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Article

Mechanism and cytotoxicity of A1874 proteolysis targeting chimera on CT26 colon carcinoma cell line

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SUMMARY

Proteolysis targeting chimeras (PROTACs) are low molecular weight compounds that enhance the degradation of specific proteins by leveraging the cell's natural ubiquitin-proteasome system. This technology offers a promising approach to overcoming the limitations of traditional drug therapies by targeting and degrading disease-causing proteins. Recent studies have demonstrated that a PROTAC called A1874 can effectively degrade bromodomain-containing protein 4 (BRD4) in various colon cancer cell lines, including HCT116. However, the effects of A1874 on CT26 colon carcinoma cell lines have not been previously investigated. This research aimed to evaluate the efficacy of A1874 on CT26 cells and to better understand the PROTAC mechanisms. We hypothesized that A1874 can degrade BRD4 in CT26 cells, thereby reducing CT26 cell viability. Our results demonstrated that treatment with 20 μM A1874 led to BRD4 degradation accompanied by 52% reduction in cell viability. However, we did not assess off-target effects of A1874, which is a limitation of our analysis and indicates that more research is needed. From a clinical perspective, this research provides valuable insights into the therapeutic potential and safety profile of A1874 for preclinical and clinical development for cancer treatments.

INTRODUCTION

Colon cancer ranks as the third most common form of cancer worldwide, representing approximately 10% of all cancer diagnoses (1). In 2020, it was estimated that there were 1.9 million new cases and 930,000 deaths related to colon cancer worldwide (1). Despite advancements in cancer treatments, which have significantly reduced risks, certain oncoproteins like bromodomain-containing protein 4 (BRD4) present challenges to traditional cancer drugs (2).

Many cancer targets are regarded as “undruggable” (3). Researchers categorize certain proteins as “undruggable” when traditional drugs struggle to interact with them. This difficulty arises because these proteins either do not have the structural features, like pockets, that allow drugs to bind effectively, or they do not perform enzyme-like actions that can be targeted by typical pharmaceuticals (2). Only about 4,500 of over 20,000 proteins encoded by the human genome are currently considered druggable (4, 5). Many cancer-specific proteins that could serve as drug targets are transcription factors that play a vital role in controlling cell growth and differentiation (6). However, inhibiting transcription factors upregulated in cancer has been difficult due to their expansive protein-to-protein interaction (PPI) interfaces or absence of deep protein pockets (7). New biotechnologies, like proteolysis targeting chimeras (PROTACs), are being developed as innovative methods to overcome these obstacles. PROTACs perform targeted protein degradation (TPD) and have shown great promise in treating various undruggable targets in cancer and significantly contributing to cancer treatments (8).

TPD is essential for cellular regulation and functions as a catalytic mechanism in which a single molecule facilitates the breakdown of multiple copies of the protein of interest (POI) (8). Traditional anticancer therapeutics, such as small-molecule-mediated therapies, have substantially inhibited oncoproteins (e.g., BRD4) (9). However, TPD offers advantages over traditional treatment by “off-switching” oncoproteins, even those previously deemed “undruggable” (9). PROTACs utilize a linker to connect a ligand for the POI with a ligand for an E3 ubiquitin ligase, creating a heterobifunctional molecule **(Figure 1A)** (10). When the PROTAC molecule undergoes molecular interaction between E3 ligase and the POI, forming POI-PROTAC-E3 ubiquitin ligase ternary complex formation, it induces polyubiquitination of the POI. Eventually, covalent binding between ubiquitin, a proteolytic marker peptide, and the POI leads to its modulation or degradation by the proteasome **(Figure 1B, C)** (11,12). Unlike traditional small-molecule inhibitors that require continuous occupancy of a target protein’s binding pocket, PROTACs can bind to target proteins without the presence of “druggable deep grooves” and do not need high affinity binding to result in degradation (13). Because of these unique properties, understanding the structural characteristics and mechanisms of PROTACs is crucial, as they can regulate previously undruggable protein targets (13).

A1874 is a novel PROTAC that is designed to specifically degrade BRD4 **(Figure 2)** (14). Due to its high expression in tumor cells and its regulation of cancer-related BRD-dependent genes, BRD4 plays a vital role in cancer development by controlling the cell cycle (15). Research suggests that blocking BRD4 can trigger cancer cell death by activating various programmed cell death mechanisms, such as apoptosis and autophagy (16). This PROTAC simultaneously binds Murine Double Minute 2 (MDM2), a negative regulator of the p53 tumor suppressor that functions as an E3 ubiquitin ligase. A1874 binding to MDM2 and BRD4 simultaneously promotes the degradation of BRD4 through the ubiquitin-proteasome pathway, leading to effective and long-lasting degradation (17). A1874 has been shown to induce cell death

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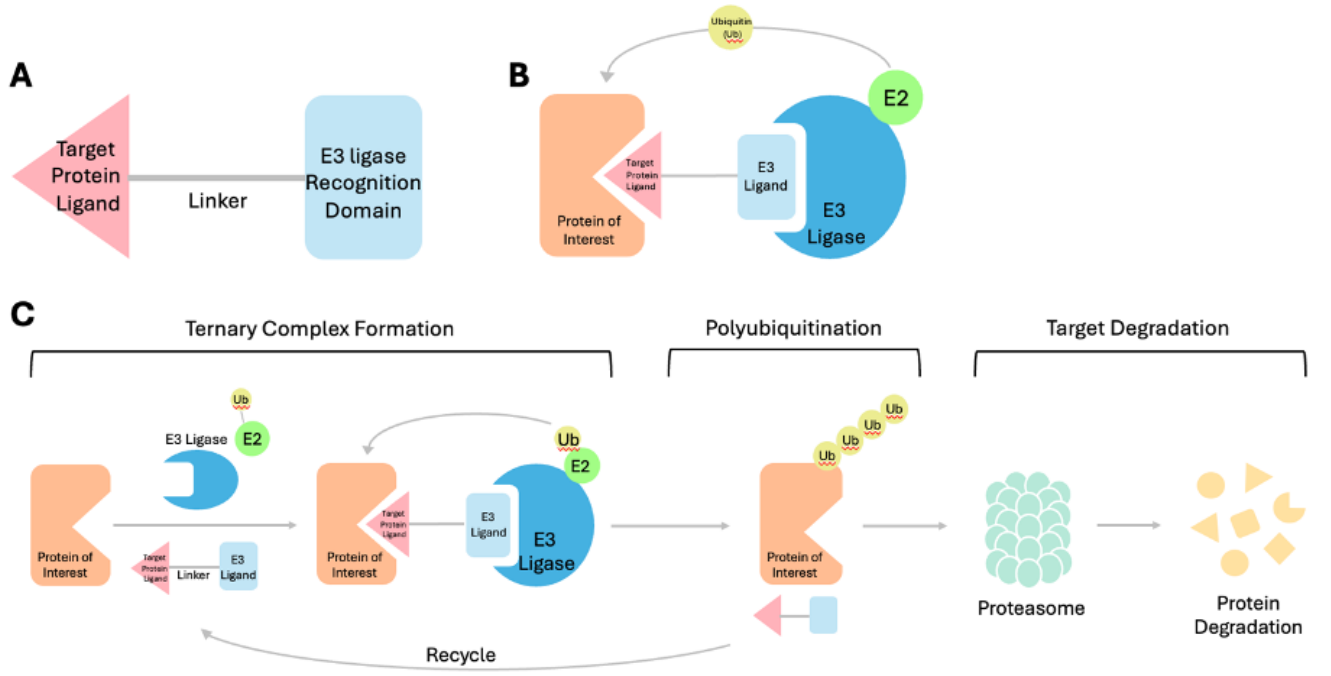

Figure 1: Mechanism of proteolysis targeting chimera (PROTAC). A) Schematic image of a basic structure of a PROTAC. B) Schematic image of a ternary complex. C) Schematic image showing the mechanism of actions for a typical PROTAC.

in colon cancer cells, potentially by inhibiting myelocytoma (MYC), a downstream protein of BRD4, and stabilizing p53 (18). Its efficacy in degrading BRD4 in immortalized HCT116 cell line has been established, which successfully inhibited cancer cell viability, proliferation, and cell cycle progression (19). However, there are no known studies on the efficacy of A1874 on the CT26 cell line in the context of degrading BRD4. In this study, we investigated the effect of A1874 on CT26 colon cancer cells. We hypothesize that A1874-induced BRD4 degradation would lead to cell death in CT26 cells. Given the lack of extensive information on A1874 treatment in CT26 cells, it is crucial to study the cytotoxicity and BRD4 concentration upon treatment. While the human colon cancer cell line HCT116 has been studied, CT26 cells are a murine colon carcinoma model derived from BALB/c mice, which are commonly used as an intermediary between cell culture studies and clinical trials of humans due to genetic similarities (14, 19). Using this model allows for the identification of interspecies differences in how A1874 affects cancer cells and provides insights into its preclinical efficacy (19). Therefore, the purpose of this research is to expand our knowledge of A1874's efficacy across species and investigate cytotoxicity with A1874 treatment. This research adds to a growing body of work that links the targeted degradation of BRD4 using the A1864 PROTAC to result in selective inhibition of colon carcinoma cells. Specifically, we found that treatment with 20 μM A1874 significantly decreased CT26 cell viability. This treatment concentration also robustly degraded the target protein BRD4 in these cells. These findings suggest A1874 is effective at degrading its target and reducing cell viability in this murine colon carcinoma model.

RESULTS

In this study, we tested whether A1874-induced BRD4 degradation would lead to cell death in CT26 colon carcinoma cell lines. We cultured CT26 mouse colon carcinoma cells and treated them with different concentrations of the PROTAC A1874. We then measured cell viability using a CCK-8 assay to see how the treatment affected cell survival. Finally, we performed Western blotting to quantify the levels of BRD4 protein in the cells after treatment to determine if A1874 caused its degradation.

Higher concentration of A1874 decreases CT26 cell viability

To determine the effect of A1874 on CT26 cell viability, we treated CT26 cells with two different concentrations of A1874 for 24 hours. Using the CCK-8 cell viability assay, when we treated CT26 cells with 5 μM of A1874, more than 93 % of CT26 cells remained viable, showing that ≤ 5 μM A1874 treatment is not lethal to CT26 cells **(Figure 3)**. On the other hand, 20 μM of A1874 treatment significantly decreased the viability of CT26 cells to 52% ($P<0.0001$, one-way ANOVA), suggesting that the higher the concentration of A1874 treatment, the more CT26 cells undergo cell death.

Treatment of A1874 induces BRD4 degradation in CT26 cells

To examine the ability of A1874 to degrade BRD4, we treated CT26 cells with the same concentrations of A1874 used to quantify CT26 cell viability. We performed an immunoblot and found that when 5 μM of A1874 is treated,

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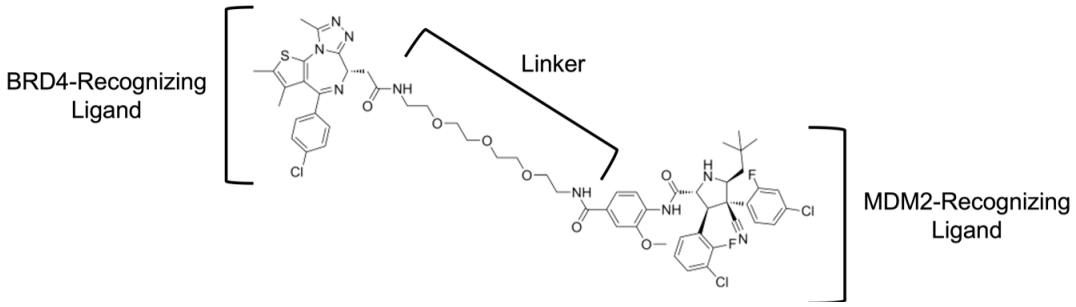

Figure 2: Structure of A1874 PROTAC. An image showing the chemical structure of A1874.

relative BRD4 expression decreases **(Figure 4)**. Higher concentration of A1874 (20 μM) treatment robustly eliminated BRD4 from CT26 cells, confirming the efficacy of A1874 in degrading the POI.

DISCUSSION

Many studies have demonstrated that TPD is highly effective in modulating disease-causing proteins that have been difficult to target with small-molecule inhibitors (20). In this study, we focused on a PROTAC targeting BRD4, a key regulator of cancer cell proliferation and programmed cell death (16). Given that BRD4 is dysregulated in numerous cancers (15), we selected A1874 to investigate the impact of A1874 on BRD4 levels and cell viability in CT26 colon carcinoma cells. We hypothesized that A1874 targeting BRD4 in CT26 cells would decrease BRD4 protein levels and inhibit colon cancer cell proliferation. When we treated CT26 cells with 20 μM of A1874, we found that A1874 nearly eliminated BRD4 in CT26 cells. The observed decrease in BRD4 protein levels suggests that A1874 binds effectively to the BRD4 protein and E3 ubiquitin ligase, targeting BRD4 for polyubiquitination and subsequent proteasome-mediated degradation (21).

A1874, functioning as a targeted protein degrader, induces BRD4 degradation in CT26 cells. This degradation disrupts transcriptional programs critical for survival, including the downregulation of BRD4-dependent anti-apoptotic genes such as Bcl-2 and Myc (15). Loss of Bcl-2, a key regulator of mitochondrial integrity, likely destabilizes the mitochondrial membrane potential, triggering cytochrome c release into the cytosol (14). This event activates the intrinsic apoptosis pathway, as evidenced by increased caspase-9 and caspase-3 activity in A1874-treated cells (14). BRD4 is known to be involved in cell death pathways, and it is possible that BRD4 inhibition by A1874 may influence the activation of caspase-3 and caspase-9. Thus, BRD4 degradation by A1874 may mechanistically link transcriptional suppression of pro-survival genes to mitochondrial dysfunction and subsequent caspase-mediated apoptosis.

Our results showed an increase in cell death as BRD4 degradation increased in CT26 cells treated with A1874. This effect coincides with reduction in BRD4 protein levels, as observed in our experimental assays. While 5 μM A1874 induced notable BRD4 degradation, cell viability remained largely unaffected. This suggests that partial BRD4 degradation alone is insufficient to compromise CT26 cell survival. However, treatment with 20 μM A1874 led to a higher level of BRD4 degradation and exhibited a significantly decrease in CT26 cell viability. This stark contrast indicates that complete BRD4 ablation may be critical for disrupting essential cellular processes in CT26 cells, ultimately leading to cell death. This observation aligns with previous studies, highlighting the crucial role of BRD4 in regulating oncogenic pathways and maintaining cancer cell viability (15). BRD4, a member of the BET family of proteins, is known to play a vital role in transcriptional regulation, particularly in controlling the expression of genes involved in cell growth, proliferation, and survival. While the data suggest a potential association between BRD4 degradation and cell death, we need further mechanistic studies to establish a causal relationship (22). Notably, the absence of BRD4 bands at higher A1874 doses in Figure 4 may reflect technical limitations or off-target effects, underscoring the need for additional validation. Future studies could explore the specific signaling pathways affected by BRD4 degradation, as well as potential synergistic effects with other therapeutic agents.

Our study provides preliminary evidence that A1874 reduces BRD4 levels and decreases cell viability in CT26 colon carcinoma cells. This observation could have implications for future research investigating the *in vivo* effects of various BRD4-targeting PROTACs. From this, the results can then be used to further guide other researchers in developing effective BRD4-targeting PROTACs. Given that BRD4 serves

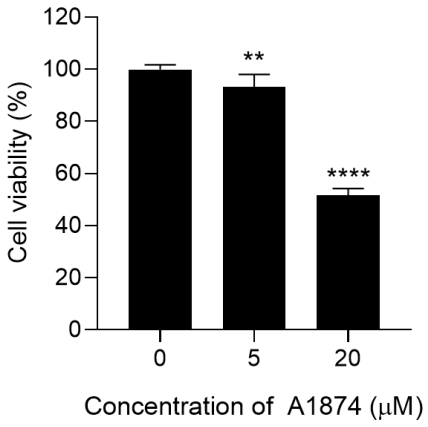

Figure 3: A1874 PROTAC decreased cell viability of CT26 colon carcinoma cell lines. Bar graph showing the percentage of viable CT26 cells after treatment of A1874 for 24 hours (n=6/group). CT26 colon carcinoma cell lines were treated with 2 different A1874 concentrations, 5 μM and 20 μM. Statistical significance levels are denoted as: **p < 0.01 and ****p < 0.0001, one-way ANOVA.

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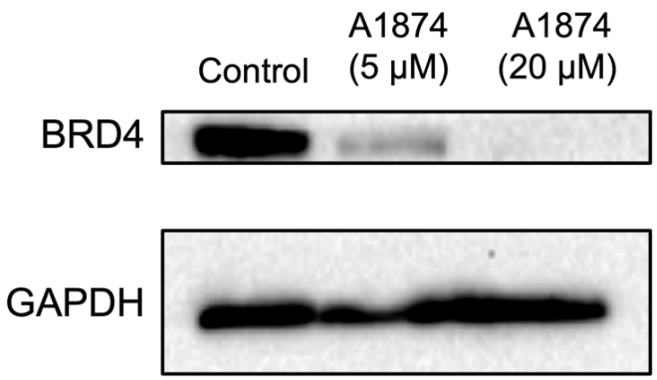

Figure 4: A1874 PROTAC decreased BRD4 protein levels in CT26 colon carcinoma cell lines. Immunoblot showing BRD4 protein levels in CT 26 cells after A1874 treatment for 24 hours. The concentration of BRD4 decreased as the treatment concentration of A1874 increased. GAPDH was used as loading control.

as a super-enhancer in numerous cancers, including breast cancer, prostate cancer, and acute myeloid leukemia, the results pave the way for further scientific exploration in this area (23).

Our findings demonstrated that A1874 reduces CT26 cell viability and decreases BRD4 levels at the tested concentration. However, the direct mechanistic link between BRD4 degradation and cell death remains to be confirmed. To investigate this gap in knowledge, future studies should employ BRD4 knockdown or knockout models to assess whether BRD4 loss alone impairs CT26 viability and whether A1874's effects persist in the absence of BRD4. Rescue experiments, such as restoring BRD4 expression in knockdown cells, could further clarify its role (24). These experiments will distinguish between on-target BRD4 and potential off-target effects of A1874, particularly at higher doses.

Beyond the promising potential of cancer therapy using PROTACs in targeting CT26 cells, the treatment with A1874 faces several challenges (25). A key issue with PROTACs is that the broad expression of E3 ligases can lead to unintended interactions, where PROTACs affect proteins other than their targets, which may cause harmful side effects such as nausea, fatigue, diarrhea, and alopecia (26, 27). Unlike traditional small-molecule inhibition, which does not affect the expression of cytoskeletal proteins and thus maintains the basic physiological activity of normal cells, tissues, and organs, the complete removal of proteins by PROTAC might impact overall health and physical function, including skin health and spinal posture (26, 28). Previous research indicates that E3 ligases are widely expressed at both cancer sites and normal tissues. More specifically, both normal and cancer cells of mammals contain E3 ubiquitin ligases such as MDM2, leading to potential side effects from their non-specific degradation of BRD4 in target cells (29). *In vivo* studies will be essential to thoroughly assess the toxicity and safety profile of A1874, ensuring its suitability for clinical applications. Further research should focus on optimizing the specificity of PROTACs, minimizing off-target effects, and extending these findings to other cancer types. If these challenges can be addressed, PROTACs hold great potential for developing novel, effective cancer therapies targeting previously undruggable oncoproteins.

MATERIALS AND METHODS

Cell culture and maintenance

CT26 mouse colon carcinoma cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (ThermoFisher) and 1% penicillin and streptomycin (ThermoFisher). The cells were maintained in a 5% CO_2 incubator (Eppendorf) at 37°C. Cells were passaged every 2-3 days when they reached 80% confluency. The old medium was removed, and the cells were detached from the culture plate using trypsin-EDTA solution (0.25%, Gibco). Then, 20% of the cells were seeded at a density of 1 x 10^5 cells/mL into a new cell culture plate with fresh medium.

Cell Viability Assay

CT26 colon carcinoma cells were seeded and cultured overnight into 96-well plates (3 x 10^4 cells per well), with 6 technical replicates per treatment group in one experiment (n=6). A1874 was dissolved in DMSO and subsequently diluted in SF media to a final DMSO concentration of 0.1%. The vehicle control was treated with 0.1% DMSO in SF media. After 24 hours of treatment, cell viability was assessed using a CCK-8 kit (Dojindo). Carefully, 10 μL of the CCK-8 solution was added to each well and the plate was incubated for 1 hour at 37°C for the reduction reaction to proceed between mitochondrial dehydrogenase from CT26 cells and CCK-8 solution, forming formazan (14). The absorbance of the media was measured at the wavelength of 450 nm using a microplate reader (Synergy H1, BioTek).

Total Protein Extraction from Cells

CT26 cells were seeded in 6-well plates at a density of 6 × 10^5 cells per well and incubated overnight. The following day, cells were treated with A1874 in serum-free medium and incubated for 24 hours before collection. After removing the old serum-free RPMI-1640 from a 6-well plate, phosphate-buffered saline (PBS) was added to wash cells and remove cell debris. Trypsin was used to detach the adherent cells by weakening the cell-cell contacts by breaking down proteins. After 3 minutes of incubation, the mixture in each well was replaced with a 1.5 mL Eppendorf tube and centrifuged at 3,000 RPM for 3 minutes to isolate cells from trypsin. After aspirating the Trypsin from the tubes, PBS was added again to resuspend the cell pellet. After removing PBS, PRO-PREP™ (iNtRON Biotechnology) buffer solution was added to completely lyse the cells. PRO-PREP™ buffer solution, containing five proteasome inhibitors, was used to prevent protein degradation, and it is made with an ionic detergent, which allows for isolating proteins. Then, tubes were centrifuged at 13,000 RPM for 5 minutes at 4°C, and supernatants containing proteins were transferred to 1.5 mL test tubes.

Bicinchoninic Acid Protein (BCA) Assay

Protein concentration was determined using a Pierce™ BCA Protein Assay Kit (ThermoFisher). Following the protocol provided by Pierce™ BCA Protein Assay Kit, a series of dilutions of known concentrations are prepared using 10 μL of albumin (BSA) protein with 10 μL of PBS diluent in a 96-well plate (non-treated) and assayed alongside the 5 μL of 6 unknown protein concentrations before the concentration

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of each unknown is determined based on the standard calibration curve. Then, 200 µL of BCA reagent was added to each well with thorough mixing and the plate was placed in the shaking incubator (Biofree) for 15 minutes. After incubation at 37 °C in the dark, the absorbance of each well was recorded at 562 nm using a microplate reader (Synergy H1, BioTek). Protein concentrations were calculated based on the standard curve equation.

Western Blotting

An equal amount of lysates extracted from the CT26 cells was denatured using a dry block heater at 98°C for 5 minutes and loaded onto an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 60 V for 30 minutes and 90 V for 1 hour. After the separation of proteins by their size, the gel was transferred onto pre-cut nitrocellulose membranes in wet conditions to be accessible for antibody detection. For transfer, a transfer sandwich composed of a fiber pad, filter papers, the gel, a membrane, filter papers, and a fiber pad was created and electroblotted for 100 minutes at 100 V. Then, membranes were rinsed with deionized water and blocked with the 5% BSA buffer for 1 hour to prevent the non-specific binding of antibodies. After blocking, the membrane was incubated overnight at 4°C with primary antibodies: rabbit monoclonal BRD4 [BL-149-2H5] (Abcam, #ab243862) antibody and rabbit GAPDH (14C10) antibody (Cell Signaling, #2118) diluted to 1:1000. On the second day, the membrane was washed with TBST Buffer for 5 minutes, repeating 3 times, and the membrane was incubated with diluted to 1:1000 specific enzyme-conjugated secondary antibody (Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP) for 1 hour at room temperature. The membrane was developed with a chemiluminescent substrate (ThermoFisher) and visualized using iBright™ CL750 Imaging System (ThermoFisher).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Data are expressed as the mean ± standard deviation (SD) as indicated. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed for comparisons among three or more groups.

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