or irinotecan in colorectal cancer. Initially, we found that NaPB inhibited the growth of HCT-116 and HT-29 cell lines in a dose-dependent manner. Although statistically not significant, HCT-116 showed to be more sensitive to NaPB than HT-29 with about two-fold difference in IC_{50}s. On the other hand, at concentrations below 4 mmol, the HT-29 cell line was more sensitive to NaPB than HCT-116 ($p<0.05$). The difference in response to NaPB may indicate that the anticancer effect of this agent is dependent on the p53 status; HCT-116 cell lines express p53 in wild-type conformation, while HT-29 harbor mutant p53; there is a G to A mutation in codon 273 of the gene that results in an arginine to histidine substitution (R273H). This missense mutation is associated with loss of wild-type activity and overexpression of a mutant p53 with oncogenic functions "gain of functions" phenotypes [29]. p53, is a tumor suppressor protein that works intracellularly to promote apoptosis or autophagy of the tumor cells [30-31], and it is the most commonly mutated gene in cancer with a mutation rate of about 50% in colorectal cancer [32, 33]. There is controversy regarding the effect of p53 status on the sensitivity of tumors to anticancer therapy. Some studies revealed that p53 overexpression, a surrogate marker of p53 mutation, is associated with increased resistance to 5-fluorouracil, oxaliplatin, and irinotecan [7, 34]; cancer cell lines with a mutation in the p53 gene were more prone to resistance than wild-type cell lines [7, 34]. While others revealed that p53-mutant cells are more sensitive to anticancer therapy [35-36]. Our findings reveal higher sensitivity to NaPB in p53 mutant cancer cell line (HT-29) than p53 wild-type cancer cell line (HCT-116). Thus, further investigations are required to identify the mechanism of the mutant-type p53-dependent antiproliferative effect of NaPB.

We also demonstrated that at low and clinically achievable concentrations, NaPB synergistically inhibited colorectal cancer cell proliferation in HCT-116 and HT-29 cell lines when combined with 5-fluorouracil, oxaliplatin, and irinotecan in a concentration-dependent manner. This synergistic effect can be explained by

several mechanisms; NaPB inhibits class I HDAC enzymes, resulting in the reprogramming of gene expression and posttranslational modifications [19]. As a consequence, NaPB therapy is associated with inhibition of cellular proliferation, metastasis, and angiogenesis, and induction of cell cycle arrest and apoptosis. It is worth to note that NaPB was able to enhance the cytotoxicity of the tested drugs against the p53 mutant cell line (HT-29); NaPB may have activated the transcriptional activity of p53 in HT-29 cell line, which as a consequence resulted in enhanced apoptosis induced by 5-fluorouracil, oxaliplatin, and irinotecan. Our findings are consistent with the goal of several studies that focus on identifying approaches to target p53 either by activating it in p53-deficient cancer cells, or changing the conformation of mutant p53 to wild-type form (reactivation) to enhance the antitumor effects of anticancer therapy [36, 37]. Our results suggest that NaPB is a potential adjuvant anticancer agent with a beneficial role in overcoming resistance to 5-fluorouracil, oxaliplatin, and irinotecan. Further *in vivo* and clinical studies are required to study the effect of combinational therapies containing NaPB in colorectal cancer, especially those expressing the mutant p53.

CONCLUSION

In conclusion, this study shows that NaPB has synergistic anticancer effect with 5-fluorouracil, oxaliplatin, and irinotecan against colorectal cancer. Future work will more fully investigate the underlying molecular mechanisms of synergism and the p53-dependent anti-proliferative activity.

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AUTHOR CONTRIBUTION

Maha S. Al-Keilani has designed the experiments, performed the data analysis, and wrote the manuscript; Dua H. Alsmadi has performed the experiments and the data analysis, and has written the manuscript.

CONFLICT OF INTERESTS

The authors declare no competing financial interest.

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