International Journal of Drug Research in Clinics

Int J Drug Res Clin, 2024, 2, e27
doi 10.34172/ijdrc.2024.e27
http://ijdrug.com

Original Article

The Inhibition of CD73 in Conjunction With the Blockade of Apoptosis Inhibitors Significantly Impedes the Advancement of Cancer via Pro-Apoptotic Mechanisms

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Article History:
Received: September 14, 2024
Accepted: September 23, 2024
ePublished: December 18, 2024

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Abstract

Background: Effective cancer treatments are among the most challenging research goals in the field. Cancer cells have diverse characteristics, including the ability to suppress antitumor immune responses and resistance to apoptosis. The upregulation of CD73 in cancer cells has been suggested in recent studies, promoting proliferation, angiogenesis, and metastasis and suppressing immune functions. On the other hand, BV6 can induce apoptosis in cancer cells by suppressing apoptosis inhibitors. Therefore, this study aimed to explore the cancer treatment potential of BV6 and anti-CD73 agents.

Methods: This study was conducted on cancer cell lines, including CT26 (colon cancer) and 4T1 (breast cancer). Cancer cells were treated with anti-CD73 small interfering ribonucleic acid (siRNA) molecules and BV6 drugs. The effect of treatment on cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and apoptosis test. In addition, the effect of treatment on the expression of target apoptosis-related genes was studied with a real-time polymerase chain reaction assay.

Results: It was revealed that delivery of anti-CD73 siRNA molecules along with BV6 to cancer cells significantly induced cell death. Although the impact of anti-CD73 siRNA monotherapy was non-significant, the combined treatment significantly decreased the expression of genes involved in cell survival while increasing the expression of apoptosis-promoting genes.

Conclusion: The findings of this study suggest the combined treatment of cancer cells using anti-CD73 siRNA molecules and BV6 as an effective anticancer intervention in vitro. Further studies should be conducted to determine its effectiveness and safety.

Keywords: BV6, CD73, Cancer immunotherapy, Combination therapy, Small interfering RNA

Please cite this article as follows: Farajzadeh M, Hosseini MS, Mozaffari M, Eshaghi FS, Alian F, Hajebrahimi S, et al. The inhibition of CD73 in conjunction with the blockade of apoptosis inhibitors significantly impedes the advancement of cancer via pro-apoptotic mechanisms. Int J Drug Res Clin. 2024; 2: e27. doi: 10.34172/ijdrc.2024.e27

Introduction

Cancer is a worldwide threat, accounting for 7.6 million lost lives annually, which is predicted to rise to over 13 million by 2030. With industrialization, lifestyle changes, and the consequent increase in environmental pollutants, the cancer rate is rising.[1] Chemotherapy, radiotherapy, surgery –or a combination of these treatments– are the most common treatments against cancers. However, these treatment methods have not had acceptable results in many patients and have limitations, such as high costs for the patient or the healthcare system, life-limiting side effects, and inefficiency in treating more advanced cancer types.[2,3] The ineffectiveness of these treatment methods has led researchers to look for novel and more effective treatment methods. Hence, numerous approaches in immunotherapy have been developed to combat cancer.

The Smac mimetic BV6 has been employed as a selective inhibitor of apoptosis protein (IAP) targeting agent. This mimetic compound imitates the natural Smac protein and effectively induces the degradation of IAPs.[4,5] Cancer cells escape apoptosis by increasing cIAP1 levels and rely on X-linked inhibitor of apoptosis protein (XIAP) expression

for their survival.[6,7] Therefore, the antagonism of either cIAP1 or XIAP could potentially lead to heightened responsiveness toward apoptotic triggers. In addition to their direct interaction with and inhibition of caspases, IAPs play a crucial role in numerous cellular processes, such as the transmission of nuclear factor Kappa B signals. Specifically, cIAP1/2 is linked to the tumor necrosis factor receptor 1 (TNFR1) complex and governs the intricate balance between the canonical and noncanonical nuclear factor kappa B signaling pathways.[8,9] Earlier investigations conducted in our laboratory have demonstrated that administering substances that sensitize cell death, such as BH3 or Smac mimetics, increases immune cell susceptibility.[10-12]

CD73 is a transmembrane glycoprotein of type I that is extensively present on the surfaces of the immune system and soft tissue cells.[13,14] CD73, functionally speaking, acts as a rate-limiting ecto-5'-nucleotidase (NT5E). This enzyme, responsible for hydrolyzing AMP, plays a crucial role in an ectoenzymatic system that governs the transformation of extracellular adenosine triphosphate into adenosine.[15] The significance of CD73 in regulating tumorigenesis, angiogenesis, and metastasis is becoming more recognized, particularly in its contribution to breast cancer (BC) progression, notably through promoting tumor immune evasion.[16,17] Consequently, targeting CD73 functions pharmacologically is observed as a highly promising strategy for treating BC.[18-20] CD73 is abundantly expressed in various types of cancer as well as by the infiltrating immune cells.[21] CD73 demonstrates notable elevation on the plasma membrane of macrophage cells, along with immunosuppressive cells such as myeloid-derived suppressor and T_{reg} cells. Extensive investigation has established a clear association between the increased manifestation of CD73 and the proliferation of cancerous cells, the advancement of metastasis, and the development of fresh blood vessels (angiogenesis).[22] In addition, tumor cells with high expression of CD73 are resistant to chemotherapy and immunotherapy. Targeted inhibition of CD73 in cancer cells and its use along with BV6 can be a therapeutic approach with a rational strategy.[23] This study evaluated the in vitro effectiveness and synergistic potential of the Smac mimetic BV6 and CD73 inhibition on cancer progression and apoptosis.

Methods

Reagents and Cell Lines

Murine colorectal carcinoma (CT26) and mammary carcinoma (4T1) cell lines were purchased from the Pasteur Institute of Iran (Tehran, Iran). Both cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco-Invitrogen, USA), 2% L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained at 37 ˚C in the incubator with 5% CO_2 and 95% humidity. Lipofectamine 2000 transfection reagent and 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit were purchased from Sigma-Aldrich (MO, USA). Anti-CD73 and control small interfering ribonucleic acid (siRNAs) were obtained from Santa Cruz (CA, USA).

Cytotoxicity Assay

The MTT assay investigated how BV6 and anti-CD73 siRNA transfection could affect the cells' viability. Seeded for 24 hours in 96-well plates, the cells were transfected with siRNA molecules (60 pm) and BV6 (27 μM for 4T1 and 31 μM for CT26). The untreated cells were the negative control, while dimethyl sulfoxide (0.2%) was the positive control. Following a 24- or 48-hour incubation period, the cell supernatant was replaced with 100 μL of MTT-containing medium and incubated for four additional hours. Lastly, each well received 100 μL of DMSO for four hours. The supernatant was removed after four hours, and 150 μL of DMSO was added. This mixture was then incubated for 30 minutes. The absorbance was measured at 570 nm and 630 nm for the sample test and reference wavelength, respectively.

Real-time Polymerase Chain Reaction

RNA extraction and cDNA synthesis were performed using RNA extraction and cDNA synthesis kits (BioFACT, Korea). Target gene expression was then measured and amplified using the LightCycler 480 real-time polymerase chain reaction (RT-PCR) system (Roche) and the SYBR Green RT-PCR master mixture (BioFACT). Standard and melting curves were drawn to verify the test's accuracy. The thermocycling condition of RT-PCR included a one-minute initial denaturation at 95 ˚C, followed by 40 cycles of amplification (including denaturation at 95 ˚C for 15 seconds, annealing at 58 ˚C for 30 seconds, and elongation at 72 ˚C for 35 seconds). Standard and melting curves were used to verify the test's accuracy. The data were analyzed using the ΔΔCT method with β-actin as the housekeeping gene.

Apoptosis Assay

The cell Death Detection ELISA kit (Sigma, USA) was used to evaluate the impact of anti-CD73 siRNA and BV6 combination therapy on the apoptosis of cancer cells. In brief, cancer cells (3×10^4) were seeded in 48-well plates and cultured for 24 hours. Subsequently, cells were treated with various therapeutic groups for 48 hours. The cells were then detached from the plate and washed twice (at 1200 rpm for 10 minutes). After one hour of exposure to lysis buffer, the cell pellet was centrifuged once more at 1200 rpm for ten minutes. The cell lysate was utilized for the apoptosis assay using the ELISA kit. The enrichment of mono- and oligo-nucleosomes in the cytoplasm of the apoptotic cells was determined based on the absorbance at 405 nm.

Statistical Analysis

The data were statistically analyzed using GraphPad Prism (version 6) software. The results were reported as

means ± standard deviations (SD), and a *P*-value of less than 0.05 was considered statistically significant.

Results

Silencing CD73 Enhances the Sensitivity of Cancer Cells to BV6-Mediated Cytotoxicity

The results of the MTT assay showed that –although statistically non-significant– silencing CD73 could decrease cancer cell viability to some extent. Meanwhile, BV6 significantly induced cell death in both cell lines. The combined treatment exhibited the greatest cytotoxicity among the therapeutic groups (Figure 1).

CD73 Molecule Expression

The efficacy of siRNA transfection in diminishing CD73 expression was assessed through RT-PCR analysis. As illustrated in Figure 2, the anti-CD73 siRNA notably decreased CD73 expression in both cell lines.

Apoptosis and Involved Genes

An ELISA-based apoptosis assay was conducted, and the expression of anti-apoptotic BCL-2 and pro-apoptotic BIM genes was recorded. The results demonstrated that although silencing CD73 could not induce significant apoptosis in cancer cells, the treatment of cells with BV6 potently enhanced apoptosis in both cell lines. However, the highest level of apoptosis was recorded through combined treatment (Figure 3a). Moreover, the results showed that treatment with anti-CD73 siRNA and BV6 decreased the Bcl-2 mRNA level, making cells

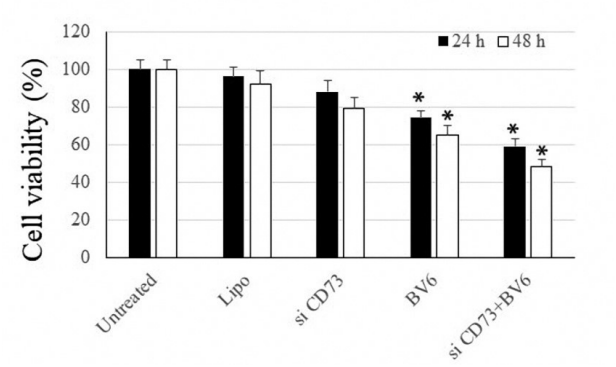

Figure 1. In Vitro, Cytotoxicity Assessed by MTT Assay for 24 Hours. *Note*. An asterisk* denotes a *P*-value less than 0.05. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

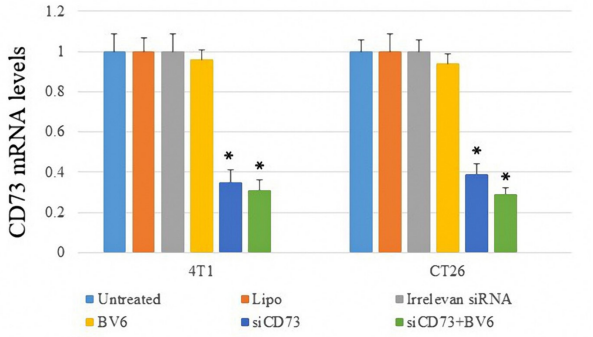

Figure 2. Real-time Evaluation of CD73 Expression in Both Cell Lines. *Note*. The asterisk* denotes a *P*-value less than 0.05

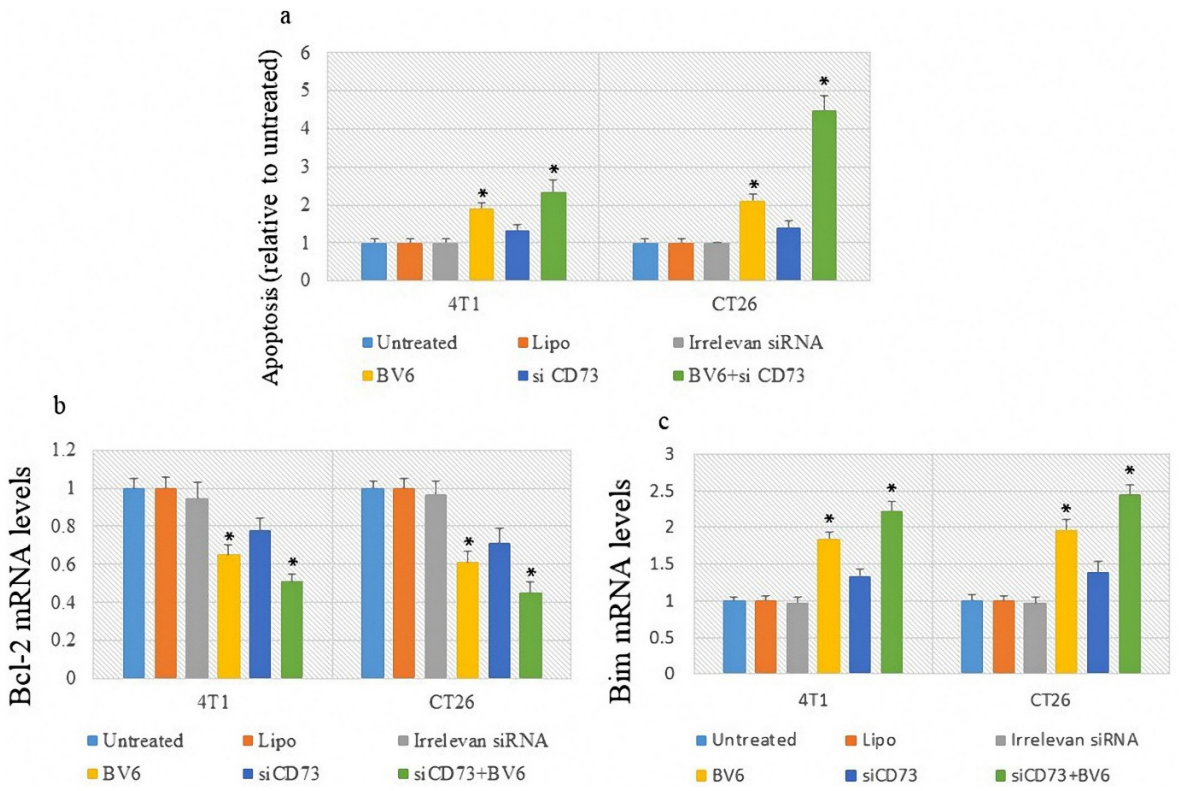

Figure 3. The Effect of Treatment on Apoptosis and the Expression Level of Apoptosis-Related Genes. *Note*. ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction. ELISA-based assay was used to evaluate apoptosis (a). Bcl-2 and Bim mRNA expression levels were evaluated using a real-time PCR assay (b and c). Asterisk* represents a *P* value less than 0.05

more sensitive to apoptosis (Figure 3b). The combined treatment significantly increased *BIM* gene expression (Figure 3c).

Discussion

Cancer has emerged as a significant challenge within the healthcare systems of numerous countries worldwide, posing a formidable threat to public health as the second leading cause of mortality.[24] BV6, a bivalent Smac mimetic, has recently been observed to elicit a conformational alteration in the structure of cIAP1. This alteration facilitates the E3 ubiquitin ligase activity within its RING domain, consequently leading to autoubiquitination and subsequent proteasomal degradation.[25,26] BV6 specifically targets the inhibitors of apoptosis (IAP), namely, cIAP1, cIAP2, and XIAP, triggering apoptosis in tumor cells.[5] BV6, acting alone, triggers apoptosis, as evident by the presence of cleaved caspase-3. Evidence suggests that Smac mimetics can also promote necroptosis in certain contexts, offering an alternative mechanism of cell death.[27] This study examined the impact of anti-CD73 siRNA-containing nanoparticles in combination with BV6 on cancer cells. Our findings revealed that the utilization of siRNA-loaded nanoparticles results in a reduction of CD73 gene expression, as well as the expression of genes associated with anti-apoptotic proteins, cell proliferation, angiogenesis, and metastasis.

Fischer et al first demonstrated the impact of BV6 on interleukin (IL)-2-activated expanded natural killer (NK) cells in sensitizing the attack on rhabdomyosarcoma cells. Additionally, they confirmed the transcriptional up-regulation of TNF-related apoptosis-inducing ligand (TRAIL) receptors.[11] Recent studies have suggested that BV6 induces cancer cells' apoptosis by degrading IAP and sensitizing the cells by producing death ligands.[28] A study by Anna et al represented that Smac mimetics and proteasome inhibitors are promising therapeutic strategies for primary diffuse large B cell lymphoma, effectively triggering cell death through mitochondrial pathways and enhancing the sensitivity to treatment.[29] In another recent study, Smac mimetics have shown the potential to induce apoptosis through noncanonical cell death pathways at elevated concentrations.[30] Some investigators sought to inhibit signaling pathways that promote cancer growth, such as both IL-6 and its receptor (glycoprotein 130), and to synergistically reduce cancer progression in vitro.[31]

The targeting of IAPs via BV6 could potentially serve as an effective strategy to inhibit cancer progression. The findings of a study indicated that BV6 triggers apoptosis in various human cancer cell types, implying that BV6 causes a reduction in cIAP1 and cIAP2 in a dose-dependent manner.[28] The radiosensitizing properties of BV6 align with previous findings that the inhibition of IAPs enhances the radiosensitivity of specific types of cancer, such as lung cancer.[32-34] Additionally, Checinska et al concluded that the application of a Smac mimetics results in a notable enhancement in cisplatin-induced caspase-3 activity and apoptosis in cancer cells in vitro.[35]

CD73 has complex and context-dependent antitumor activity. Upregulated CD73 contributes to immune evasion by generating high levels of adenosine, suppressing T cell activity, and promoting tumor growth.[36] Consequently, targeting CD73 with specific inhibitors is being explored as a therapeutic strategy to enhance antitumor immunity by counteracting the suppressive effects of adenosine on the immune system.[37,38] The coordinated activity of CD73 in conjunction with CD39—another ectonucleotidase—plays a crucial role in regulating the balance between extracellular adenosine triphosphate and adenosine, thereby maintaining overall immune homeostasis.[38] Numerous studies have now proved the significant involvement of CD73 in several aspects of immunity and inflammation, including both antitumor immune responses and the evasion of immune surveillance by tumors. By blocking CD73 through antagonistic chemicals or monoclonal antibodies, tumor immunosurveillance could be potentially restored, delaying tumor progress and metastasis through T-cell- and NK-cell-dependent mechanisms, respectively.[39] In a study conducted by Gao et al, it was demonstrated that CD73 plays a role in facilitating the growth and movement of cervical cancer cells in humans, regardless of its enzymatic function. In line with the results of our study, their findings indicated that CD73 could potentially enhance the EGFR/Akt and VEGF/Akt pathways, thereby promoting proliferation and migration, independently of its enzymatic activity. These results offer novel perspectives on the regulatory role of CD73 in cancer cells and propose CD73 as a potential target for therapeutic interventions in cervical cancer.[40]

Yang et al found that the activity of CD73 can be suppressed by tiamulin, leading to the inhibition of BC growth and lung metastasis.[41] In another study and a mouse rectal cancer model, Tsukui et al reported that the inhibition of CD73 amplifies the localized and systemic impacts of radiotherapy on coral.[42] Our research findings support the notion that suppressing the expression of the *CD73* gene is crucial in regulating the spread and growth of rectal cancer cells. Yu et al concluded that CD73 enhances the proliferation of human BC cells via the AKT/GSK-3B/ß-catenin/cyclinD1 signaling cascade,[43] which conforms to our research findings on the significance of suppressing *CD73* gene expression in regulating BC progression. Zhou et al observed that the upregulation of CD73 enhances the ability of T-47D human BC cells to invade and adhere to the extracellular matrix (ECM). The findings propose that controlled adenosine production, along with changes in EGFR and IL-8 expression resulting from CD73 overexpression, could contribute to the promotion of BC metastasis induced by CD73.[44]

In another recent study, Jin et al explored a new CD73-targeting antibody-drug conjugate for suppressing lung cancer and enhancing the antitumor immune responses.[45] Likewise, Turcotte et al demonstrated that the presence of CD73 is linked to an unfavorable prognosis in high-

grade serous ovarian cancer.[46] Miyazaki et al have presented evidence suggesting that the presence of tumor ECM metalloproteinase inducer and heightened stromal CD73 levels are associated with a poor prognosis in cases of external auditory canal carcinoma.[47] All these results corroborate our study findings, highlighting the significance of CD73 in tumor immune responses.

Conclusion

Data obtained from all the stages of our study confirm the tumor inhibitory effects of the combined targeting of CD73 and treatment of BV6. In this study, the researchers first targeted this synergistic cycle and used this novel therapeutic combination. The development of a powerful nanocarrier system with conjugated trimethyl chitosan-alginate for this compound is another strength of this study, which increases the effectiveness of the treatment. However, future in vivo studies are required to prove the effectiveness of this therapeutic approach.

Ethics statement

This study was ethically approved by Tabriz University of Medical Sciences (Ethical number: IR.TBZMED.VCR.REC.1399.453).

Disclosure of funding source

This study is financial y purported by the grant number 66443 from the Tabriz University of Medical Sciences.

Conflict of interests declaration

The researchers have no conflict of interests to declare.

Acknowledgments

We would like to thank Tabriz University of Medical Sciences for the financial support of this study (grant number: 66443).

Data availability statement

The data related to the study are available upon request from the corresponding author upon reasonable request.

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Writing–review & editing: Maryam Farajzadeh, Mohammad-Salar Hosseini, Farhad Jadidi-Niaragh.

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