Article

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Synergistic Inhibition of Colorectal Cancer Cells by Autocrine Motility Factor Peptide and Glycyrrhetinic Acid

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Background: Anti-cancer peptides are a powerful drug concept that induces cancer cell death through growth inhibition and membrane disruption, providing broad efficacy. The autocrine motility factor (AMF) interacts with the AMF receptor, regulating cancer cell motility, proliferation, metastasis, and angiogenesis through autocrine and paracrine pathways. However, studies verifying the synergistic effect of the combined use of anti-cancer drugs extracted from plants and AMF treatment are insufficient.
Methods: The effects of AMF-derived peptide sequences were evaluated in HT29 and SW620 colorectal cancer (CRC) cell lines. The study assessed the impact of AMF peptides on cell proliferation, colony formation, the Nicotinamide Adenine Dinucleotide Phosphate/Reduced Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$/NADPH) ratio, and reactive oxygen species (ROS) generation in these CRC cells. Additionally, the combined effect of AMF peptides and glycyrrhetinic acid (GA), a compound derived from licorice plants, was investigated by analyzing cell proliferation, colony formation, ROS production, and cell cycle progression in CRC cells.
Results: AMF peptides significantly inhibited CRC cell growth ($p < 0.05$), decreased colony formation ($p < 0.05$), and increased the $NADP^+$/NADPH ratio ($p < 0.05$) and ROS production ($p < 0.001$). When combined with GA, AMF peptides enhanced GA's effects on CRC cells, further suppressing cell growth ($p < 0.05$) and colony formation ($p < 0.05$) while increasing ROS generation ($p < 0.05$).
Conclusion: The synergy between AMF peptides and GA, derived from licorice plants, suggests the potential for combined peptide-phytochemical therapy for treating CRC.

Keywords: autocrine motility factor peptide; colorectal cancer; glycyrrhetinic acid; reactive oxygen species

Introduction

In the realm of peptide therapeutics, anti-cancer peptides (ACPs) derived from various natural sources offer a potent mechanism for inducing cancer cell death through membrane disruption, providing broad-spectrum efficacy irrespective of metabolic activity or drug sensitivity. However, advancing the utilization of ACPs faces challenges such as cytotoxicity and inadequate targeting persistence [1,2]. Glucose-6-phosphate isomerase (GPI) facilitates the conversion of glucose-6-phosphate to fructose-6-phosphate in glycolysis. Autocrine motility factor (AMF), the secreted form of GPI, interacts with the autocrine motility factor receptor (AMFR), stimulating cancer cell motility, proliferation, metastasis, and angiogenesis through autocrine and paracrine pathways [3–5]. Elevated serum levels of AMF and AMFR are strongly correlated with poor prognosis in patients with different types of cancer, making them prime candidates for cancer therapy [6,7]. However, AMF has also demonstrated an ability to selectively inhibit cancer cell growth by inducing apoptosis, although its effectiveness varies depending on the specific AMF types and dosages used, as well as the particular cancer cell types involved [8–10]. This is intricately linked to the modulation of glucose-6-phosphate dehydrogenase (G6PD) expression and reactive oxygen species (ROS) production [9]. G6PD, a critical enzyme in the oxidative phase of the pentose phosphate pathway, contributes to the generation of Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)

and ribose-5-phosphate. Elevated G6PD expression in cancers is closely associated with chemoresistance, DNA synthesis and repair, cell cycle regulation, redox equilibrium, and metastasis [11–13]. GPI is a 63 kDa dimer characterized by distinguishable large and small domains. The catalytic site, crucial for phosphosugar isomerization, resides within a cleft between these domains and involves residues from adjacent subunits [14,15]. This site likely shares common parts of the AMF region responsible for cytokine function, encompassing the segment spanning amino acid residues 117–288 of the AMF protein. Another noteworthy region, spanning amino acids 325–339, is responsible for inducing inflammatory cytokine secretion from synoviocytes in rheumatoid arthritis [16]. In this study, we evaluated the impact of AMF peptides encompassing amino acids 201–230 on the proliferation of colorectal cancer (CRC) cells, including highly adherent HT29 and metastatic SW620 cells. The AMF peptides demonstrated inhibitory effects on CRC cells, indicating a capacity comparable to that of AMF in inducing the apoptosis of cancer cells by downregulating G6PD and elevating ROS levels. Glucocorticoids are widely acknowledged for their therapeutic efficacy across various conditions; however, their usage is constrained by potential side effects [17]. Phytochemicals like glycyrrhetinic acid (GA), guggulsterone, boswellic acid, withaferin A, and diosgenin exhibit steroid-like activity and represent alternative therapeutic options [18]. Among these, GA, derived from licorice plants, has garnered considerable attention for its anti-inflammatory and anti-tumoral properties across diverse cancer types [19–21]. This study introduces a novel strategy by combining the synergistic effects of AMF peptides and GA, leveraging the selective anti-tumor effect of AMF peptides for CRC treatment.

Materials and Methods

Cell Culture and Materials

The HT29 (KCLB 30038) and SW620 (KCLB 60068) CRC cell lines were purchased from the Korea Cell Line Bank (Seoul, Korea). They were verified by short tandem repeats and tested negative for mycoplasma. The cell lines were cultured in Dulbecco's Modified Eagle Medium (11965118, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (26140079, Thermo Fisher Scientific) and 1% penicillin/streptomycin (15140122, Thermo Fisher Scientific). The cells were incubated in a humidified incubator with 5% CO_2 at 37 °C. The AMF peptide sequences (95% purity) used in this study (Fig. 1A) were synthesized and purchased from Biostem (Suwon, Korea). The anti-AMFR antibody (SC-166358) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

MTT Assay

The HT29 and SW620 cell lines (8×10^3 cells/well) were seeded in a 96-well plate (30096, SPL Life Sciences Co., Pocheon-si, Korea) and incubated for 24 hours. Subsequently, the cells were treated for 48 hours with either an AMF peptide (1–10 µg/mL), GA (50–150 µM), or a combination of both. Cell growth was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (M6494, Thermo Fisher Scientific) according to the manufacturer's instructions. Optical density (O.D.) at 570 nm was measured using the BioTek Cytation 7 Cell Imaging Multi-Mode Reader (Agilent Technologies, Inc., Santa Clara, CA, USA). The O.D. values were calculated to determine relative cell proliferation (%) = O.D. value of experimental sample/O.D. value of experimental control (untreated cells) × 100%.

Colony Growth Assay

The HT29 and SW620 cell lines (3×10^3 cells/well) were seeded in a 24-well plate (30024, SPL Life Sciences Co.) and incubated for 24 hours. Subsequently, the cells were treated with AAP (derived from A549 cells) (5 and 10 µg/mL) (Fig. 1) alone and with 5 µg/mL of AAP in combination with 75 µM GA. After colony formation, the cells were stained with 0.5% crystal violet (V5265, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour and then detected images with an iPhone 12. The stained cells were dissolved using a 0.1% sodium dodecyl sulfate solution (L3771, Sigma-Aldrich), and the absorbance was measured at 600 nm using the BioTek Cytation 7 Cell Imaging Multi-Mode Reader. The O.D. values were calculated to determine relative colony growth (%) = O.D. value of experimental sample/O.D. value of experimental control (untreated cells) × 100%.

$NADP^+$ and NADPH Assay

The HT29 and SW620 cell lines were treated with the AAP (2.5 and 10 µg/mL) peptide for 24 hours, and total Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$) and NADPH levels were measured in the lysates (2×10^6 cells) using the NADP/NADPH assay (N510, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Measurements were obtained using the BioTek Cytation 7 Cell Imaging Multi-Mode Reader.

Reactive Oxygen Species and Immunofluorescence Assay

The HT29 and SW620 cell lines (8×10^3 cells/well) were seeded in a 96-well plate and incubated for 24 hours before being treated with the AAP (2.5 and 5 µg/mL) peptide for an additional 24 hours. ROS generation was measured using 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; 10 µM, D399, Thermo Fisher Scientific) and detected using the BioTek Cytation 7 Cell Imaging Multi-Mode Reader. The fluorescence intensity value was measured

with the BioTek Cytation 7 Cell Imaging Multi-Mode Reader, and the relative fluorescence intensity was compared between the groups. The HT29 and SW620 cell lines (1×10^4 cells/well) were seeded in a 96-well plate, incubated for 24 hours, treated with AMF peptide for 8 hours, and the relative 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA) intensity was evaluated to assess ROS generation for 36 hours using the BioTek Cytation 7 Cell Imaging Multi-Mode Reader.

Cell Cycle Assay

The HT29 and SW620 cell lines (8×10^3 cells/well) were seeded in a 6-well plate (30006, SPL Life Sciences Co.) and incubated for 24 hours. Subsequently, the cells were treated with AAP (5 µg/mL), GA (75 µM), and AAP combined with GA for 24 hours. The cell cycle was analyzed using the Muse® Cell Cycle Kit (MCH100106, Millipore Co., Billerica, MA, USA) according to the manufacturer's instructions and detected using a BD FACSymphony™ A1 (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

All analyses of the experimental data groups were performed using one-way analysis of variance (ANOVA) followed by the Brown-Forsythe and Bartlett's test or the unpaired *t*-test using GraphPad Prism version 10 software (GraphPad Software, Inc., San Diego, CA, USA). *p*-values of <0.05 were considered statistically significant.

Results

AMF Peptides Exhibit Differential Inhibitory Effects on CRC Cell Proliferation

The AMF sequences represent regions derived from A549 (lung cancer), AsPC-1 (pancreatic cancer), DU145 (prostate cancer), HeLa (cervical cancer), MCF-7 (breast cancer), HepG2 (liver cancer), HT29 (colon cancer), and SKOV3 (ovarian cancer) cells [8–10]. Four different AMF peptides, each containing 201–230 amino acid residues of the AMF protein, were labeled AAP (derived from A549 cells), HGP (derived from HepG2 cells), HTP (derived from HT29 cells), and SKP (derived from SKOV3 cells) (Fig. 1A). Their effect on the proliferation of HT29 and SW620 CRC cells was assessed using the MTT assay. AAP and SKP at various concentrations significantly decreased the viability of CRC cells in a dose-dependent manner ($p < 0.01$), which was greater compared to HGP and HTP. However, IC_{50} values were not obtained from AAP treatment up to 10 µg/mL (Fig. 1B). Subsequently, we focused on AAP due to its superior effects; 5 µg/mL AAP decreased the growth of both CRC cell lines by 20% compared to the untreated controls ($p < 0.01$). In a colony-forming assay, AAP inhibited CRC cell growth ($p < 0.05$) (Fig. 1C). Considering that the ability of therapeutic ACPs to specifically target tumors is pivotal for their effectiveness [1,2], we investigated how the anti-AMFR antibody influenced the targetability of AAP to CRC cells by interacting with the AMFR. When co-administered with AMFR antibody and AAP, the cell growth reduction caused by AAP was restored by the AMFR antibody ($p < 0.05$), indicating that AAP activity was mediated through its interaction with the AMFR, which is recognized as a crucial target for the development of cancer treatments (Fig. 1D).

AMF Peptide Stimulates Increased ROS Production

AAP increased the $NADP^+$/NADPH ratio while the ratio was significantly reduced by combined treatment with the AMFR-antibody ($p < 0.05$), suggesting that AAP can cause ROS production via NADPH oxidases (Fig. 2A). H_2DCFDA staining indicated a time-dependent increase in ROS production over 36 hours in both CRC cells treated with AAP ($p < 0.0001$), suggesting a shared underlying mechanism contributing to AAP effects in various cancer cell types (Fig. 2B). Similarly, the enhanced H_2DCFDA fluorescence provides corroborating evidence for the impact of AAP-mediated increases in ROS production (Fig. 2B). In the pursuit of developing sophisticated ACPs with precise tumor-targeting capabilities, the AMF peptide emerges as a highly promising alternative. Its potency is underscored by its effects, which are akin to that of the AMF protein, and its stemness derived from diffusible AMF [4,5]. Consequently, AAP, with its distinct binding affinity toward the oncogenic AMFR, positions itself as a noteworthy candidate for targeted therapy in CRC.

AMF Peptide and GA Synergistically Suppress the Proliferation of CRC Cells

We shifted our focus to assessing the efficacy of AAP in conjunction with GA. GA stands out due to its practicality, affordability, and clinical safety, prompting extensive research into various GA derivatives aimed at enhancing effectiveness and bioavailability. GA's anti-cancer efficacy lies in its association with mitochondrial permeability transition, targeting multiple apoptotic factors and deactivating nuclear factor (NF)-κB, which has anti-inflammatory, pro-apoptotic, and pro-autophagic properties [19,22]. After determining GA's activity against CRC cell growth using the MTT assay, AAP and GA were co-administered, which demonstrated superior efficacy compared to GA alone ($p < 0.05$) (Fig. 3A). In a colony-forming assay, treatment with 75 µM GA and 5 µg/mL of AAP nearly suppressed the formation of surviving CRC cell colonies ($p < 0.01$) (Fig. 3B). These results further underscore the aggressive nature of AMF, which is contained within AAP and represents the minimal catalytic site region of GPI/AMF. ROS production following a 6-hour treatment with 2.5 and 5 µg/mL AAP alone or in combination with 50 µM GA further substantiated the synergistic effect. A significant increase in ROS levels was observed in HT29 and SW620 cells with AAP and GA compared to untreated control cells,

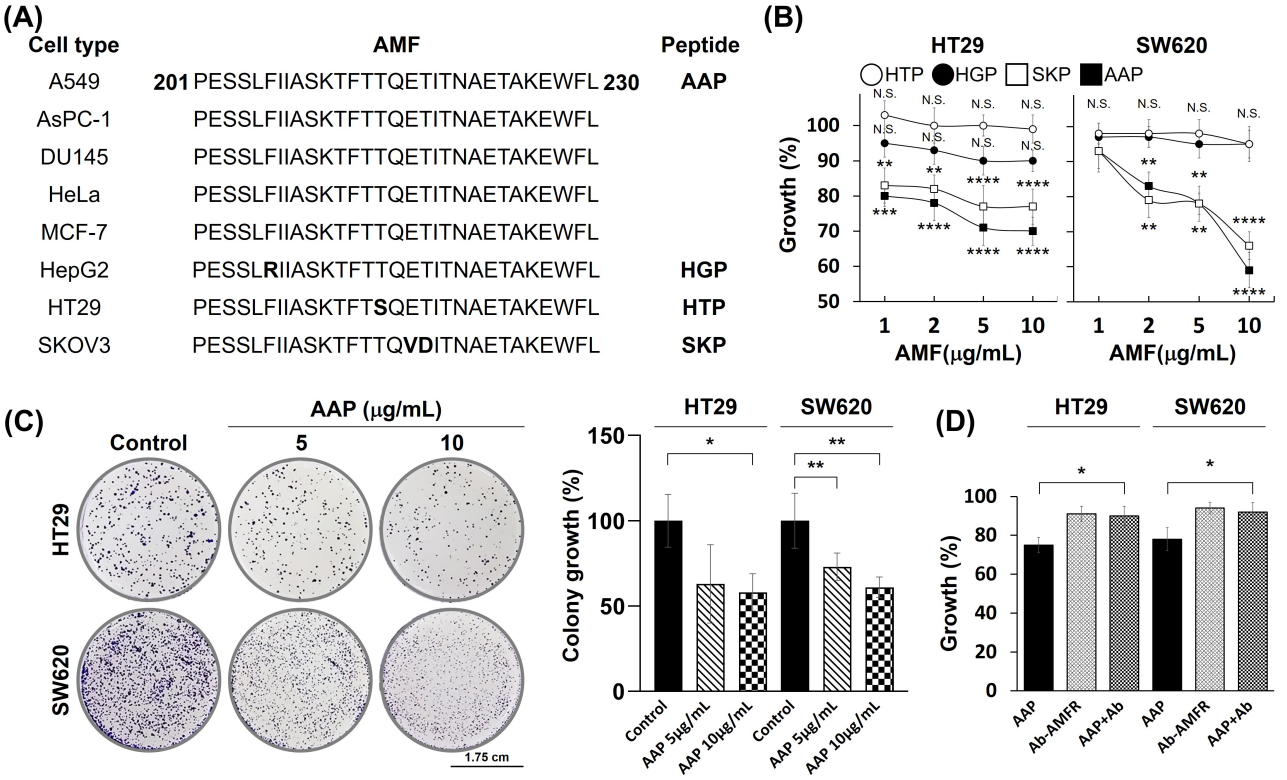

Fig. 1. The effect of autocrine motility factor (AMF) peptides on colorectal cancer (CRC) cell proliferation. (A) AMF sequences in various types of cancer cells and four distinct peptides derived from AMF. (B) CRC cell proliferation was measured after 48 hours of exposure to AMF peptides (1 to 10 μg/mL), and significance was analyzed by comparing the results with the untreated group. (C) Effect of AAP (5 and 10 μg/mL) on colony formation in CRC cells. The experimental control (untreated cells) was set to 100% and labeled after testing for significance. (D) Comparison of CRC cell proliferation following treatment with AAP (5 μg/mL), anti-autocrine motility factor receptor (AMFR) antibody (1 μg/mL), and AAP combined with the AMFR antibody. Data are representative of three biological replicates. Statistical significance is indicated by *p*-values, with asterisks denoting significance levels (*$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$ or ****$p < 0.0001$; N.S., not significant). AAP, derived from A549 cells; HGP, derived from HepG2 cells; HTP, derived from HT29 cells; SKP, derived from SKOV3 cells.

whether administered individually or in combination ($p < 0.05$) (Fig. 3C). Overall, the increased ROS levels may be closely associated with enhanced growth inhibition and the near-complete elimination of CRC cells, where AAP sensitizes cells to the impact of GA, leading to the activation of apoptosis and autophagy [23]. Cancer cells thrive on moderately elevated levels of ROS due to their enhanced antioxidant systems, making them sensitive to stimuli that further increase ROS production. Various therapeutic strategies aim to increase ROS levels to disrupt cancer cells' redox adaptation [24]. In this context, it is plausible to propose that the observed increase in ROS levels resulting from the synergy between AAP and GA may have exceeded the tolerable threshold of CRC cells. In the cell cycle analysis, GA was shown to effectively arrest cells in the G0/G1 phase. When combined with GA, AAP further increased the G0/G1 phase ratio, suggesting that their synergy partly arises from disrupting cell cycling ($p < 0.05$) (Fig. 3D).

Discussion

AMF, known for its role in cancer progression, exhibits the selective inhibition of specific cancer cell types, offering insight into competitive cellular dynamics. G6PD and ROS levels are crucial factors in AMF's aggressive behavior [8,9]. Cellular competition, influenced by transcriptional and metabolic control, maintains cellular fitness and survival. Metabolic pathway alterations, including ROS signaling-associated mitochondrial activity, protein synthesis, and glycolysis, significantly impact the competitive status [10]. AMF-induced elevated ROS levels suggest its role as a key signaling factor in cancer cell fate. The GPI protein segment spanning amino acids 117–288 is crucial for both the enzymatic and cytokine functions of AMF. Another region (amino acids 325–339) induces inflammatory cytokine secretion in rheumatoid arthritis [15,16]. Our study revealed the growth-inhibitory activity of the AMF 201–230 peptide, consistent with that of the full AMF pro-

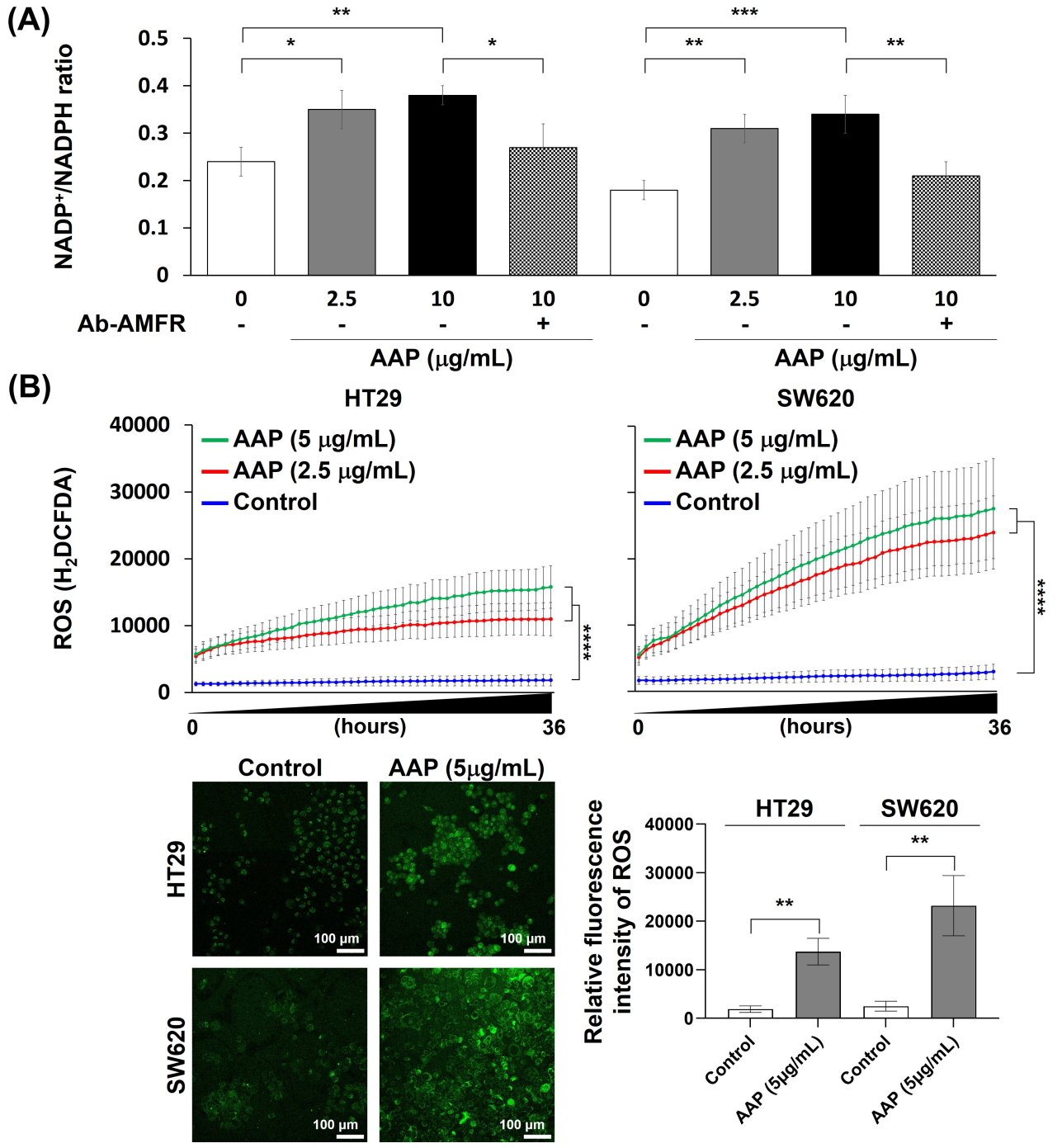

Fig. 2. Effect of AAP treatment on CRC cells. (A) Assessment of the Nicotinamide Adenine Dinucleotide Phosphate/Reduced Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$/NADPH) ratio after treatment with AAP (2.5 to 10 μg/mL), anti-AMFR antibody (1 μg/mL), and AAP combined with the AMFR antibody. (B) Comparison of reactive oxygen species (ROS) production in CRC cells after 36 hours of AAP treatment (2.5 and 5 μg/mL). Visualization of 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA)-incorporated CRC cells via fluorescence microscopy after AAP treatment (5 μg/mL) and a histogram of relative ROS fluorescence intensity (scale bar: 100 μm). Data are representative of three biological replicates. Statistical significance is indicated by *p*-values, with asterisks denoting significance levels (*$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$ or ****$p < 0.0001$).

tein. AAP can potentially be used to develop therapeutic peptides against CRC, and the AMF peptide can be further optimized for therapeutic efficacy.

Therapeutic peptides, especially ACPs, show promise in drug development, triggering cancer cell death even in chemoresistant tumors. However, the challenges include

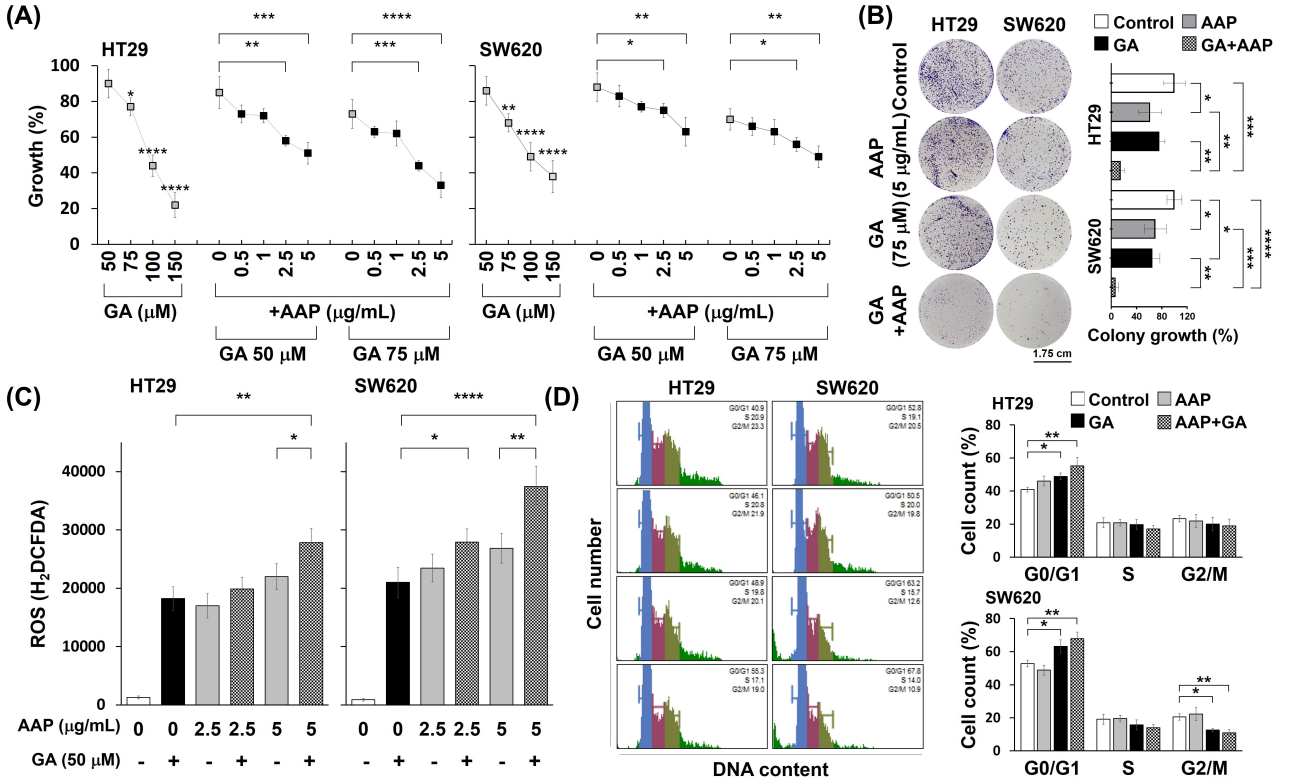

Fig. 3. Effect of AAP in combination with glycyrrhetinic acid (GA) on CRC cells. (A) Synergistic inhibition of CRC cell growth in combined AAP (0.5 to 5 μg/mL) and GA (50 to 150 μM) treatment. The experimental control (untreated cells) was set to 100% and labeled after testing for significance. (B) Synergistic effect of AAP (5 μg/mL) and GA (75 μM) on CRC cell colony formation (left panel). Comparison of colony formation between treatment with GA alone and in combination with AAP (right panel). (C) Comparison of ROS production in CRC cells after treatment with AAP (2.5 and 5 μg/mL) alone or in combination with GA (50 μM) for 36 hours. (D) Cell cycle analysis (left panel) and graphical representation (right panel) of CRC cells treated with either AAP (5 μg/mL), GA (75 μM), or AAP combined with GA. Data are representative of three biological replicates. Statistical significance is indicated by *p*-values, with asterisks denoting significance levels (*$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$ or ****$p < 0.0001$).

potential toxicity to healthy cells and the need for precise targeting. Researchers are developing tumor-targeting peptides to improve specificity, often at the cost of reduced anti-tumor efficacy. The ideal solution would combine precise targeting with potent anti-cancer effects [1,2]. The AMF peptide emerges as a promising candidate for CRC treatment, disrupting balanced redox states in cancer cells. It likely retains its parent protein's behavior, binding to overexpressed AMFR in many cancers [6,7], suggesting specific tumor-targeting capability without non-specific membrane disruption.

GA, derived from licorice, is a triterpene saponin with diverse pharmacological properties, including significant anti-tumor effects. It inhibits the PI3K/AKT pathway, suppresses NF-κB activation, and reduces inflammatory cytokine release. Techniques like solid dispersion have addressed GA's poor aqueous solubility [25]. It shows promise in combating multidrug resistance, enhancing its potential as a therapeutic agent. Overcoming chemoresistance is crucial in cancer treatment, involving multiple processes such as drug transport, oncogenesis, tumor suppression, mitochondrial activity, apoptosis, DNA repair, autophagy, and epithelial-mesenchymal transition [26]. Combination therapies incorporating various agents have been explored to address chemoresistance. Synergistic inhibition by the AMF peptide and GA holds promise as an alternative CRC treatment with a potentially reduced risk of chemoresistance. Further research, including animal studies and clinical trials, is necessary to evaluate the effectiveness and safety of this approach and confirm these initial findings.

Conclusion

The potential of AMF-comparable peptides to selectively inhibit CRC cells by modulating ROS levels, penetrate targeted CRC cells by binding to the AMFR, and act synergistically with GA to increase cytotoxic ROS levels represents a promising approach for advancing CRC therapy.

Availability of Data and Materials

All data are available from the corresponding authors upon reasonable request.

Author Contributions

SGK, TVD, SL, and HSP performed the experiments and contributed to the analysis and interpretation of the data. KR contributed to the analysis and interpretation of the data. HSP and HK conceived the study design and contributed to the analysis and interpretation of the data. All authors contributed to the writing of the manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content. All authors gave final approval of the version to be published. All authors participated fully in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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