Evaluating the Anti-proliferative and Apoptotic Role of Atrial Natriuretic Peptide in Colon Cancer Cell Lines

Alakesh Das, Dikshita Deka, Antara Banerjee and Surajit Pathak*

Faculty of Allied Health Sciences, Chettinad Hospital & Research Institute, Chettinad Academy of Research and Education, Chennai- 603103, India

E-mail/Orcid Id:
AD, dasalakesh0@gmail.com, https://orcid.org/0000-0003-2595-9389; DD, dekadikshita14@gmail.com, https://orcid.org/0000-0001-5087-9023; AB, antarabanerjee@care.edu.in, https://orcid.org/0000-0002-5519-6878; SP, drsurajitpathak@care.edu.in, https://orcid.org/0000-0002-7306-1272

Abstract: The use of small peptides and conventional anticancer drugs is gaining importance in oncology as small peptides may help to increase the chemo or radiation sensitivity. The present study aimed to study the impact of atrial natriuretic peptide (ANP) in reducing colon cancer cell proliferation in primary and metastatic colon cancer cell lines. The proliferation of colon cancer cell lines (SW480 and SW620) was analysed by CCK-8 assay and cell damage was analyzed by lactate dehydrogenase activity. Catalase activity assay was performed to measure the oxidative stress and antioxidant defense mechanisms in SW480 and SW620 colon cancer cell lines. Subsequently, up or downregulation of cancer-specific gene expressions such as BAX, Caspase-3, BCL-2, CDK-6, and PCNA genes were assessed after the treatment of small peptide ANP in SW480 and SW620 colon cancer cell lines. The ANP treatment decreased the colon cancer cell proliferation, by upregulating the apoptosis-related gene expression (Caspase-3, BAX), downregulated the anti-apoptotic gene (BCL-2) expression, and proliferation-related genes (CDK-6, PCNA) in SW480 and SW620 colon cancer cell lines, and the differences were found to be statistically significantly. Further, increased levels of catalase in the colon cancer cell lines after ANP-treatment suggested the therapeutic role of ANP. Subsequently, the LDH analysis showed the potential of ANP in inducing colon cancer cell damage. Collectively, the current study clearly shows that ANP is a potential molecule in reducing uncontrolled cancer cell growth. However, additional research using animal models and other colon cancer cell lines is needed to validate its potential usage in clinical studies.

Introduction

The use of small peptides along with conventional treatment approaches for colon cancer may improve the effectiveness of the treatment and address the difficulties linked to traditional therapeutic options like chemoresistance and radioresistance (Thundimadathil, 2012; Osman et al., 2019; Flickinger et al., 2022). This strategy exploits the distinctive characteristics of peptides, including their specificity, capacity to target particular cellular pathways and favorable safety profiles, to enhance the effectiveness of conventional approaches (Wang et al., 2022; Cachot et al., 2021; Nath et al., 2024; Halder, 2024). Peptides can disrupt the cellular processes that contribute to drug resistance, such as efflux pumps (e.g., P-glycoprotein) or pathways that promote cell survival (Deng et al., 2022; Halder et al., 2022; Solairaja et al., 2023). Peptides are found to increase the vulnerability of cancer cells to chemotherapy drugs by altering these processes (Kalimuthu et al., 2018). The pathogenesis of colon cancer is a complex phenomenon that encompasses a series of sequential events, driven by a combination of genetic and epigenetic factors (Tender et al., 2021; Rajamäki et al., 2021). A significant proportion of colorectal malignancies originate from the development of a polyp, a growth that emerges on the inner mucosal lining of the colon or rectum (Chen et al., 2021; Ahadi et al., 2021; Srvec et al., 2018; Mehta et al., 2023; Kesavan et al., 2023). Consequently, there has been
a significant increase in interest in investigating small molecules like peptides in various types of cancer (Hadianamrei et al., 2022). Atrial natriuretic peptide (ANP) is a 28 amino acid peptide that is part of a group of hormones produced by the heart and blood vessels. Multiple analysis indicates that ANP has a role in innate immunity by promoting the generation of superoxide anions, the synthesis of leukotriene B4, and the elevation of CD11 expression in polymorphonuclear neutrophils (PMN) (Takahashi et al., 2020; Colini Baldeschi et al., 2018; Liang et al., 2023). Analysis has shown that ANP alters the characteristics of the potassium current in human stomach cancer AGS cells, specifically in terms of steady-state activation features (Li et al., 2021; Sun and Li, 2022; Li et al., 2016). Specifically, the use of ANP at different doses can either reduce or enhance the stable activation characteristic of potassium current in these cells, suggesting that the activity of the cells is influenced by ANP in a manner that depends on the dosage of the peptide (Li et al., 2021; Sun and Li, 2022; Li et al., 2016; Zhang and Lin, 2020; Delghanbanadaki et al., 2021). Moreover, the natriuretic peptide receptor A (NPR-A) has recently been identified as a promising focus for cancer investigation. Furthermore, comprehending the impact of ANP on cancer cell behavior requires a thorough understanding of the differential expression and synthesis of the peptide, as well as their role in determining the expression of the peptide receptors in cells (Sun and Li, 2022; Xu et al., 2022; Mezzasoma et al., 2021; Hajikazemi et al., 2018). Studies utilizing athymic mouse models have shown that a combination of cardiac hormones effectively eradicates a significant proportion of human pancreatic adenocarcinomas and breast malignancies (Xu et al., 2022; Aggarwal et al., 2022; Kozlowski and Kozlowski, 2021; Sun et al., 2006; Mehta et al., 2023; Saha and Yadav, 2023; Das et al., 2021). The results emphasise the potential of cardiac hormones as anticancer drugs, offering a new and innovative strategy for treating cancer. Therefore, the present work aimed to determine the role of ANP in reducing uncontrolled cell growth and increase apoptosis in colon cancer cell lines.

Materials and methods

Cell line expansion and peptide used for the study

The colon cancer cell lines, SW480 (primary colon cancer cell line) and SW620 (metastatic colon cancer cell line) were acquired from NCCS in Pune, India (All cell lines are mycoplasma-free and a Short tandem repeat analysis was done in NCCS-Pune to check the cross-contamination) (Supplementary material 1). DMEM supplemented with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin was used to culture the colon cancer cells which was further maintained at 37°C in 5% CO₂. The ANP (Sequence of ANP: SLRRSCFGGMRIGAQSGLCNSFRY), with a purity >90%, was purchased from PRIVEEL PEPTIDES, Chennai, India.

SW480 and SW620 colon cancer cell lines were selected for the present study as they originate from the same patient, representing a model of disease progression from an early to advanced stage of colon cancer. Furthermore, these cell lines are also thought to be responsive to different therapeutic agents, making them appropriate models for assessing the activity of ANP.

Cell viability studies after treatment of different doses of ANP in SW480 and SW620 colon cancer cell lines

To study the viability of colon cancer cells after the treatment of ANP, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) analysis was performed and the optimal dose of the peptide was selected for further experiments. 2 × 10⁵ cells per well of both the SW480 and SW620 colon cancer cells were seeded in a 96-well plate. After 24 h incubation, the cells were treated with various concentrations of ANP (1 ng/mL, 250 ng/mL, 500 ng/mL, 750 ng/mL, 1 μg/mL). Subsequently, as per the standard protocol, the MTT solution (5 mg/mL) was added to the cells and kept for incubation at 37 °C for 4 h. Further, the absorbance was taken at 546 nm using an ELISA reader (Robonik, Readwell TOUCH ELISA Plate Analyser).

Cell Counting Kit-8 (CCK-8) Assay

The Cell Counting Kit-8 (CCK-8) was utilized to assess the proliferation of colon cancer cells. A total of 2,000 cells were seeded in each well of a 96-well plate. Following 24 hours of cell seeding, the cells were exposed to the selected concentrations of ANP for 24, 48, and 72 hours. Further, the absorbance at 450 nm was measured using a microplate reader.

Lactate dehydrogenase Assay

To measure the cell damage capacity of ANP, lactate dehydrogenase (LDH) activity assay was performed using the EZcount™ lactate dehydrogenase cell assay kit (Cat. No: CCK036, HiMedia) as per the manufacturer’s procedure.

Catalase activity analysis

To measure the catalase activity, the assay was performed using a standard protocol as documented by Hadwan and Abed, 2016 with slight modification (Hadwan and Abed, 2016). Briefly, about 3 ml of the reaction mixture was prepared using H₂O₂ (15 mM), phosphate buffer (50 mM), and sample of about 0.1 ml. OD was measured spectrophotometrically (Model No.:
UV-1800 240V) at 240 nm for an interval of 60 seconds for 3 minutes. Analysis was done and the activity was calculated as well as expressed as µM/min/mg protein.

**Gene expression analysis by qPCR**

RNA extraction was performed from ANP-treated cells, followed by quantification of total RNA using NanoDrop. Fold changes were calculated for apoptotic genes (Caspase-3, BAX, BCL-2) and proliferative genes (CDK-6, PCNA). The detailed sequences were used as depicted in Das et al., 2023 (Das et al., 2023). The CT values were normalized with the housekeeping gene GAPDH. The fold change was determined through relative quantification (RQ) values calculated as $2^{\Delta\Delta CT}$.

**Statistical analysis**

Statistical analysis was carried out by student’s t-test using GraphPad V8.4.2 software. All experiments have been performed in triplicates and the data were considered statistically significant with $p < 0.05$ and represented as asterisks (* ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and ns=non-significant.

**Results**

**Cell viability studies after treatment of different doses of ANP in SW480 and SW620 colon cancer cell lines**

Colon cancer cells were treated with various doses of ANP (1 ng/mL, 250 ng/mL, 500 ng/mL, 750 ng/mL, 1 µg/mL). 500 ng/mL and 250 ng/mL concentrations of ANP were selected for further analysis as the lower doses were not reducing the viability of the colon cancer cells. The ANP peptide showed lower efficacy in killing cancer cells at 24 and 48 hours; however, it exhibited cytotoxic effects at 500 ng/mL, and 250 ng/mL concentrations after 72 hours. Therefore, the 72-hour time period was selected for further investigation for the current study.

**Cell counting kit-8 (CCK-8) assay**

CCK-8 was used to analyse the proliferation capability of untreated colon cancer cell lines and ANP-treated colon cancer cell lines. Following 72 hours of treatment, ANP reduces the cell growth compared to untreated SW480 and SW620 colon cancer cell lines, the same was also observed at 24 and 48 hours although the differences were not significantly marked.

**Lactate dehydrogenase (LDH) activity**

The potential of ANP in inducing cellular damage was analysed by measuring the percentage of LDH release in both SW480 and SW620 colon cancer cell lines. In the present study, 500 ng/mL concentration of ANP showed a significant increase in LDH release in SW480 colon cancer cell line.

**Catalase activity analysis**

Our findings indicate that ANP has the potential to increase the catalase activity in SW480 and SW620 colon cancer cell lines. However, it was noted that 500 ng/mL concentration of ANP could significantly increase catalase activity in both SW480 and SW620 colon cancer cell lines.

**Cancer-specific Gene Expression Analysis**

In the present study, the impact of ANP was observed on the expression of pro-apoptotic markers, namely Caspase-3 and BAX, in the SW620 colon cancer cell line treated with 500 ng/mL concentration of ANP. It was found that their expression was significantly increased. Moreover, in the SW480 colon cancer cell line, 500 ng/mL concentration of ANP, the expression of BCL-2 was significantly downregulated. Additionally, ANP showed a significant decrease in the expression of CDK-6 and PCNA in the SW480 colon cancer cell line when treated with both 250 ng/mL, 500 ng/mL concentrations.

![Figure 1. Bar charts illustrating the cell viability analysis of A) SW480 and ANP-treated SW480; B) SW620 and ANP-treated SW620 colon cancer cell lines.](image-url)
of the small peptide. Similarly, in SW620 colon cancer cells, both 250 ng/mL, 500 ng/mL concentrations of the peptide resulted in a significant reduction in CDK-6 expression. However, only 500 ng/mL concentration of ANP could downregulate the expression of PCNA in the SW620 colon cancer cell line.

Figure 2 (A-B). Bar graph illustrating the variances in proliferation among SW480, SW620, and ANP-treated SW480 and SW620 colon cancer cell lines at 24, 48 and 72 hours.

Figure 3 (A-B). Bar graph representing the percentage of LDH release in A) SW480 and ANP-treated SW480 colon cancer cell lines; B) SW620 and ANP-treated SW620 colon cancer cell lines.
Figure 4 (A-B). Figure representing the impact of ANP in catalase activity in A) SW480 and ANP-treated SW480; B) SW620 and ANP-treated SW620 colon cancer cell lines.

Figure 5. Expression profile of apoptotic and cell proliferation markers of ANP on A) SW480 and ANP-treated SW480 colon cancer cell lines and B) SW620 and ANP-treated SW620 colon cancer cell lines.
Discussion
The resistance of colon cancer cells to radiotherapy and chemotherapy is a substantial obstacle in the management of colon cancer (Kopsida et al., 2024). Multiple processes contribute to this resistance, so reducing the proliferation of cancer cells is more challenging. Researchers are now investigating new methods to overcome resistance to colon cancer drug resistance, including combination therapies, targeted therapies, and peptide therapy (Ren et al., 2021; Kopsida et al., 2024). Peptides with anticancer properties have become a promising candidate in the treatment of colon cancer in combination with conventional treatments, providing a new strategy for the treatment of cancer (Das et al., 2023; Lima et al., 2022).

An important function of the peptides in the treatment of colon cancer is centered on their capacity to trigger apoptosis in cancerous cells (Chantawannakul et al., 2021; Karami Fath et al., 2022). Peptides can interfere with essential cellular activities in cancer cells by regulating the specific targets, resulting in reducing the growth of the cancer cells. The efficacy of ANP in inhibiting cell proliferation has been widely shown in several types of cancer, including human pancreatic adenocarcinoma, breast, and prostate cancer (Mao et al., 2022; Dehghanbanadaki et al., 2021; Tan et al., 2020; Chakrovorty et al., 2021). The natriuretic peptide receptor A (NPR-A) has recently been identified as a promising focus for cancer treatment. NPR-A is a desirable therapeutic target for treating both inflammation and cancer (Dehghanbanadaki et al., 2021; Tan et al., 2020; Xu et al., 2022; Mezzasoma et al., 2021; Hajikazemi et al., 2018; Rami et al., 2023; Kulkarni et al., 2023).

In a non-cancerous state, cell proliferation is a highly controlled process that maintains an equilibrium between cell growth and cell death (Tan et al., 2020; Wei et al., 2021; Boga and Bisgin, 2022). However, in the case of cancer, this equilibrium is disturbed, resulting in unregulated cellular proliferation and division. The rapid proliferation of cancer cells contributes to tumor growth and the formation of malignant lesions (Oláh et al., 2018). The cell proliferation analysis in the present study showed a decrease in the proliferation of the SW480 and SW620 colon cancer cell lines when treated with the ANP peptide in a time-dependent manner indicating that the peptide may interfere with specific pathways or mechanisms essential for survival and proliferation and drug resistance of cancer cells. Under normal physiological conditions, LDH is retained within the cell. However, when cells undergo damage or death, such as due to exposure to cytotoxic agents like chemotherapeutic drugs or peptides, the integrity of the cell membrane is disrupted leading to the release of LDH to the surrounding environment. The results in the present study showed that peptide treatment has elevated the LDH levels in the SW480 colon cancer cell line with the 500 ng/mL concentration of the peptide indicating that the peptide may be causing some form of stress or damage to these cells, potentially inhibiting their growth or viability. However, there was no significant difference observed in the SW620 colon cancer cell line. Additionally, a reduction in catalase levels was observed in the untreated group, suggesting higher susceptibilities to oxidative damage, which has been shown to be essential in various cellular functions (Zińczuk et al., 2019). The enhanced levels of catalase were found in both SW480 and SW620 colon cancer cell lines when treated with 500 ng/mL concentration of ANP.

The levels of Caspase-3, BAX, and BCL-2 expression may be involved with the progression of colon cancer. An increased level of BCL-2 or a reduced level of BAX is linked to negative prognosis (Kunac et al., 2022; Zhou et al., 2018). The upregulation of the pro-apoptotic and downregulation of the anti-apoptotic genes in SW480 and SW620 colon cancer cell lines suggests that the treatment of ANP for a duration of 72 hours triggers apoptosis. The reduced expression of the CDK-6, and PCNA showed the role of the ANP on colon cancer cell proliferation and DNA replication.

The probable mechanistic pathways through which ANP might exert its effects on cell proliferation and apoptosis in colon cancer cell lines could involve multiple interconnected signaling cascades. ANP, acting through its receptor, the natriuretic peptide receptor-A (NPR-A), initiates a series of intracellular events. One probable pathway is the cGMP-dependent signaling cascade, where ANP binding to NPR-A leads to the activation of guanylate cyclase, resulting in the production of cyclic guanosine monophosphate (cGMP) (Mezzasoma et al., 2020). Elevated levels of cGMP can modulate various downstream effectors, including protein kinases and ion channels, ultimately influencing cell proliferation and survival (Roy et al., 2021). Additionally, ANP may also interfere with key regulatory pathways such as the PI3K/Akt and MAPK/ERK pathways, which are known to play pivotal roles in cell growth, proliferation, and apoptosis (Palabiyik et al., 2019).

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Conclusion

In summary, the current investigation has shown that ANP may exhibit the anti-cancer characteristics by reducing the proliferation and increasing cell apoptosis in SW480 and SW620 colon cancer cell lines through upregulating pro-apoptotic genes and subsequently downregulating the anti-apoptotic and cell-proliferating genes. To confirm ANP’s anti-colon cancer potential and use it in small-scale clinical trials, however, more in vitro testing on different colon cancer cell lines and in vivo study on colon cancer-induced mice models are needed.

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Conflict of interest

The authors declare that there is no conflict of interest.

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