The Impact of Simultaneous Epigenetic and Epitranscriptomic Intervention in Breast Cancer Cells

Sevinç YANAR[*], Asuman DEVECİ ÖZKAN[**], Merve Gülşen BAL ALBAYRAK[***], Zeynep BETTS[****]

Abstract

Aim: Breast cancer remains a significant cause of mortality worldwide, necessitating the development of innovative therapeutic approaches. Epigenetic and epitranscriptomic regulation have emerged as promising avenues for novel treatments. Sodium Butyrate (NaB) and Meclofenamic Acid (MFA) have gained attention for their respective roles in epigenetic and epitranscriptomic modulation. NaB, a histone deacetylase inhibitor, serves as a critical regulator of chromatin remodeling and gene expression. MFA has been identified to be a potent inhibitor of the FTO enzyme. This inhibitory potential marks its role in epitranscriptomic regulation. This study aimed to investigate the potential effects of MFA and NaB, individually and in combination, on the MCF7 breast cancer cell line.

Method: In order to investigate the cytotoxic and apoptotic effects of the combination treatment of MFA and NaB, cell viability assay, Annexin V analysis and Acridine Orange/DAPI staining were executed.

Results: The results revealed that the combination treatment unexpectedly exhibited antagonistic effects. This was evidenced by a remarkable increase in cell viability and a decreased apoptotic response compared to individual treatments. The strongest antagonistic effect was observed when the cells were treated with 100 µM MFA and 2 mM NaB for a period of 48 hours (CI = 88.3).

Conclusion: This study, for the first time, sheds light on the complex interaction between meclofenamic acid and sodium butyrate that reveals an unexpected antagonistic effect on MCF7 breast cancer cells. These findings challenge conventional concepts of synergistic interactions and underscore the complexity of drug combinations in breast cancer treatment.

Keywords: Breast cancer, meclofenamic acid, sodium butyrate, combined therapy, antagonism.

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[*] Ph.D., Res. Asst., Sakarya University Faculty of Medicine, Department of Histology and Embryology, Sakarya, Türkiye. E-mail: sevincyanar@sakarya.edu.tr ORCID https://orcid.org/0000-0002-6438-7385
[**] Assoc. Prof. Dr., Sakarya University Faculty of Medicine, Department of Medical Biology, Sakarya, Türkiye. E-mail: deveci@sakarya.edu.tr ORCID https://orcid.org/0000-0002-3248-4279
[***] Ph.D., Res. Asst., Kocaeli University Faculty of Medicine, Department of Medical Biology, Kocaeli, Türkiye. E-mail: mervegulsenbal@gmail.com ORCID https://orcid.org/0000-0003-2444-4258
[****] Asst. Prof. Dr., Kocaeli University Faculty of Science, Department of Biology, Kocaeli, Türkiye. E-mail: duman.zeynep@gmail.com ORCID https://orcid.org/0000-0003-2391-7543
[*****] Asst. Prof. Dr., Manchester Institute of Biotechnology, Faculty of Science and Engineering, University of Manchester, Manchester, UK. E-mail: duman.zeynep@gmail.com ORCID https://orcid.org/0000-0003-2391-7543

Meme Kanseri Hücrelerinde Eş Zamanlı Epigenetik ve Epitranskriptomik Müdahalenin Etkisi

Öz

Amaç: Dünya çapında önemli bir ölüm nedeni olmaya devam etmekte olan meme kanseri için yenilikçi tedavi yaklaşımlarının geliştirilmesi gerekmektedir. Epigenetik ve epitranskriptomik düzenleme, yeni tedaviler için umut verici yollar olarak ortaya çıkmıştır. Sodyum Butirat (NaB) ve Meklofenamik Asit (MFA), epigenetik ve epitranskriptomik modülasyondaki ilgili rollerinden dolayı dikkat çekmektedir. Bir histon deasetilaz inhibitörü olan NaB, kromatin yeniden yapılanması ve gen ekspresyonunda kritik bir düzenleyici olarak görev yapmaktadır. MFA'nın ise FTO enziminin güçlü bir inhibitörü olduğu tespit edilmiştir. Bu inhibitör potansiyel, epitranskriptomik düzenlemedeki rolünü göstermektedir. Bu çalışma, MFA ve NaB'nin ayrı ayrı ve kombinasyon halinde MCF7 meme kanseri hücre hattı üzerindeki potansiyel etkilerini araştırmayı amaçlanmıştır.

Yöntem: MFA ve NaB kombinasyon tedavisinin sitotoksik ve apoptotik etkilerini araştırmak amacıyla hücre canlılığı analizi, Annexin V analizi ve Akridin Orange/DAPI boyaması yapılmıştır.

Bulgular: Sonuçlar kombinasyon tedavisinin beklenmedik şekilde antagonistik etki gösterdiğini ortaya çıkarmıştır. MFA ve NaB'ın tek başına uygulamasına kıyasla kombinasyon halinde uygulanması hücre canlılığında kayda değer bir artışa ve apoptotik yanıtın azalmasına neden olmuştur. En güçlü antagonistik etki, hücreler 48 saat boyunca 100 μM MFA ve 2 mM NaB ile inkübe edildiğinde gözlemlenmiştir (CI= 88,3).

Sonuç: Bu çalışma, ilk kez, meklofenamik asit ile sodyum bütirat arasındaki karmaşık etkileşime ışık tutmuş ve MCF7 meme kanseri hücreleri üzerindeki beklenmedik antagonistik etkisini ortaya koymuştur. Bu bulgular, geleneksel sinerjistik etkileşim kavramlarına meydan okumakla birlikte meme kanseri tedavisinde ilaç kombinasyonlarının karmaşıklığının altını çizmektedir.

Anahtar Sözcükler: Meme kanseri, meklofenamik asit, sodyum bütirat, kombinasyon tedavisi, antagonizm.

Introduction

Breast cancer ranks as the primary cause of cancer-related deaths among women worldwide[1]. Despite there have been breakthroughs in therapies, the need for different and novel therapeutic approaches is important due to the complexity of breast cancer biology and drug resistance. In this pursuit, a promising avenue for innovative therapeutic approaches is the investigation of epigenetic and epitranscriptomic controls[2]. These layers of biological control operate beyond the genetic code and represents a largely unexplored source of therapeutic targets. The ability to modulate gene expression and cellular behavior via these mechanisms opens new avenues for the development of treatments that can precisely target the multifaceted nature of breast

cancer pathology and potentially overcome the limitations imposed by conventional therapies[3].

Sodium Butyrate (NaB) is a short-chain fatty acid and naturally occurs in the gut as a result of the fermentation of dietary fibers. NaB functions as an inhibitor of histone deacetylase (HDAC), which is an important regulator of chromatin remodeling and gene expression[4]. It disrupts the equilibrium between histone acetylation and deacetylation, causing to an accumulation of acetylated histones. This modification results in a more open chromatin structure and facilitates the transcription of genes, including those involved in cell cycle arrest, apoptosis, and tumor suppression[4]. Studies have shown that NaB can induce apoptosis and inhibit the proliferation of some cancer cell lines such as colorectal[5], cervical[6] and breast cancer[7]. This potential highlights its usage as a therapeutic agent throughout a wide range of cancer types. NaB also plays important roles in repairing DNA double-strand breaks, inhibiting oxidative stress besides modulating various cellular mechanisms[8,9].

Meclofenamic Acid (MFA), on the other hand, is traditionally known as a non-steroidal anti-inflammatory drug (NSAID) that inhibits cyclooxygenase enzymes. This inhibition results in reduced production of prostaglandins, which are involved in the processes of inflammation, pain, and fever, as also tumor growth and metastasis[10]. Beyond its anti-inflammatory properties, MFA has been identified as a potential inhibitor of FTO enzyme, which marks its role in epitranscriptomic regulation. FTO demethylates N6-methyladenosine (m6A) sites on RNA, a modification that affects RNA metabolism, such as stability and translation efficiency[11]. Recent findings have underscored the pivotal role of m6A modifications in cancer development. Studies have indicated that m6A demethylase FTO contributes to tumor progression by inducing aberrant m6A modifications in some cancer types. Inhibition of FTO by MFA can cause to altered expression of cancer-related genes and reduced proliferation of cancer cells[10,12–14]. Specifically, studies have shown MFA's capacity to suppress the growth of prostates and lung cancer cells, primarily attributed to its action on the FTO enzyme. Research has also indicated its anti-carcinogenic properties across various cancers, such as cervical, breast, and small cell lung carcinoma[10,12–14]. By inhibiting FTO, MFA impacts the epitranscriptomic regulation of cancer-related genes via alterations in m6A RNA methylation. This modulation of RNA methylation status disrupts the normal stability and translation of oncogenic mRNAs and consequently leads to a decrease in cancer cell viability.

The anticancer effects of NaB and MFA, which are attributed to their different mechanisms of action (epigenetic modulation via HDAC inhibition and epitranscriptomic regulation via FTO inhibition, respectively), provide a compelling reason for their combined use. Moreover, to the best of researcher's knowledge from the existing literature, such a comprehensive study investigating the combined impact of NaB and MFA on breast cancer cells, integrating both epigenetic and epitranscriptomic mechanisms, remains largely unexplored. Therefore, in this study we investigated the combined effect of NaB and MFA on MCF7 breast cancer cells and explored the potential of simultaneous epigenetic and epitranscriptomic intervention as a synergistic therapeutic strategy. By integrating the effects of HDAC and FTO inhibition, we aimed to uncover a comprehensive understanding of how these interventions affect breast cancer cell survival and proliferation, paving the way for future therapeutic innovations.

Material and Methods

Cell Culture and Drugs

Human breast cancer cell MCF7 was purchased from ATCC (Rockville, USA). DMEM (Sigma-Aldrich, USA) was used to grow the cells. 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin (Gibco, USA), and 100 µg/mL streptomycin (Gibco, USA) were also added. The cultures were kept in the incubator (Thermo Fisher, USA) with 5% CO_2 humidity and 37°C.

For the preparation of meclofenamic acid (Merck, Germany), 200 mg chemical has been dissolved in 1 mL of DMSO (Sigma-Aldrich, USA). To prepare sodium butyrate (Sigma Aldrich, USA), 1 mg of NaB was dissolved in water to yield 1 mM stock solution.

Cell Viability Assay

The effect of MFA on the viability of MCF7 cells was examined by WST-1 assay (Roche Applied Science, USA). Initially, MCF7 cells were plated on 96 well plates (5000 cells/well). Various concentrations of MFA (40, 60, 80, 100, 120, and 150 µM) were administered for periods of 24 and 48 hours. After that, 10 µL of WST-1 reagent (Roche Applied Science, USA) was dispensed into each well, followed by a 2-hour incubation at 37°C in darkness. After incubation, a microplate reader was used to measure cell viability at a 450 nm wavelength (Thermoscientific, MA, USA). The experiment was repeated 3 times.

Drug Combination Studies

In the combination experiments, cells were exposed to 80 and 100 μM of MFA using three distinct concentrations of NaB (1, 2, and 4 mM) for periods of 24 and 48 hours. The effect of these combination treatments on cell proliferation was investigated through WST-1 assay, following the method outlined above. To determine the synergistic impact of MFA and NaB, the Chou–Talalay method for calculating the combination index (CI) was employed[15], based on the outcomes of the WST-1 assay.

To determine the synergistic impact of MFA and NaB, the Chou–Talalay method was used to calculate the combination index (CI)[15], based on the outcomes of the WST-1 assay. According to this analysis, a CI value of less than 1 indicates synergism, a CI value equal to 1 indicates additivity, and a CI value greater than 1 shows antagonism.

ANXA5 Enzyme-Linked Immunosorbent Assay (ELISA)

To investigate the apoptotic effects of MFA and NaB, either individually or in combination, the levels of free Annexin A5, namely ANXA5, were assessed. ANXA5 binds to phosphatidylserine groups on the cell surface[16]. This evaluation was conducted by using ANXA5 ELISA (Abbexa, UK) kit. MCF-7 cells, at a density of $5x10^4$ cells/well, were seeded in a 6-well plate. Subsequently, the cells were subjected to treatments with either individual or combined concentrations of MFA (80 and 100 μM) and NaB (2 mM) for 48 hours. After the treatment period was completed, the cell culture supernatant was collected and subjected to analysis using the Human ANXA5 ELISA Kit following the manufacturer's instructions.

Acridine Orange and 4′,6-Diamidino-2-phenylindole Dihydrochloride Staining

To assess variations in cellular and nuclear morphology following the treatments with MFA and NaB, either alone or in combination, Acridine Orange (AO) and 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining were performed. MCF-7 cells, at a density of $4x10^5$ cells/well, were seeded in a 6-well plate that contained slides. Subsequently, the cells were exposed to individual or combined concentrations of MFA (80 and 100 μM) and NaB (2 mM) for 48 hours. After the treatment period, the cells were fixed using a 4% paraformaldehyde solution, and the fixed cells were then stained with AO (Sigma Aldrich, USA) for 30 minutes and DAPI (Sigma Aldrich, USA) for 5 minutes. After staining, cells were observed, and images were taken using fluorescence microscopy (Olympus, Japan).

Statistical Analysis

Statistical analyses were done by GraphPad Prism version 9.1.0 (La Jolla, USA). The results were shown as the mean ± standard deviation from three separate experiments. For comparisons involving multiple groups, ANOVA was performed, followed by Tukey's test for post-hoc analysis. Moreover, the combination index (CI) for MFA in combination with NaB was analyzed using CompuSyn version 1.0 software.

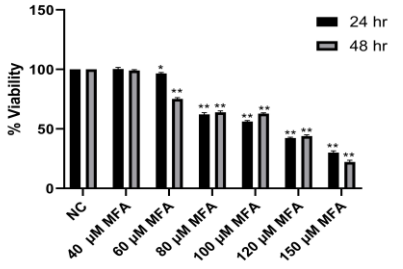
Results

Impact of MFA on MCF7 Cell Viability

The effect of MFA on the growth of cells was evaluated to determine appropriate concentrations for future studies. The findings showed that MFA inhibited the growth of MCF7 cells in a dose- and time-dependent manner (Figure 1). Concentrations greater than or equal to 60 µM showed a statistically significant decrease in cell viability after both 24 and 48 h ($p<0.05$). Specifically, treatment with 80 and 100 µM of MFA with incubation of 24 h resulted in cell viability of 62% and 63%, respectively. Treatment with these concentrations for 48 h caused 64% and 56% cell viability, respectively. Therefore, 80 and 100 µM MFA concentrations were used for combination studies.

The effects of NaB on MCF7 cell viability were demonstrated in researcher's earlier study[17].

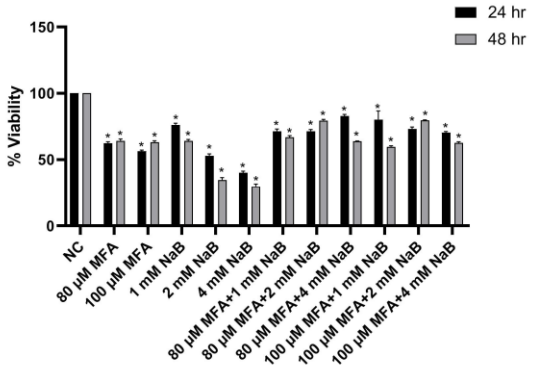
Figure 1. Effect of meclofenamic acid on MCF7 cell viability (*$p<0.05$, **$p<0.01$)



Antagonistic Effects of MFA and NaB

When examining viability among the combination groups, a decrease in cell viability was observed compared to the control group. However, when comparing viability to individual drug treatments, a notable increase draws attention. Viability surpassed 50% in both time intervals and across all combinations, even exceeding 80% in some groups. All these increases were statistically significant (Figure 2).

Figure 2. The effect of combined treatment of MFA and NaB on MCF7 cell viability (*p<0.01)



To investigate any synergistic impact of MFA and NaB, CI was utilized. CI data were obtained through Compusyn software (Table 1). The results indicated antagonistic effects for all combinations. Particularly noteworthy are the considerably high CI values observed in cells treated with 80 µM MFA+2 mM NaB and 100 µM MFA+2 mM NaB for 48 hours (53.3 and 88.3, respectively).

Table 1. CI values of MFA and NaB combinations applied to MCF7 cells

		CI		
Time (h)	**MFA (µM)**	**1 µM NaB**	**2 µM NaB**	**4 µM NaB**
24hr	80	2.080	2.719	7.273
	100	4.621	3.380	4.162
48hr	80	2.794	53.349	5.419
	100	1.433	88.304	4.817

Furthermore, the dose reduction index (DRI) was computed using Compusyn software. DRI values were categorized as follows: DRI < 1, DRI = 1, and DRI > 1, denoting unfavorable dose reduction, no dose reduction, and favorable dose reduction, respectively. The results revealed that nearly all combinations caused DRI values under 1 (Table 2).

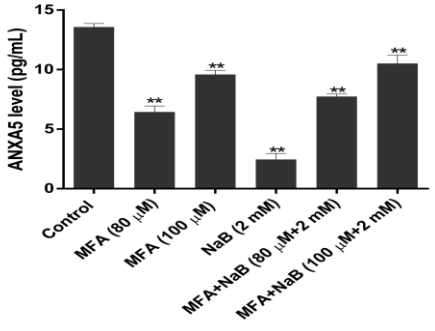
Table 2. DRI values of MFA and NaB combinations applied to MCF7 cells

		1 µM NaB		2 µM NaB		4 µM NaB	
Time (h)	**MFA(µM)**	**DRI MFA**	**DRI NaB**	**DRI MFA**	**DRI NaB**	**DRI MFA**	**DRI NaB**
24hr	80	0.6945	1.5634	0.6945	0.7817	0.3975	0.2102
	100	0.2988	0.7846	0.5082	0.7079	0.5801	0.4101
48hr	80	0.6346	0.8207	0.0207	0.1963	1.0000	0.2263
	100	1.9261	1.0940	0.0121	0.1833	1.2469	0.2490

Effect of MFA and NaB Individually and in Combination on ANXA5 Levels

The impact of the two combinations of MFA and NaB with the highest CI values (80 µM MFA+2 mM NaB and 100 µM MFA+2 mM NaB) on the free ANXA5 level in MCF7 cells, along with their application, was assessed through ELISA analysis. The results unveiled a significant elevation in the free ANXA5 level in cells that were subjected to the combination treatments compared to those treated with the drugs alone (Figure 3). Moreover, the notably enhanced levels of free ANXA5 in cells exposed to combination therapies compared to individual drug treatments, particularly in the 100 µM MFA+2 mM NaB group, highlight the potential antagonistic effect of these combinations.

Figure 3. The effects of MFA and NaB, either alone or in combination, on ANXA5 levels in MCF-7 cells. ANXA5 protein levels were assessed following treatment with 80 µM and 100 µM MFA and 2 mM NaB for 48 hours, either individually or in combination. The data presented are representative of triplicate experiments (**p<0.05).

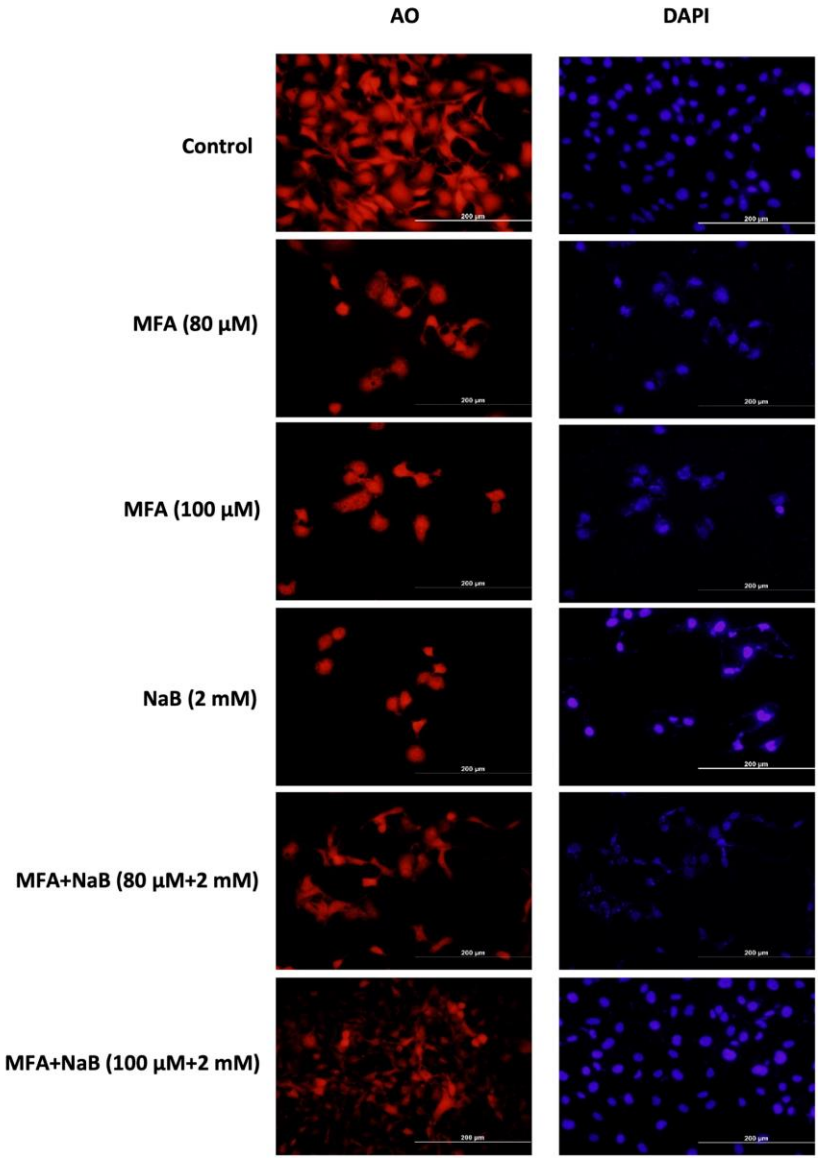


Morphological Alterations Induced by Combined Treatment

Treatment with MFA and NaB individually induced apoptotic morphological alterations such as membrane blebbing and cell shrinkage. Additionally, DAPI staining revealed

nuclear fragmentation and irregular nuclear blebbing in cells treated with MFA and NaB alone. Notably, cells treated with the combination of drugs showed a decrease in apoptotic changes compared to those treated with the drugs individually. In particular, it was noted that the number of cells displaying apoptotic morphology was significantly less in the group treated with 100 µM MFA and 2 mM NaB (Figure 4). The observed reduction in apoptotic changes in cells treated with the combination of drugs aligns with the findings indicating antagonistic effects between MFA and NaB.

Figure 4. The impact of MFA and NaB, individually and in combination, on the morphology of MCF-7 cells after 48 hours of treatment



Discussion

Contrary to researcher's initial expectations, the combination of MFA and NaB exhibited antagonistic effects on MCF7 cell viability. While individual treatments with MFA or NaB demonstrated dose-dependent cytotoxicity, the combination thereof led to a notable increase in cell viability, surpassing even that of the control group. This unexpected outcome challenges the notion of synergistic interactions between these compounds. CI analysis[15], confirmed the antagonistic nature of MFA and NaB combination, with notably high CI values observed across various concentration combinations. These suggest that the simultaneous administration of MFA and NaB may interfere with each other's cytotoxic mechanisms, leading to a compromised efficacy in inhibiting MCF7 cell proliferation.

Antagonistic drug combinations involve actions that interfere either at the same target or at different targets within related pathways that regulate the same target[18]. One mechanism for antagonism at the same target involves mutual interference occurring at the same site. Another mechanism for antagonism at different targets within related pathways, such as in this case, involves counteractive actions that impede the normal functions of the partner drug[18]. An example is the antagonistic combination of cytarabine with 17-AAG. In this combination, 17-AAG counteracts the cytotoxic effects of cytarabine by triggering G1 cell-cycle arrest, thereby it hinders the integration of cytarabine into cellular DNA[19]. Studies findings of the antagonistic interactions between MFA and NaB may parallel the mechanism observed in this study, where induction of G1 cell-cycle arrest by one agent could impede the action of the other. This suggests a potential interference with cell cycle progression as a contributing factor to the observed antagonistic relationship in this study.

The dose reduction index (DRI) further supported the antagonistic interaction, indicating unfavorable dose reductions for both MFA and NaB in combination treatments. It is noteworthy that the DRI is lower for MFA compared to NaB in both cases, indicating a more pronounced reduction in the effective dosage of MFA is achieved when combined with NaB. Compared to researcher's previous study, where they observed a synergistic effect between MFA and topotecan, they current investigation revealed low DRI values indicative of an antagonistic relationship[20]. This stark contrast underscores the dynamic nature of drug interactions and may serve as further evidence that MFA may have different effects in different combinations, highlighting the need for tailored approaches in treatment regimens.

There are combination studies in the literature investigating potential therapies for breast cancer, revealing antagonistic relationships between the tested agents. Some research indicated that certain HDACs, apart from NaB, might exhibit antagonistic interactions with other medications in breast cancer treatment. Investigations into HDAC inhibitors combined with cisplatin or cambinol[21,22]. demonstrated reduced efficacy when used together. Similarly, studies examining the combination of tamoxifen with simvastatin or celecoxib with a range of chemotherapeutic agents revealed decreased therapeutic outcomes in comparison to individual treatments[23]. Notably, interactions between HDAC inhibitors and cisplatin as well as between celecoxib and doxorubicin were also consistently antagonistic across some breast cancer cell lines[24]. Additionally, an HDAC inhibitor valproic acid caused antagonistic interactions when it was combined with a newly developed PARP1 inhibitor AZD2461[25]. These findings underscore the importance of carefully tailoring combination therapies to optimize treatment outcomes and minimize potential antagonistic interactions in breast cancer management.

Consistent with the observed antagonistic effects on cell viability, the combination of MFA and NaB resulted in a decreased apoptotic response when compared to individual treatments. Increased levels of free Annexin A5 were observed in cells treated with combinations in comparison to treatments alone. The ANXA5 ELISA enabled precise quantification of free ANXA5 levels in the cell culture supernatant, which reflects the extent of apoptotic induction. Contrary to expectations, the results revealed a significant and dose-dependent increase in free ANXA5 levels in cells subjected to combination treatments, particularly notable in the 100µM MFA+2mM NaB group. This elevation in free ANXA5 levels supported the potential antagonistic effect, where the combination of MFA and NaB may interfere with the apoptotic process induced by individual drug treatments. Wawruszak et al. observed a similar outcome in their study, demonstrating that the combination of the drugs paclitaxel (PAX) and cambinol (CAM) in triple-negative breast cancer cell lines resulted in antagonistic effects. This combination reduced the effectiveness of inhibiting cell growth and inducing apoptosis[26].

Study results contradict findings from studies exploring combinations of MFA with other drugs, where synergistic effects were often observed[20,27]. Similarly, NaB has shown synergistic interactions with various agents in previous research, indicating its potential in combination therapy[17,28]. These synergies are often attributed to complementary mechanisms of action, such as histone acetylation modulation, RNA methylation

regulation, and apoptosis induction. As a result, it highlights the potential for enhanced therapeutic efficacy in combination therapy approaches. Studies have explored the combination of MFA with a variety of agents, including traditional chemotherapeutic drugs, targeted therapies, and natural compounds. For instance, MFA has been evaluated in combination with cisplatin, gefitinib, and paclitaxel. These combinations demonstrated synergistic cytotoxic effects and enhanced apoptosis induction in cancer cells[29,30]. The combination of MFA and simvastatin inhibited the growth and invasion of human prostate cancer cells through the AKR1C3 mechanism[27]. Furthermore, MFA mitigates the accumulation of ROS, inhibits excessive autophagy, and protects hair cell-like HEI-OC1 cells from the damage caused by cisplatin[31].

Similarly, NaB has been investigated in combination with various agents, including other HDAC inhibitors, chemotherapeutic drugs, and dietary compounds. Preclinical studies have shown synergistic anticancer effects when NaB is combined with HDAC inhibitors such as vorinostat (SAHA)[32] or chemotherapeutic drugs like cisplatin[33]. These combinations resulted in enhanced histone acetylation and apoptosis induction in cancer cells. Additionally, NaB has been evaluated in combination with dietary compounds such as resveratrol[34] and quercetin[17], which demonstrated synergistic effects on cell proliferation inhibition and apoptosis induction in breast cancer cells. Studies combining epigenetic modifiers, such as histone deacetylase (HDAC) inhibitors like NaB, with other epigenetic or epitranscriptomic regulators have shown promising results in preclinical models of cancer.

The contrasting results between this study and previous research highlight the importance of considering the specific mechanisms of action and molecular interactions of epigenetic and epitranscriptomic regulators in combination therapy design. While MFA and NaB individually modulate gene expression and RNA modifications, their simultaneous administration may cause interference in these regulatory pathways, resulting in antagonistic effects on cell viability and apoptotic pathways.

Moving forward, it will be crucial to conduct further mechanistic studies to elucidate the underlying molecular mechanisms of the antagonistic interactions between MFA and NaB., Exploring alternative combinations of epigenetic and epitranscriptomic regulators with different agents may offer new perspectives to optimize combination therapy regimens in breast cancer treatment.

Conclusion

In conclusion, this study provided valuable insights into the intricate interactions between MFA and NaB and their impact on MCF7 breast cancer cells. While the combination of these compounds exhibited antagonistic effects on cell viability and apoptotic induction, further researches are needed to be done to investigate the underlying mechanisms and optimize therapeutic strategies for breast cancer management.

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