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EFFECT OF BIOINSPIRED SYNTHETIC ANTI-MICROBIAL PEPTIDES ON COLORECTAL CANCER CELLS (SW620)

BY

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ABSTRACT

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Title: Effect of Bioinspired Synthetic Antimicrobial Peptides on Colorectal Cancer Cells (SW620)

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Colorectal cancer (CRC) is the world's third most common cancer and the second biggest cause of cancer-related deaths. Despite significant advances in CRC therapeutic intervention, cytotoxicity, drug resistance, and adverse effects remain a major issue to be major problems, necessitating the urgent development of novel therapeutic agents. The use of Bioinspired Short Anti-Microbial Peptides (BSAMPs) as anticancer peptides could pave the way for the development of innovative anticancer treatments with better therapeutic characteristics. Anti-microbial peptides (AMPs) are a class of small peptides that widely exist in nature, and they are an essential part of the innate immune system of different organisms. AMPs have a wide range of inhibitory effects against bacteria, fungi, parasites, viruses, and even cancer cells. This study investigated the anticancer and anti-inflammatory role of novel BSAMPs in human colorectal cancer cells (SW620). The results demonstrated that BSAMPS inhibited the proliferation of CRC cells by inducing apoptosis through the activation of the intrinsic mitochondrial pathway. Moreover, treatment with BSAMPs downregulated the activation of STAT3, EGFR, Akt, and NF-kB, followed by decreased expression of c-Myc and β-catenin. This is the first time, that BSAMPs structurally derived from naturally occurring AMPs have been recognized for their anticancer potential. These pleiotropic effects of BSAMPs on apoptosis, cell proliferation, survival, differentiation, and inflammation highlight their role as an alternative therapy in cancer treatment.

DEDICATION

All praises to Allah, who gave me patience and strength to finish my thesis.

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LIST OF ABBREVIATIONS

1. Anticancer peptides (ACPs)
2. Anti-microbial peptides (AMPs)
3. American Cancer Society (ACS)
4. B-cell lymphoma-extra-large (Bcl-xL)
5. Beta-catenin (β-catenin)
6. BCL2-associated X protein (Bax)
7. Bio-inspired Short Anti-Microbial Peptides (BSAMPs)
8. Bovine lactoferricin (LfcinB)
9. Colorectal cancer (CRC)
10. Cyclin-dependent kinase inhibitor 1 (p21 Waf1/Cip1)
11. C-myelocytomatosis oncogene product. (c-Myc)
12. Database of Anti-microbial Activity and Structure of Peptides (DBAASP)
13. Death-inducing signaling complex (DISC)
14. Dulbecco's modified Eagle's medium (DMEM)
15. Epidermal Growth Factor Receptor (EGFR)
16. Endoplasmic reticulum (ER)
17. Fas-associated death domain (FADD)
18. Fetal Bovine Serum (FBS)
19. Gastrointestinal (GI)
20. Half-maximal inhibitory concentration (IC50)
21. Host defense peptides (HDPs)
22. Inflammatory bowel disease (IBD)
23. Janus Kinases (JAKs)
24. Mismatch repair (MMR)

25. Mitochondrial outer membrane permeabilization (MOMP)

26. Mitochondrial membrane potential (MMP).

27. Nonsteroidal anti-inflammatory medications (NSAIDs)

28. Nuclear Factor Kappa-light-chain-enhancer activated B (NF-kB(p65))

29. Phospho- Nuclear Factor Kappa-light-chain-enhancer activated B p-NF-kB(p65)

30. Phospho- Signal Transducer and Activator of Transcription 3 (p-Stat3)

31. Phosphatidylinositol 3-kinase (PI3K)

32. Phosphatidylserine (PS)

33. Poly adenosine diphosphate-ribose polymerase (PARP)

34. Phospho- Epidermal Growth Factor Receptor (p-EGFR)

35. Signal Transducer and Activator of Transcription (STAT)

36. Transforming Growth Factor β (TGF-β)

37. Vascular endothelial growth factor (VEGF)

1. INTRODUCTION

Colorectal cancer (CRC) is the third most frequent carcinoma diagnosed (6.1%) and the second most common cause of mortality/death (9.2%) worldwide. The mortality rate from rectal and colon carcinoma is expected to grow by 60 to 71.5% by 2035 (Sawicki et al., 2021). Lifestyle, excess weight, and food patterns are supposed to increase the morbidity of CRC significantly. Physical activity appears to have a protective role against CRC, and regular screening and early diagnosis increase the chance of treatment and survival (Chan & Buczacki, 2021).

A combination of surgery, radiation therapy, immunotherapy, targeted therapy, and chemotherapy can slow the spread of CRC; however, severe side effects, drug resistance, and toxicities limit the short-and long-term outcomes in patients. Therefore, there is a pressing need for novel and alternative therapies with low cytotoxic effects and less drug resistance (Jafari, Babajani, Sarrami Forooshani, Yazdani, & Rezaei-Tavirani, 2022).

Anti-microbial peptides (AMPs) have received much interest as a potential therapeutic option for treating a variety of malignancies, including CRC, as they are more selective towards tumor cells because of the increased presence of negatively charged phosphatidylserine on their outer surfaces compared to normal cells and are less toxic and may help in overcoming drug resistance (Grissenberger, Riedl, Rinner, Leber, & Zweytick, 2020; Hankins, Baldridge, Xu, & Graham, 2015; J. Lei et al., 2019; Ran, Downes, & Thorpe, 2002; Scocchi, Mardirossian, Runti, & Benincasa, 2016; Wodlej et al., 2019). These amphipathic and cationic peptides are short chains of 5-50 amino acids with a molecular weight of less than 10 kDa. AMPs act as the first line of defense in plants, invertebrates, and higher species due to broad activity against bacteria, yeasts, fungi, viruses, and other pathogenic

microbes, thus also referred to as ‘host defense peptides’, further supporting their role as potential immunomodulators (Castro & Fontes, 2005; Kardani & Bolhassani, 2021; J. Li, Hu, Jian, Xie, & Yang, 2021; Pasupuleti, Schmidtchen, & Malmsten, 2012; Scott et al., 2007). Anticancer peptides (ACPs), a subgroup of AMPs composed of 10-60 amino acids, have been shown to inhibit tumor cell proliferation, migration, and angiogenesis with minimum chances of causing drug resistance (Hicks, 2016; Hoskin & Ramamoorthy, 2008; Kunda, 2020) (Xie, Chen, & Fang, 2020). In addition, several physicochemical features of ACPs impact their stability, penetration, and targeting efficacy. Prior to the lab investigation, various databases and other servers for *in silico* and computational prediction of AMPs/ACPs were built (Q. Y. Zhang et al., 2021).

Anti-microbial peptides’ poor stability in biological environments and their interactions with biochemical elements in complex environments restrict their use. Encapsulating AMPs into functioning carrier systems can preserve them from degradation, modify their release and bioavailability profile, and target their tissue distribution. The other approaches to overcome low activity, stability, and side effects of natural AMPs are shortening the length of peptides, generating chimera molecules and creating structural scaffolds, which lead to the design of novel bioinspired AMPs (BSAMPs) (Fjell, Hiss, Hancock, & Schneider, 2011; Ji, Li, Zhang, Zhang, & Cao, 2014; C. K. Wang et al., 2014). In this study, we aim to investigate the effect of novel BSAMPs on human CRC cells (SW620) and define their role as alternative therapeutic agents in cancer treatment.

2. LITERATURE REVIEW

2.1. Etiology of CRC

Colorectal Cancer (CRC), also known as bowel cancer, colon cancer, or rectal cancer, affects the colon and rectum. In the United States, roughly 104,270 cases of colon cancer and 45,230 cases of rectal cancer were reported in 2021 by the American Cancer Society (ACS). While 20-30% of CRC is familial, 5-10% is inherited, and 60-70% occurs sporadically. CRC is a disease that primarily affects the elderly, and symptoms may not appear for a long time after the inception of the disease. CRC patients have a 5-year survival rate of around 90% if found early and localized, but only a few individuals (4 out of 10) are diagnosed early, and once distant metastasis begins, the 5-year survival rate reduces to 10% (M. Arnold et al., 2017). Changes in bowel patterns such as diarrhea, constipation, tenesmus (a feeling that the intestines are not emptying correctly), rectal bleeding (dark brown or black feces), stomach pain, and bloating are CRC symptoms. Around 37% of patients diagnosed with CRC have blood in their feces or rectum, 56% have a bowel blockage, 25% have peritonitis (inflammation of the stomach lining), and 18% have a rupture in their colon. Several risk factors, including family and inherited variables, environmental, and lifestyle-related risk factors, such as physical inactivity, obesity, smoking, exposure to environmental contaminants, and alcohol consumption, may enhance the likelihood of developing colorectal polyps or CRC(M. Arnold et al., 2017) (Haggar & Boushey, 2009). Some countries such as New Zealand, Australia, and some regions of Europe, Canada, and the United States have higher rates of CRC than others due to multiple factors mentioned above, such as diets and lifestyle. Countries with the lowest cancer rates include some regions of South America and Africa, China, and India. The global rise in CRC, mainly due to lifestyle changes and gene mutations, could be managed by

appropriate dietary adjustments, regular physical activity, and maintaining a healthy weight, combined with focused screening programs and early therapeutic interventions.

Both men and women are affected by CRC at equal rates. CRC is the third most common type of cancer in both men and women in the United States(M. Arnold et al., 2017; Haggar & Boushey, 2009). While the top two types of cancer in women are breast and lung cancers, prostate and lung cancers are more common in men. The level of mortality for CRC is relatively high, causing deaths in half of the diagnosed cases. The high mortality rate of CRC is mainly attributed to its late diagnosis as most patients are not diagnosed until the late stages when cancer reaches to metastasis stage.

Nearly all colon and rectal cancers begin as a polyp, a growth on the colon's inner surface Figure 1. Noncancerous cells inside the colon can undergo hyperproliferation forming adenomatous polyps, a type of benign tumor. The polyp then develops into severe dysplasia and adenocarcinoma (C. N. Arnold, Goel, Blum, & Boland, 2005). As long as these polyps are noncancerous, they may not cause any complications, and patients may not experience any symptoms. Regular checkups are required to control CRC, which may help detect cancer early before the lumps turn into cancerous cells.

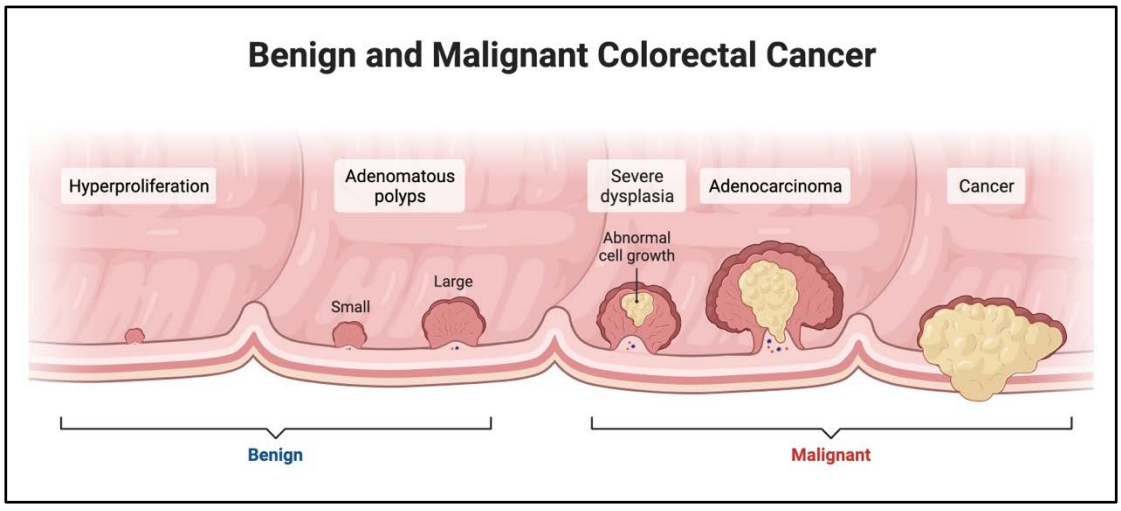

Figure 1. An overview of the progression of colorectal cancer from benign to malignant, including hyperproliferation, adenomatous polyps, severe dysplasia, adenocarcinoma, and cancer. The figure was created using the BioRender software.

Cancer death rates are higher in countries with poor air quality. According to Jagai et al. (Jagai et al., 2017), exposure to general low environmental quality, mainly air quality, may raise cancer risk and mortality. The study found that people living in places with poor environmental quality had a greater frequency of developing cancer. Similar to affecting the mortality rate for diseases such as stroke and heart disease, the quality of the environment raises the mortality rate for cancer patients (Turner et al., 2020).

In another study, exposure to environmental pollutants has been shown to increase CRC risk. This study found that water purification using chlorine may be a leading cause of cancer development, especially for people living in industrialized areas. The presence of mutagenic compounds created by chlorine interactions with natural substances released by the degradation of plants in the source water could explain the mutagenic activity of chlorinated water.

2.2. Inflammation and CRC

Long-standing chronic inflammation is considered one of the hallmarks of cancer (Hanahan & Weinberg, 2011) Figure 2. Inflammation is characterized by interactions between immune cells, inflammatory cells, chemokines, cytokines, and pro-inflammatory mediators, leading to tumor cell proliferation, abnormal growth, and invasion. CRC has long been recognized as one of the best examples of a tumor intimately linked to chronic inflammation, presenting earlier in the tumor development process. Inflammatory bowel disease (IBD), such as Crohn’s disease or ulcerative

colitis, puts patients at a higher risk of CRC as they share inflammatory components (Jess, Frisch, & Simonsen, 2013). In fact, CRC risk is predicted to be 4- to 20-fold higher in persons with IBD (Stidham & Higgins, 2018). Thus, reducing inflammation in such patients can lower the incidence of CRC.

Anti-inflammatory medications such as Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to reduce the risk of CRC incidence linked to inflammation (Rothwell et al., 2010). According to a large body of observational and interventional trial evidence, aspirin and other NSAIDs appear to protect against the development of colonic adenomas and cancer. Adenomatous polyps, also known as adenomas, are seen in most patients who develop CRC, and NSAIDs and aspirin can slow their progression into cancer. Regular aspirin and other NSAIDs intake are linked to a 20%-40% reduction in colonic adenomas and the incidence and mortality due to CRC (Maniewska & Jezewska, 2021; Rothwell et al., 2010).

A study found that people with mutations in DNA mismatch repair genes (Lynch syndrome) had a lower risk of CRC when given aspirin and ibuprofen (Ait Ouakrim et al., 2015). DNA mismatch repair (MMR) hereditary genes cause germline mutations and increase the likelihood of developing CRC. Using aspirin helps reduce the risk of even for those individuals with this gene mutation.

However, some patients can experience complications after using NSAIDs. Long-term use of NSAIDs medications has been associated with more complications. Some patients also experience gastric ulcers as a result of taking NSAIDs. In addition, different factors such as old age and a history of previous gastrointestinal (GI) injuries may increase the chances of complications from NSAIDs intake.

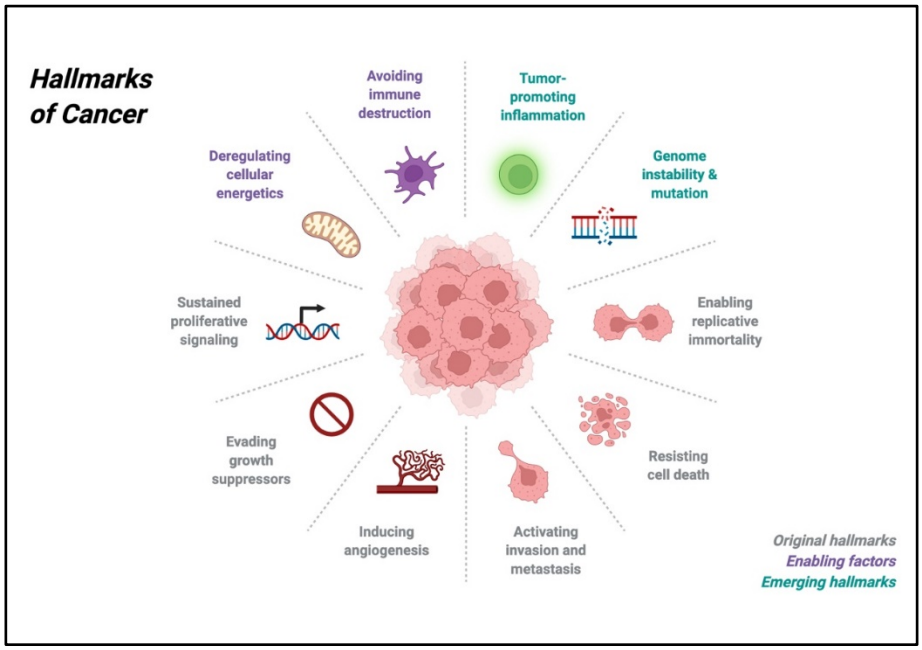

Figure 2. An overview of hallmarks of cancer. The figure was created using the BioRender software.

2.3. Apoptosis and CRC

Apoptosis is a programmed cell death mechanism that occurs both in physiological and pathological conditions in multicellular organisms. It is an orderly process that allows the disposal of unwanted cells when triggered by an appropriate stimulus. Thus, any dysfunction or deregulation of the apoptotic mechanism can lead to the development of various pathologies, including cancer. Morphological hallmarks of apoptosis include chromatin condensation, nuclear fragmentation, membrane blebbing, loss of membrane integrity, and cell shrinkage. A wide variety of external and internal stimuli such as hypoxia, exposure to certain drugs, chemicals or radiation, infectious agents, and various pathological states (Brown, Yang, & Ray, 2014) are responsible for triggering apoptosis which occurs through two pathways known as the intrinsic (mitochondrial) and extrinsic (death receptor) pathways.

The intrinsic apoptotic pathway is activated in response to internal cellular

stress or internal stimuli such as DNA damage, hypoxia, or endoplasmic reticulum (ER) stress, as shown in Figure 3. This pathway is regulated by complex interactions between the pro-apoptotic Bcl-2 family proteins such as Bad, Bax, Bim, Bid, Bik, Noxa, Puma, Hrk and the anti-apoptotic Bcl-2 family proteins such as Bcl-xl, Bcl-2, Bcl-w, and McI-1 (Wong, 2011). The activation of pro-apoptotic effector proteins such as Bax and Bak leads to mitochondrial outer membrane permeabilization (MOMP). The proteins released through the MOMP subsequently drive the activation of executioner caspases 3 and 7, thus leading to apoptosis (Cavalcante et al., 2019; Jacqueline Ho, 2014). Once activated, Caspases cleave many structural and integral proteins responsible for maintaining the integrity of the cell, and the result is nuclear condensation, DNA fragmentation, and plasma membrane blebbing (Boice & Bouchier-Hayes, 2020).

While the extrinsic apoptotic pathway is activated in response to external stimuli or a pro-death signal originating from the extracellular environment. This pathway is initiated when death ligands such as tumor necrosis factor (TNF) and Fas ligand (FasL) bind to death receptors type 1 TNF receptor (TNFR1) and Fas. This ligand binding results in the formation of a protein complex known as the death-inducing signaling complex (DISC) that allows the recruitment of adaptor proteins such as Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD) on which caspase-8 aggregates, leading to its activation. Activated caspase-8 cleaves and stimulates the activity of executioner caspases 3 and 7, thus leading to the initiation of apoptosis (Cavalcante et al., 2019; Jacqueline Ho, 2014; Widmann, 2007; Wong, 2011).

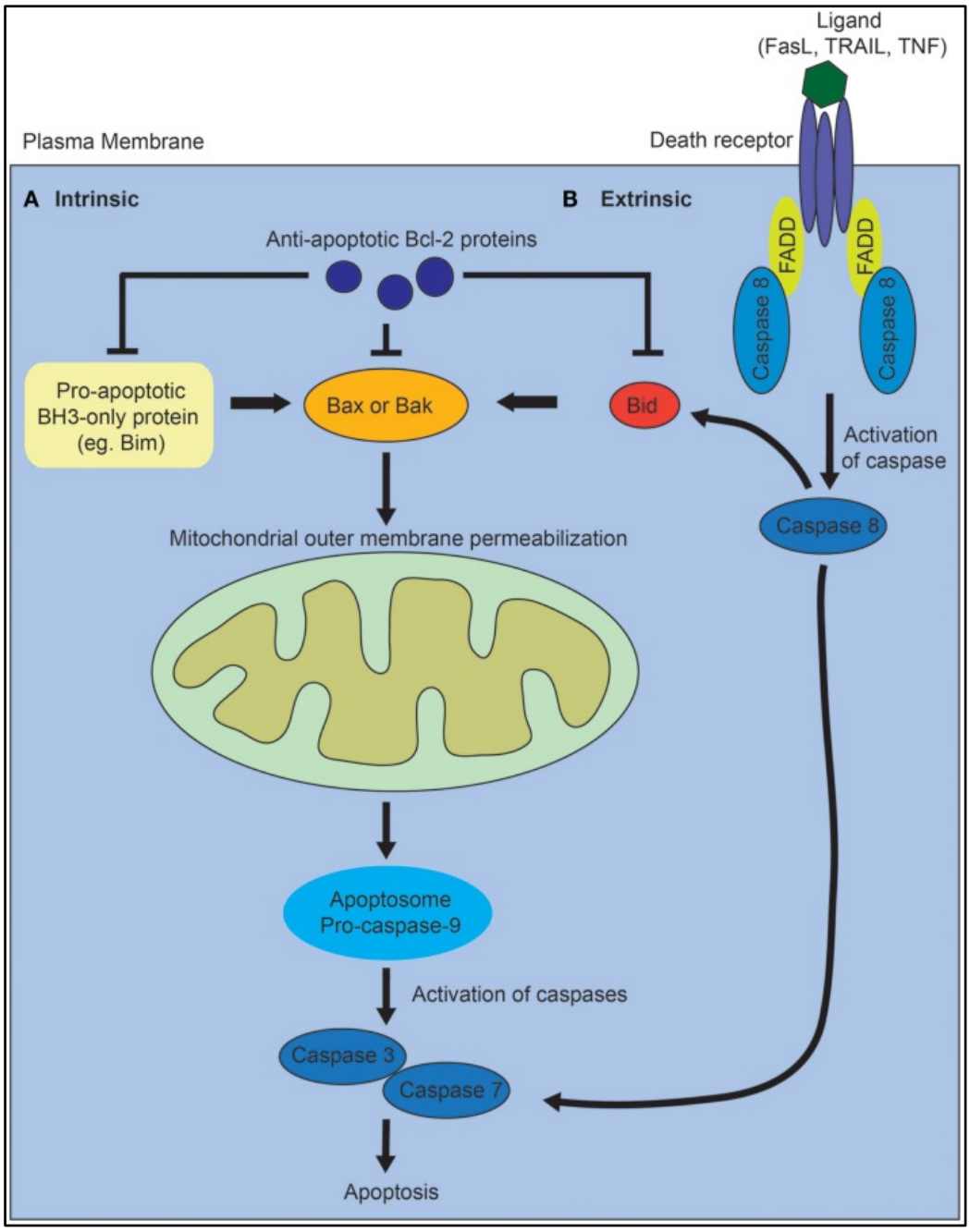

Figure 3. Extrinsic and intrinsic pathways of apoptosis. Source:(J. Ho, 2014)

2.4. Current Treatment Strategies

Improvement in the treatment of CRC has increased the survival rate of patients (Xie et al., 2020). Removing the cancerous tumor and metastases through surgery is the most effective treatment of CRC. Some patients are treated by chemotherapy and radiotherapy to ensure shrinkage of the tumor, which helps curb their tumors' growth. The patient may also undergo chemotherapy and radiotherapy before or after surgery to ensure that the tumor is reduced entirely and to stabilize it (Xie et al., 2020). Chemotherapy remains the primary treatment option for disseminated metastatic disease and non-resectable tumors (Daaboul & El-Sibai, 2018). Treatment of CRC through different stages is mentioned below.

Treatment of stage 0 CRC: The tumor is occasionally restricted to the internal colon lining in stage 0 colorectal cancer. All that is required is the surgical procedure for the removal of cancer. In most cases, a colonoscopic local excision or polypectomy is sufficient. In more developed tumors, a partial colectomy is needed (Daaboul & El-Sibai, 2018).

Treatment of malignant polyps: "Malignant polyps are adenomas that have been identified histologically, to be adenocarcinomas which have invaded through the muscularis mucosa into the basic submucosa. For the low-risk stage I rectal cancer, both endoscopic resection and transanal excision can be used" (Daaboul & El-Sibai, 2018).

Treatment of stage I CRC: Stage I CRC refers to cancer that has not spread beyond the colon layers to the other surrounding organs. Surgical excision of cancer in stage I is frequently curative. Transanal excision and endoscopic resection are occasionally used for treating low-risk stage I rectal cancer (Daaboul & El-Sibai, 2018).

Treatment of stage II CRC: The significance of adjuvant treatment in stage II

CRC is unknown. Surgical treatment must aim for a broad excision of the tumor, the affected bowel segment, and the removal of the lymphatic system draining the affected region (Daaboul & El-Sibai, 2018).

Treatment of stage III CRC: Any tumor size with metastases to regional lymph nodes characterizes stage III colon cancer. A partial colectomy is performed to remove the affected portion of the colon and the surrounding lymph nodes, preceded by adjuvant chemotherapy (Daaboul & El-Sibai, 2018).

Treatment of stage IV CRC: In resectable metastases or locally recurrent disease, curative surgical intervention is always the preferred standard of care. Systemic chemotherapy remains the primary treatment option for disseminated metastatic disease and non-resectable tumors. Fluoropyrimidines are the cornerstone of all guidelines for treating metastatic CRC(Daaboul & El-Sibai, 2018).

Though chemotherapy has been the primary treatment strategy for the past two decades, new therapeutic options such as targeted monoclonal antibodies, immunotherapy, and cytokine targeting have begun to show promise in preclinical and clinical trials. IL-6 has been identified as a key cytokine that promotes tumor cell proliferation and inhibits apoptosis (Waldner, Foersch, & Neurath, 2012) . Clinical and experimental evidence clearly suggests that IL-6 signaling has a role in developing both sporadic and colitis-associated colorectal cancer (CAC), thus could be a potential target for CRC therapy (Atreya et al., 2000; Matsumoto et al., 2010). In CAC mouse models, suppressing or eliminating IL-6 or its receptor reduced the tumor burden (Bollrath & Greten, 2009). Therefore, blocking IL-6 in IBD patients may prevent or delay the onset of CAC (Miyabayashi et al., 2015).

In recent years, the other most important shift in cancer treatment has been Immunotherapy(Grierson, Lim, & Amin, 2017; Stein, Moehler, Trojan, Goekkurt, &

Vogel, 2018). Immune checkpoint inhibitors, which work by blocking checkpoint proteins from binding with their counterpart proteins, thereby allowing T cells to kill cancer cells, are taking the lead in cancer immunotherapy (Grierson et al., 2017; Stein et al., 2018). The most well-known inhibitory checkpoint mechanisms are CTLA-4 (cytotoxic T lymphocyte-associated molecule-4), PD-1 (programmed cell death receptor-1), and PD-L1 (programmed cell death ligand-1). In individuals with advanced CRC and mismatch-repair failure, therapy with an inhibitor of the costimulatory molecule PD-1 has been shown to be effective (Chu et al., 2015). In advanced CRC, monoclonal antibodies targeting specific growth factors, or their receptors are also being used alone or in combination with chemotherapy (Cunningham et al., 2004; Hurwitz et al., 2004; Mody, Baldeo, & Bekaii-Saab, 2018; Vincenzi et al., 2006).

Even though modern treatments have improved overall survival rates, their effectiveness is limited, with substantial adverse side effects recorded. This raises concerns about using these forms of treatment in CRC patients. Furthermore, a surge in drug-resistant cancer cells reduces the usefulness of available therapies. These drug-resistant tumors have made the treatment of CRC very challenging. Although it is possible to control the adverse effects if the polyps are detected early, late detection means that patients have no appropriate treatment that can be viable for them. Therefore, further research is necessary for effective and safe alternative therapies for CRC treatment.

2.5. Signaling Pathways Deregulated in CRC

Multiple molecular changes and genetic alterations cause the dysregulation of signaling pathways leading to increased cells proliferation, migration, invasion, and inhibition of apoptosis, which contribute to CRC development and metastasis. The essential signaling pathways that are dysregulated in CRC include Nuclear Factor

Kappa-light-chain-enhancer activated B (NF-kB), epidermal growth factor receptor (EGFR), PI3K/AKT, Wnt, vascular endothelial growth factor (VEGF), JAK/STAT, TGF/SMAD, Notch, Hedgehog, Hippo and immune checkpoint signaling (Ahmad et al., 2021; Bertrand, Angus, Partis, & Sigounas, 2012; Jeong, Ro, & Choi, 2018; Koveitypour et al., 2019; Lopez, Harada, Vasilakopoulou, Shanbhag, & Ajani, 2019; Tiwari, Saraf, Verma, Panda, & Jain, 2018; Vinson, George, Fender, Bertrand, & Sigounas, 2016; H. B. Wang et al., 2017).

NF-kB transcription factor plays an essential role in immune responses, inflammation, cell proliferation, and cell death (Hassanzadeh, 2011). Dysregulation of the NF-kB signaling pathway is involved in tumor development and progression and resistance to anticancer therapies suggesting its importance as a potential therapeutic target in CRC (Hassanzadeh, 2011).

JAK-STAT signaling pathway plays a role in immunity, tumor formation, and cell division. This pathway transmits information to the cell nucleus, enabling oncogenes' transcriptional activation and repression of tumor suppressor genes. This pathway comprises three components, Janus Kinases (JAKs), Signal Transducer and Activator of Transcription (STAT) proteins, and binding receptors. Disruption of JAK-STAT may lead to cancer formation and other diseases affecting the immune system (Slattery, Lundgreen, Kadlubar, Bondurant, & Wolff, 2013). The TGF-β signaling pathway is paradoxically involved in the susceptibility and advancement of colorectal cancer. In the normal intestinal epithelium, TGF-β acts as a tumor suppressor by inhibiting cell proliferation and inducing apoptosis. However, in the late stages of CRC, TGF-β levels increase, promoting invasion and metastasis, acting as a tumor promoter. It is one of the most frequently changed cellular signaling pathways in human malignancies (Bellam & Pasche, 2010).

Epidermal Growth Factor Receptor (EGFR) is a receptor protein found in stromal and epithelial cells in smooth muscle cells (Pabla, Bissonnette, & Konda, 2015). EGFR family members play an essential role in normal cell growth and development. EGFR signaling is involved in apoptosis, cell differentiation, and cell division. Abnormal changes in the EGFR signaling cascade, such as gene alterations, gene amplification, and protein overexpression, contribute to increased proliferation, survival, and metastasis of transformed cells (Lindsey & Langhans, 2015).

AKT, also known as protein kinase B, is a group of 3 threonine protein kinases responsible for apoptosis, glucose metabolism, and cell proliferation (Pandurangan, 2013). The Akt pathway activates the growth of tumors through tensin homolog, PTEN, and phosphatase. Tensin homolog is a negative Akt regulator and is found in about 60-70% of cancer. Phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt) signaling is likely to aid colon cancer therapy. 20% of colon cancer tumors have the mutation for the β-catenin gene, which influences downstream signaling in the P13K/Akt pathway. Natural and synthetic drugs target this pathway which reduces the burden of the tumor.

The Notch pathway regulates cell death during the development process. Its ligands have transmembrane proteins which prevent signaling transfer to surround cells (Bray, 2006). Although the Notch signal's intracellular transduction is very basic, requiring no secondary messengers, it is involved in a wide range of developmental events, and its failure has been linked to many malignancies.

Irregularity in DNA damage repair pathways also contributes to cancer development. DNA damage results from endogenous reactive oxygen species, error in replication or recombination, carcinogens such as ionizing radiation, UV light, polycyclic aromatic hydrocarbons, heterocyclic aromatic amines, mycotoxins, aristolochic acid, nitrosamines, and particulate matter (Barnes, Zubair, John, Poirier, &

Martin, 2018) and some anticancer medications. A schematic presentation of PI3K/Akt, RAS/MAPK, JAK/STAT signaling pathways is provided in Figure 4.

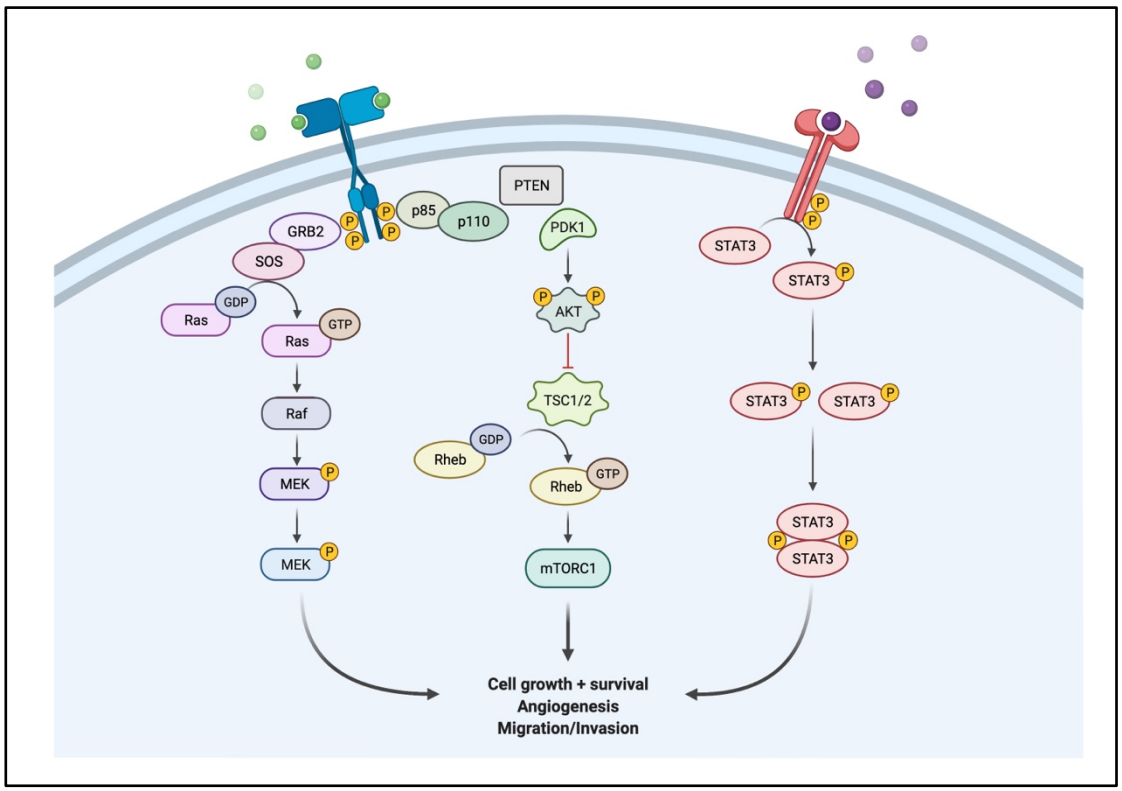

Figure 4. A schematic representation of PI3K/Akt, RAS/MAPK, and JAK/STAT signaling pathways in cancer. This figure depicts the PI3K/Akt, RAS/MAPK, and JAK/STAT signaling pathways which regulate cell growth/survival, angiogenesis, and cell migration. Growth factors bind to Receptor tyrosine kinases (RTKs), followed by activation of Ras/Raf/MAPK, PI3K/Akt/mTOR, and JAK/STAT pathways to promote diverse hallmarks of cancer development.

2.6. Anti-microbial Peptides

Anti-microbial peptides (AMPs), also known as host defense peptides, are tiny cationic or amphipathic molecules produced by prokaryotic and eukaryotic organisms that play an important role in innate immunity against viruses, bacteria, fungi, and mycoplasma and also exhibit immunomodulatory activities by affecting cytokine production, wound healing, angiogenesis, apoptosis of immune cells and as

immunoadjuvants (Brogden, 2005; Dos Santos-Silva et al., 2021; Hancock & Diamond, 2000; Klotman & Chang, 2006; Kuwano, Tanaka, Shimizu, & Kida, 2006; Niyonsaba et al., 2007; Pasupuleti et al., 2012; Raheem & Straus, 2019; Scott, Davidson, Gold, Bowdish, & Hancock, 2002; Tani et al., 2000). Cationic antitumor peptides are considered promising targets for cancer therapy due to their simple structure and specific cytotoxicity to cancer cells (Mulder, Lima, Miranda, Dias, & Franco, 2013). Different mechanisms of action of these cationic antitumor peptides have been described in Figure 5. Although anti-microbial peptides (AMPs) have primarily been studied as potential alternatives to antibiotics for treating infectious diseases, their use as anticancer peptides (ACPs) in cancer therapy, either alone or in combination with other conventional drugs, has been considered a possible therapeutic strategy to investigate. Several AMPs that act as ACPs can cross cell membranes and kill cancer cells (Table 1). The presence of negatively charged phospholipid phosphatidylserine (PS) on tumor cells' surfaces is induced by high levels of reactive oxygen species and hypoxia, which alter the tumor microenvironment and cause membrane phospholipid dysregulation (Baxter, Lay, Poon, Kvansakul, & Hulett, 2017). Tumor cells lose the asymmetry of phospholipid distribution between the outer and inner layers of the plasma membrane, exposing PS on the outer layer, helping easy binding by ACPs (Felicio, Silva, Goncalves, Santos, & Franco, 2017). In addition to increased PS expression, high levels of zwitterionic phosphatidylethanolamine molecules, deregulated glycolipid glycosylation, and membrane glycoproteins with repeated regions of O-glycosylation, as well as over-expression of heparan sulfate proteoglycans, all contribute to an increase in negative net charge on cancer cell membranes, making them a selective target for ACPs with increased electrostatic binding (Baxter et al., 2017; Raman & Kuberan, 2010). Other characteristics that

increase the susceptibility and selective cytotoxic activity to ACPs include the presence of filopodia and microvilli on the surface of tumor cells. The increased number of microvilli increases the contact area, further contributing to increased affinity for ACPs (Piotrowska, Sobczak, & Oledzka, 2017). Internalization of ACPs compromises the fluidity and integrity of tumor cell membranes, resulting in cell lysis (Kunda, 2020). An important class of ACPs, Cathelicidins, have been demonstrated to have anticancer effects in a variety of malignancies via a variety of mechanisms, mainly through activation of specific cell surface receptors, membrane channels, and intracellular targets (Mader, Mookherjee, Hancock, & Bleackley, 2009; Ren et al., 2012; Wu et al., 2010). Human defensins (α and β) have been shown to have cytolytic activity against several cancer cells by forming dimers on cell membranes thereby inducing apoptosis via an extrinsic (cytoplasmic) pathway involving the Fas death receptor, or an intrinsic (mitochondrial) pathway involving the release of cytochrome c from the mitochondria and activation of death signals (Chavakis et al., 2004; Phan et al., 2016; Y. S. Wang et al., 2009; Xu et al., 2008). Bovine lactoferricin (LfcinB), another important class of ACPs and LTX-315 which is designed from the more active structure of LfcinB have shown antitumor activity against several cancer cells (Eliassen et al., 2006; Furlong, Ridgway, & Hoskin, 2008; Haug et al., 2016; Sveinbjornsson, Camilio, Haug, & Rekdal, 2017). More details about these and other ACPs are reviewed in (Baxter et al., 2017; Chu et al., 2015; Deslouches & Di, 2017; Jin & Weinberg, 2019; Tornesello, Borrelli, Buonaguro, Buonaguro, & Tornesello, 2020).

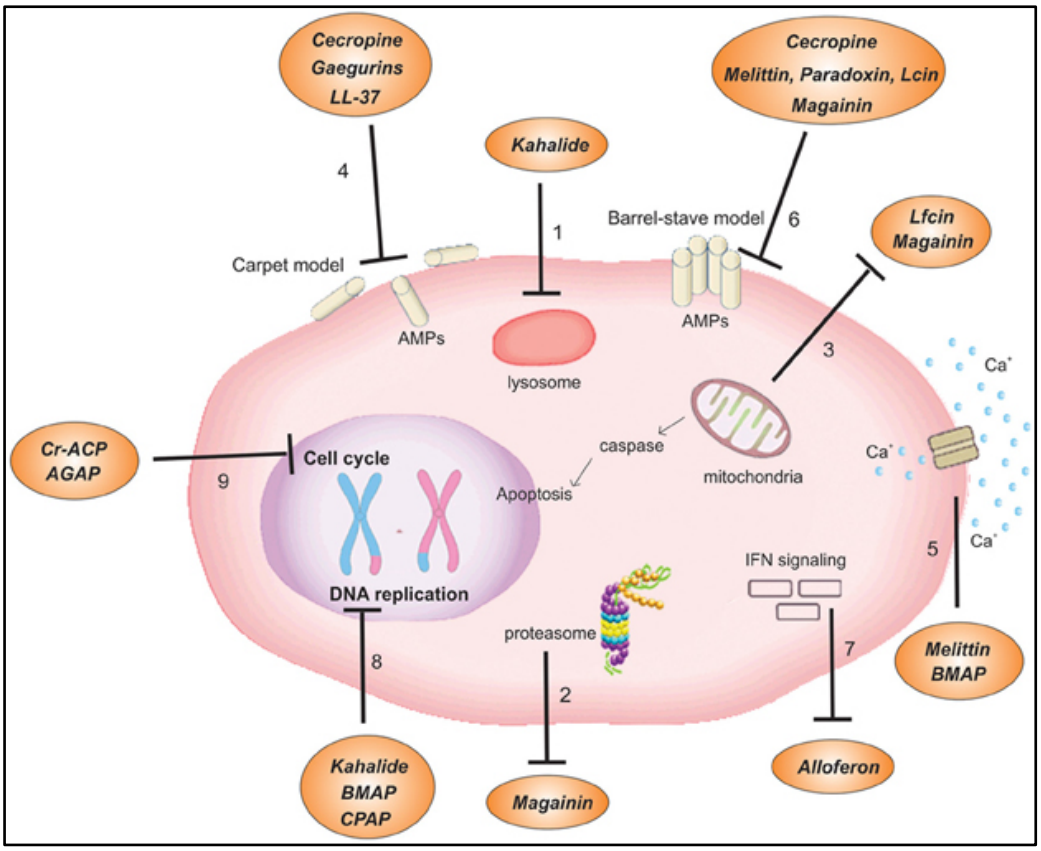

Figure 5: Mechanisms of action of anticancer peptides. Source:(Mulder et al., 2013).

Table 1. Anti-cancer peptides and their cancer targets

ACPs	**Structure**	**Cancer target**	**References**
L-K6	α-helical	MCF-7	(C. Wang et al., 2017)
LL-37	α-helical	HCC MCF-7 LoVo HCT116	(Ren et al., 2013; C. Wang et al., 2017; H. Zhang et al., 2022)
MdCecA	α-helical	leukemia	(Hui, Leung, & Chen, 2002)
Polybia-MPI	α-helical	PC-3 Biu87 EJ	(K. R. Wang et al., 2008)
MG2	α-helical	RT4 MDA-MB-231	(Lehmann et al., 2006) (Anghel, Jitaru, Badescu, Badescu, & Ciocoiu, 2013)
MPLfcinB6	β-pleated sheet	Jurkat CEM	(Hilchie, Vale, Zemlak, & Hoskin, 2013)
LfcinB-P13	β-pleated sheet	SMMC772 L02	(Meng et al., 2017)
Aurein	α-helical	T98G	(Dennison, Whittaker, Harris, & Phoenix, 2006)
HNP-1	β-pleated sheet	PC-3	(Gaspar, Freire, Pacheco, Barata, & Castanho, 2015)
TAT-RasGAP317-326	unknown	STC	(Heulot et al., 2017)

LFB	unknown	STC	(B. Li et al., 2019)
CB1	unknown	HTC STC	(X. Li et al., 2016)
DRS-B2	α-helical	PC3 DU145 LnCap	(Dos Santos et al., 2017)
hBD3	mixed structure	HTC STC	(Phan et al., 2016)

Abbreviations: HCC: hepatocellular carcinoma; SMMC772, HCT and L02: Liver cancer; STC: Soft tissue cancer; MCF-7 and MDA-MB-231: breast cancer; Lovo and HCT116: colorectal cancer. MG2: Magainin 2; LL-37: human cathelicidin peptide; Cecropin A: MdCecA; RT4, Biu87, and EJ: bladder cancer; Jurkat and CEM: T-leukemia; T98G: brain Cancer; LFB: Limnonectes fujianensis Brevinvin; CB1: Cecropin B1; LfcinB: Bovine lactoferricin.; HNP-1: human neutrophil peptides. PC-3, DU145 and LnCap: Prostate cancer; HNP-1: Human neutrophil peptide; DRS-B2: Dermaseptin B2. hBD3: Human b-defensin-3.

Despite AMPs ability to directly inhibit or kill microbial pathogens, there are certain limitations regarding their usage. The interaction of AMPs with host cells can weaken or result in the loss of activity for some AMPs (Starr, He, & Wimley, 2016). Also, some AMPs tend to get digested and absorbed into the bloodstream resulting from their high absorbance within the body solvent (Heymich, Srirangan, & Pischetsrieder, 2021). Concurrently, most of the AMPs possess a short half-life due to rapid degradation by proteolytic enzymes in the blood plasma and fast hepatic and renal clearance (Vlieghe, Lisowski, Martinez, & Khrestchatisky, 2010). Moreover, serum protein binding, proteolytic degradation, and lack of selectivity impede the systemic activity of AMPs (Jenssen, Hamill, & Hancock, 2006). Another challenge is the

permeability of the microbial membranes that might reduce the effectiveness of the peptides against pathogens, and some AMPs might lead to hemolytic activity (Jun Lei et al., 2019).The stability of AMPs is subject to internal and external variables resulting in unaccounted side effects that reduce their efficacy(Huan, Kong, Mou, & Yi, 2020). Based on these limitations aforementioned, it can be deduced that more combinations of peptides and techniques are required to overcome the limitations exhibited by AMPs. Secondly, to overcome the proteolytic degradation, protease-resistant peptides such as cyclized AMPs and peptide analogs can be used. Encapsulating AMPs into functioning carrier systems such as nanoparticles can preserve them from degradation, modify their release and bioavailability profile, and target their tissue distribution. The other approaches to overcome low activity, stability, and side effects of natural AMPs are shortening the length of peptides, generating chimera molecules and creating structural scaffolds, which lead to the design of novel bioinspired AMPs (BSAMPs) (Fjell et al., 2011; Ji et al., 2014; C. K. Wang et al., 2014). For the peptide selectivity and to assess the effect on host cells, different hemolysis and bactericidal assays can be utilized (Jun Lei et al., 2019). These methods exemplify future findings and directives towards microbial resistance to AMPs.

Two BSAMPs have been developed so far, in collaboration with the research group of the Co-Supervisor, and selected to be used in this study, that have never been investigated for their anti-cancer effects in CRC, which we referred to as peptide C (Sequence: GVLCCGYRCCSKWGWCGTTK) and peptide E (Sequence: CWWMTRRAWR) throughout the thesis for an easy nomenclature.

Thus, this study aims to examine the antiproliferative and anti-inflammatory effects of novel BSAMPs on CRC cells (SW620) and elucidate the molecular mechanisms by which they induce CRC cells death.

3. RATIONALE/HYPOTHESIS/OBJECTIVES

RATIONALE: The current treatments for CRC such as surgery, radiation therapy, immunotherapy, targeted therapy, and chemotherapy can slow the spread of CRC; however, severe side effects, drug resistance, and toxicities limit the short-and long-term outcomes in patients. Therefore, there is a pressing need for novel and alternative therapies with low cytotoxic effects and less drug resistance. In this regard, AMPs have received much interest as a potential therapeutic option for treating a variety of malignancies, including CRC, as they are more selective towards tumor cells compared to normal cells and are less toxic and may help in overcoming drug resistance.

Hypothesis: The synthetic AMPs exhibit anticancer properties through the activation of inflammatory and apoptotic pathways.

Objective: The specific aims for the study are to:

1- Investigate the effects of BSAMPs on cell proliferation, cell cycle, and apoptosis of CRC cells (SW620).
2- Assess the potential anti-inflammatory effect of BSAMPs on CRC cells (SW620)
3- Identify the molecular mechanisms of AMP-induced CRC cell death

4. MATERIAL AND METHODS

4.1. Reagents and antibodies

DMEM (1X), FBS, and PenStrep were purchased from Thermo Scientific (California, USA), and engineered BSAMPs (GVLCCGYRCCSKWGWCGTTK and CWWMTRRAWR) were purchased from NovoPro Inc (Shanghai, China). Two BSAMPs were used in our study, which we referred to as peptide C (Sequence: GVLCCGYRCCSKWGWCGTTK) and peptide E (Sequence: CWWMTRRAWR) throughout the thesis for an easy nomenclature. The annexin V-FITC apoptosis staining/detection kit was purchased from Abcam (Cambridge, UK). Antibodies Bcl-xl, c-Myc, p-Akt, Akt, p-EGFR, EGFR p-Stat3, Stat3 p-NF-kB(p65), NF-kB(p65), Cleaved caspase-3, Caspase-3 β-actin, Cleaved PARP, PARP, p21Waf1/Cip1, p27Kip1 IKBP, Bcl-2, Bax, and β-catenin were purchased from Cell Signaling Technology (Beverly, USA).

4.2. Cell culture

Human colon cancer cell lines (SW620) were purchased from the European Collection of Authenticated Cell Cultures (ECACC). CCD 841 CoN (CRL-1790) cell line was purchased from ATCC, USA, and used as a normal colon cell line. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The culture media was supplemented with 10% Fetal Bovine Serum (FBS) and 5% penicillin-streptomycin (Pen Strep). The cells were incubated in a standard humidified incubator at 37^{o}C with 5% CO_2.

4.3. Cell viability assay

CellTiter-Glo Luminescence Assay (Promega, Madison, WI) was used to determine the half-maximal inhibitory concentration (IC50) and dose-response curves for each of the

BSAMPs. This assay is a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. Cells were seeded at a concentration of 2,000 cells/well in a 96-well plate. Cells were treated with peptides following a 2-fold serial dilution treatment in 5% FBS-DMEM medium. The cell viability was measured after 24 h using the CellTiter- Glo reagent. The dose-response curves were plotted using the GraphPad Prism 9, following a non-linear Regression (four-parameter, least-squares fit) method. IC50 values were determined by a four-parameter, non-linear regression method.

4.4. Clonogenic assay

BioPioneer CellMAXTM Clonogenic assay or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. SW620 cells were seeded at 1,000 cells/well in a 6-well plate. Cells treated with different doses 0 (control), 200, and 400 µM of peptide C and peptide E for 24 h incubate for 10 days. The media was changed every 4 days. On day 10, cells were rinsed with PBS after discarding the media. Fixation was done with 4% paraformaldehyde and staining of clones was done with a mixture of 0.5% crystal violet for 45 minutes. The wells were rinsed with water and left for drying at room temperature for 24h. Counting of clones was done on the following day by using ImageJ software.

4.5. TUNEL assay

DeadEnd™ Fluorometric Terminal deoxynucleotide transferase dUTP nick and labeling (TUNEL) System (Promega) was used to measure fragmented nuclear DNA to assess cell apoptosis. Cells were seeded on chamber slides and treated with peptides C and E. The slides were then fixed with 4% paraformaldehyde with PBS for 25 minutes at 4^{o}C. The slides were then permeabilized with 0.2% Triton-X 100 and later treated with equilibration buffer for 10 minutes. After equilibration, recombinant terminal deoxynucleotidyl transferase (rTDT) incubation buffer was added to the cells

and incubated for 1 hour in a humidified chamber (37^{o}C). The reactions were terminated by adding 2X SSC to the cells for 15 minutes at room temperature. The cells were then washed with PBS and mounted with Vectashield DAPI (Vector Lab Cat. # H-1200) to stain the nuclei. Coverslip was added to the slide, and the TUNEL positive cells as green fluorescence were observed under an EVOS microscope.

4.6. Cell cycle analysis by Flow Cytometry

This assay was used to estimate the percentages of a cell population in the different phases of the cell cycle after treatment with peptides C and E. This assay reveals the distribution of cells in three major stages of the cell cycle (G1 vs S vs G2/M) and enables the detection of apoptotic cells with fractional DNA content. CRC cells (SW620) were seeded in T-75 cell culture flasks and incubated for 24 h, followed by treatment with peptides for 24 h of additional incubation. Cells without treatment of peptides C and E were used as a positive control. The cells were then washed, trypsinized, and centrifuged at 1000 rpm for 5 min at 25^{o}C. The resulting cell pellet was fixed with cold 70% ethanol and stored at 4^{o}C overnight. The cells were then analyzed using flow cytometry.

4.7. Annexin V/PI staining

This assay was utilized to detect early apoptotic cells upon treatment with peptides C and E and differentiate apoptotic cells from necrotic cells. Simultaneous staining with Propidium Iodide (PI) was also performed. Cells were seeded and treated with peptides C and E in a tissue culture flask. Treated cells were then harvested and washed with cold PBS (4^{0}C) .The cells were then resuspended in a binding buffer, and 100 ul of the cell solution was transferred to a falcon tube. Subsequently, Annexin V-FITC and PI were added to the treated cells and incubated at room temperature in the dark for 15

minutes. The cells were centrifuged and resuspended in binding buffer and analyzed by flow cytometry.

4.8. Measurement of Mitochondrial Membrane Potential (MMP)

To determine the effect of peptides C and E on MMP in SW620 cells, the JC1 stain kit was used as described previously (Prabhu et al., 2017). The membrane-permeant JC-1 dye is an indicator of mitochondrial membrane potential and is widely used in apoptosis studies to monitor mitochondrial health. According to the manufacturer's instruction, cells were stained for 10 minutes with JC-1 stain in the dark. The loss in MMP was determined using flow cytometry as a reduction in red fluorescence. The mean values were plotted in the graph for loss of MMP representation.

4.9. Western blot

The effect of peptides C and E on cell proliferation, apoptosis, and inflammation was investigated using Western blotting to examine the expression of respective protein markers. Cells were seeded in 6-well culture plates and treated with different concentrations of peptides C and E. After 24 h; the cells were washed using ice-cold PBS (4^0C). RIPA lysis buffer was then added to the cells (RIPA buffer, Protease Inhibitor Cocktail, PMSF lysis and Sodium orthovanadate). Different amounts of the protein (15 and 20 µg) were loaded on 8%, 10%, and 12 % SDS-PAGE. Blots were blocked for 1 hour at room temperature with 5% blocking buffer (TBST containing 5% Skimmed Milk powder) and then incubated overnight at 4^oC with the respective antibodies. Subsequently, the blots were washed with TBST 3 times and then incubated with anti-mouse (anti-mouse HRP conjugated secondary antibody) and anti-Rabbit (anti-Rabbit HRP conjugated secondary antibody) at room temperature for 1 hour. Immobilon Western Chemiluminescent HRP substrate detection reagent (Millipore) was used to detect the protein bands. All immunoblots were imaged using ChemiDoc (BioRad, USA).

4.10. Statistical analysis

The statistical analysis was performed using GraphPad Prism 9 software, version 5.0 (Sandiego, CA, USA). Statistical differences between mean ± SD values were evaluated by one-way ANOVA (nonparametric) Dunnet multiple comparison test. Differences between groups with $p < 0.05$ were considered statistically significant.

5. RESULTS

5.1. BSAMPs inhibit cell proliferation and colony formation of SW620 CRC cells

The first objective of this study was to determine the effect of BSAMPs (peptide C and E) on the viability of SW620 cells. CCD841 was used as a normal control cell line. SW620 and CCD841 cell lines were treated with an increasing concentration of BSAMP for 24 h. Assessment of cell viability was performed using CellTiter- Glo solution. Figure 6 shows the effect of peptide C and E treatment of SW620 cells at 24 h. Results show a significant decrease in the cell viability in a dose-dependent manner when treated with 0 (control), 100, 200, and 400 μM of peptide C and E, respectively. The half-maximal inhibitory concentration (IC50) was achieved at 24 h for SW620 cells. IC50 for SW620 cells was observed in 188.402 μM of peptide C and 111.102 μM of peptide E, whereby the cell viability decreased from 100% to 60.1% ± 0.5 and 32.7% ± 0.9, respectively for peptide C. In contrast, the cell viability decreases from 100% to 25.21% ± 0.5 and 10.2% ± 0.9, respectively for peptide E.

To determine the effects of BSAMP on normal cells, CCD841 cells were treated with the same doses of BSAMP as mentioned above for 24 h. After the treatment period, CellTiter- Glo solution was added to the cells to assess the cell viability. Results showed that peptides C and E do not have any inhibitory effects on CCD841as there were no differences observed in the cell viability compared to the control and BSAMP treated wells Figure 6.

Next, we investigated whether peptides inhibit the colony-forming ability of SW620 cells. First, SW620 cells were seeded (2000 cells/well) in complete medium in six-well plates and allowed to adhere for 24 h. The medium was then replaced with a complete medium containing respective peptides (C and E) at different doses 0 (control), 200 and 400 μM), and the ability of SW620 cells to form colonies was monitored over the next 10 days, as described in Materials and Methods section. Our results show that compared to

control (0 μM), treatment of SW620 cells with 200 and 400 μM of peptide C and E significantly inhibited the colony formation, as shown in Figures 7A, B, and C.

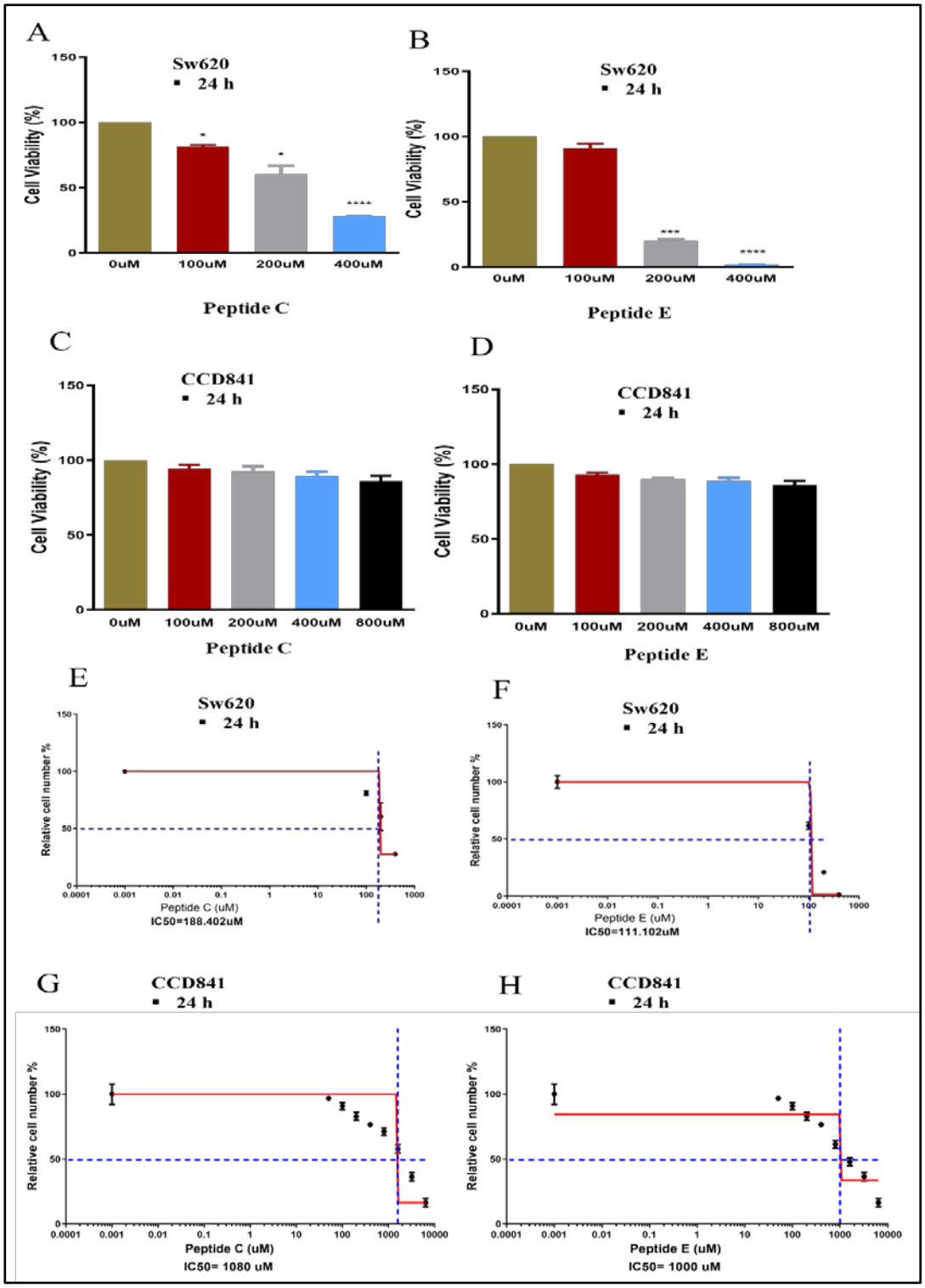

Figure 6. (A-B) Effect of Peptides C and E on cell viability of SW620 cells (C-D) CCD841 (normal colon epithelial cells) after treatment with 0 (control), 100, 200, 400,

*800 µM of peptide C and E for 24 h. The graphs display the mean± SD (standard deviation) of three independent experiments with replicates. *p<0.05, ***p<0.001, ****p<0.0001.(E-F) The curve of IC50 value and the inhibition % of Peptides C and E on SW620 cells. Each data point represents an average of three independent experiments. (G-H) The curve of IC50 value and the inhibition % of Peptides C and E on normal CCD841 cells. Each data point represents an average of three independent experiments.*

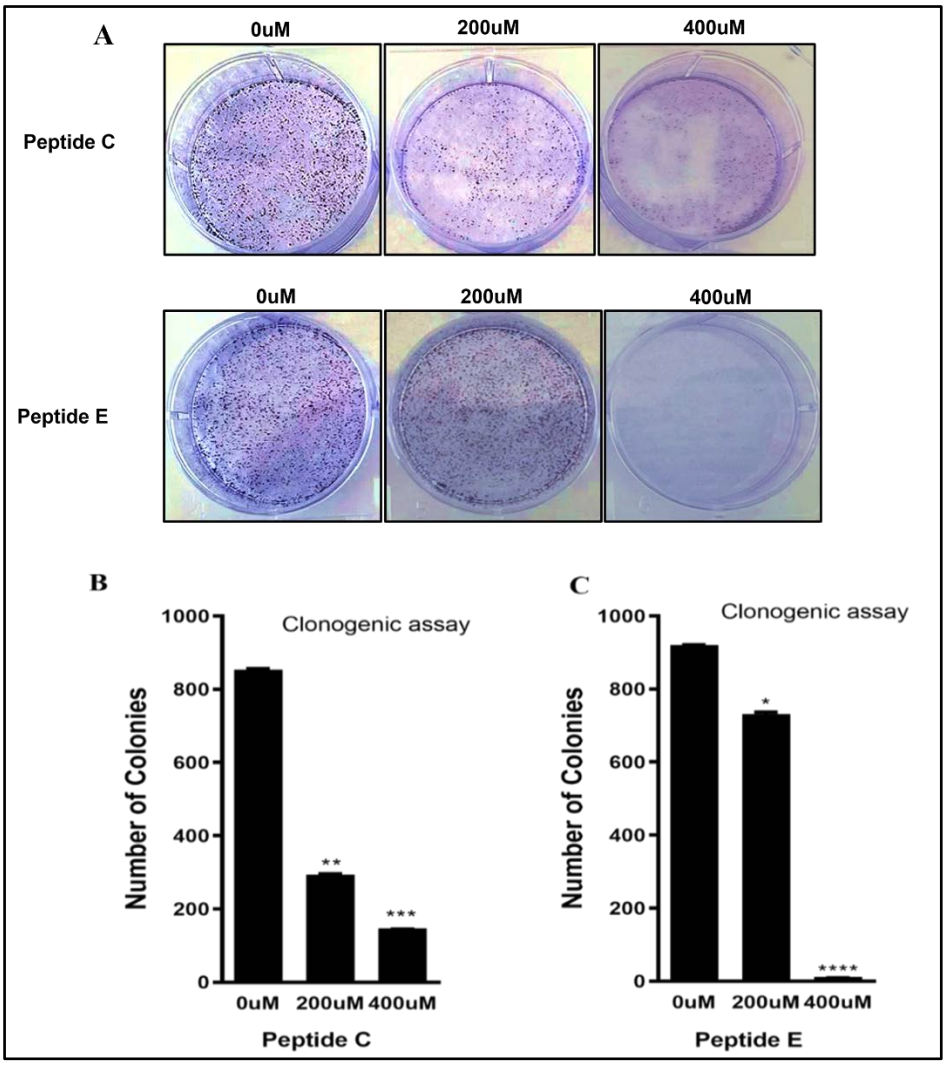

Figure 7. A) Clonogenic assay was performed in SW620 cells after treatment with doses 0 (control), 200, 400 µM of peptide C and peptide E for 24 h. Top panel shows microscope captured images of representative colonies for peptide C and E. B) Quantification of clonogenic data for peptide C using Image J software Error bar represents mean± SD. C) Quantification of clonogenic data for peptides using Image J software. Error bar represents mean± SD.

5.2. BSAMPs caused cellular DNA damage, cell cycle arrest, mitochondrial membrane depolarization and induced apoptosis in SW620 cells

To evaluate the ability of BSAMPs (C and E) in causing cellular DNA damage in SW620 cells, a TUNEL assay was performed. SW620 cells were seeded (20000 cells/well) in complete medium in 4-well chamber slides and allowed to adhere for 24 h. The medium was then replaced with a complete medium containing respective peptides (C and E) at different doses 0 (control), 200, and 400 μM for 24 h. The TUNEL assay was performed for apoptosis detection, which occurs in response to DNA damage. Our results show that both peptides (C and E) induced apoptosis in a dose-dependent manner compared to control, as shown in Figures 8A and B. TUNEL positive cells were quantified using Image J software. DAPI (blue) was used to stain the nucleus. The apoptotic cells (green) were detected under the confocal microscope.

Next, we wanted to investigate the effect of peptides C and E on cell cycle arrest as this is also a cause for cell apoptosis. The cell cycle phase distribution of SW620s cells treated with peptide C and E at 24 h is depicted in Figure 9. The distribution of different cell cycle phases for SW620 was altered in a dose-dependent manner after incubation with increasing doses of peptides C and E, 0 (control), 200, 400 μM for 24 h. The control condition showed more accumulation of cells in G0/G1 (71.4%) with approximately the same proportion of the cells in S and G2/M (14.8 %). After exposure to 200 and 400μM of peptide C and E (23.3% to 27.5%- 38.7 %to 50%) of SW620 cells were in the SG2/M phase. Our result revealed that the most effective concentration of peptides C and E that induced the highest apoptosis in SW620 cells also caused the accumulation of cells at G2/M phase of the cell cycle. G2 phase in the cell cycle is where DNA repair might occur in cells, along with preparation for mitosis in the M phase (DiPaola, 2002). To further confirm the cell cycle arrest, we performed a western

blot analysis of two crucial cell cycle regulators, p21 and p27. The representative Western blot results of these cell cycle regulators (p21Waf1/Cip1 and p27Kip1) in SW620 Cell 24 h treated with different doses of peptide C and E: 0 (control), 200, and 400 μM Figure 10a,10b. and their quantitative analysis is summarized in Figures 10 Ba, 10Bb. The protein expression levels of (p21 Waf1/Cip1 and p27Kip1) were increased in peptide C and E compared to control.

The loss of mitochondrial membrane potential with subsequent release of apoptosis-inducing factors causing caspase activation and nuclear condensation also contributes to apoptosis. SW620 cells were treated with peptide C and E 0 (control), 200, and 400 μM labeled with JC-1 stain and measured using flow cytometry. It was observed that MMP loss was 7.8% in untreated SW620 cells and increased significantly upon treatment with peptide C and E and reached a value of 26.7% -24.9 % with 200 μM and 50.7%-37 .8% with 400 μM peptide C and E Figure 11.

To confirm that the growth inhibition of SW620 cells occurs via the apoptosis cell death pathway, an annexin V-FITC/PI dual staining experiment was performed using the flow cytometry technique. The cell surface of healthy cells is composed of lipids distributed in the inner and outer plasma membrane. Phosphatidylserine (PS) is a lipid restricted in the inner plasma membrane and is exposed only to the cytoplasm. During apoptosis, PS is exposed to the outer plasma membrane, and it can bind to Annexin V, a calcium-binding protein. Furthermore, Annexin V can also stain necrotic cells due to their ruptured membranes. However, co-staining of Annexin V with propidium iodide (PI) can distinguish between necrotic and apoptotic cells as PI is membrane-impermeant in apoptotic cells but not in necrotic cells where the plasma membrane collapses (Crowley et al., 2016). Early stages of apoptosis when the cell membranes are still intact stain positive for Annexin V-FITC but negative for PI (Annexin V-FITC+ve/PI−ve).

However, late apoptotic cells are observed to be double-positive (Annexin V-FITC+ve/PI+ve), and in the necrotic stage, the plasma membrane integrity is lost, and cells stain negative for Annexin and positive for PI (Annexin V-FITC-ve/PI+ive). Peptide C and E -induced apoptosis was evaluated by annexin V and PI staining in SW620 cells. The mean percentages of total apoptosis are plotted in the bar graph in Figure 12. As shown, peptides C and E significantly induce apoptosis in the SW620 cell line ($P<0.05$). Following 24h treatment with 0 (control), 200, and 400μM of Peptides C and E, there is an increase in apoptosis in a dose-dependent manner Figure 12. In the cells treated with Peptide C, the fraction of cells with total apoptosis were increased significantly (16.51%) following treatment with 200 μM. There was a further increase in total apoptosis (91.36%) when treated with 400 μM of peptide C compared to control (1.86%). A similar trend was observed with peptide E where the % cells with total apoptosis were increased significantly (34.2%) following treatment with 200 μM, and there was a further increase in total apoptosis (44.3%) when treated with 400 μM of peptide E as compared to control (1.86%).

To get more insights into the molecular mechanisms of apoptosis induced by BSAMPs in SW620 cells, we measured the expression of antiapoptotic and proapoptotic proteins by western blot. Figure 13 shows that the anti-apoptotic protein Bcl-2 and Bcl-xl expression levels were decreased, while the expression of the pro-apoptotic protein Bax was increased. Moreover, Peptide C and E markedly increased the activities of cleaved caspase-3 and cleaved PARP in SW620 cells in a concentration-dependent manner. These results indicated that Peptide C and E induced SW620 cell apoptosis through different mechanisms like cellular DNA damage, cell arrest, mitochondrial membrane depolarization, and activation of caspases, suggesting mitochondrial-mediated intrinsic apoptosis as a cause of cell death.

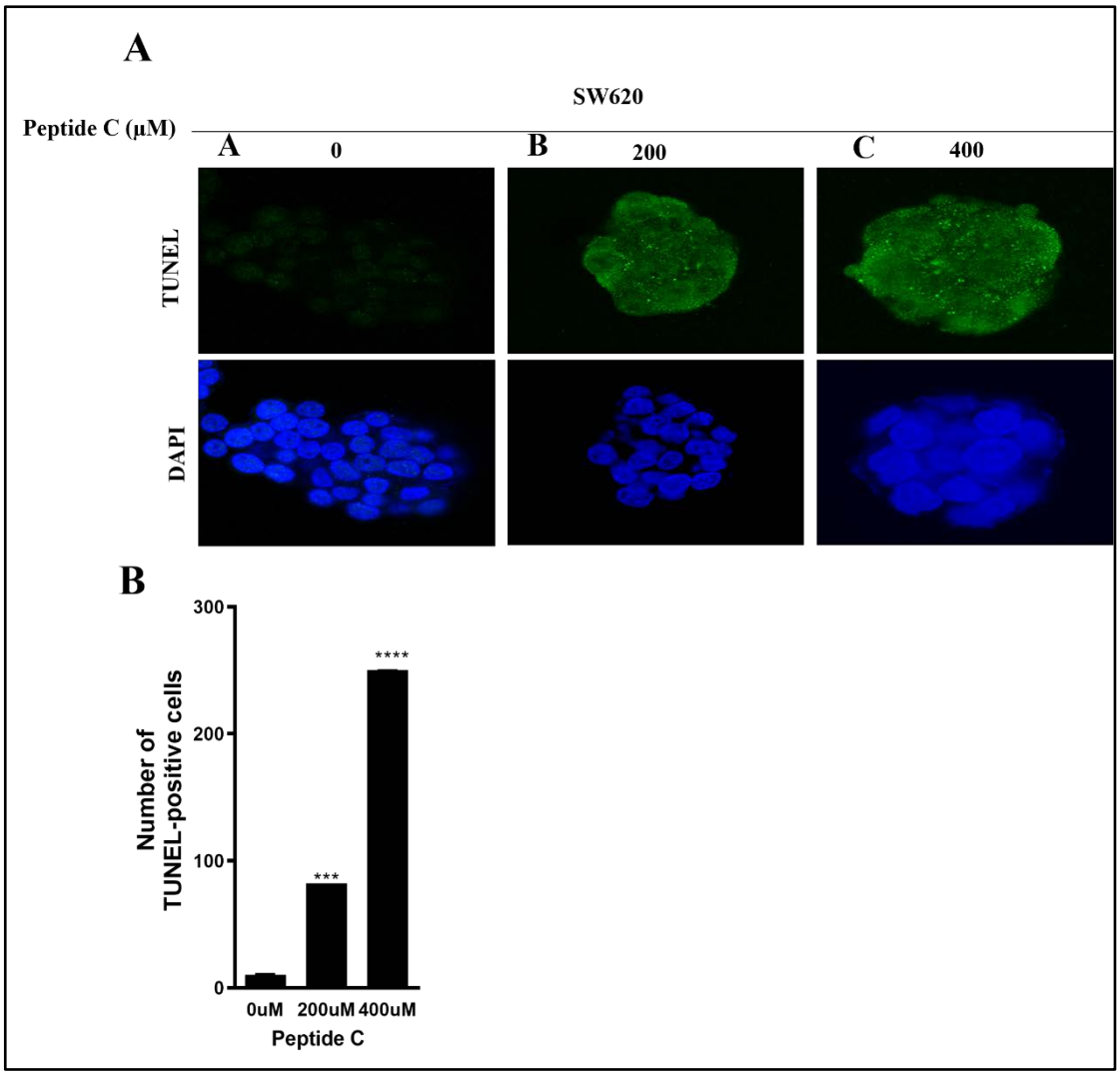

Figure 8a. TUNEL assay was performed in SW620 cells after treatment with different doses 0 (control), 200, 400 µM of peptide C for 24 h. (A) Top panel shows representative confocal images, and the (B) lower panel shows the quantification of TUNEL positive cells using Image J software. Error bar represents mean± SD.

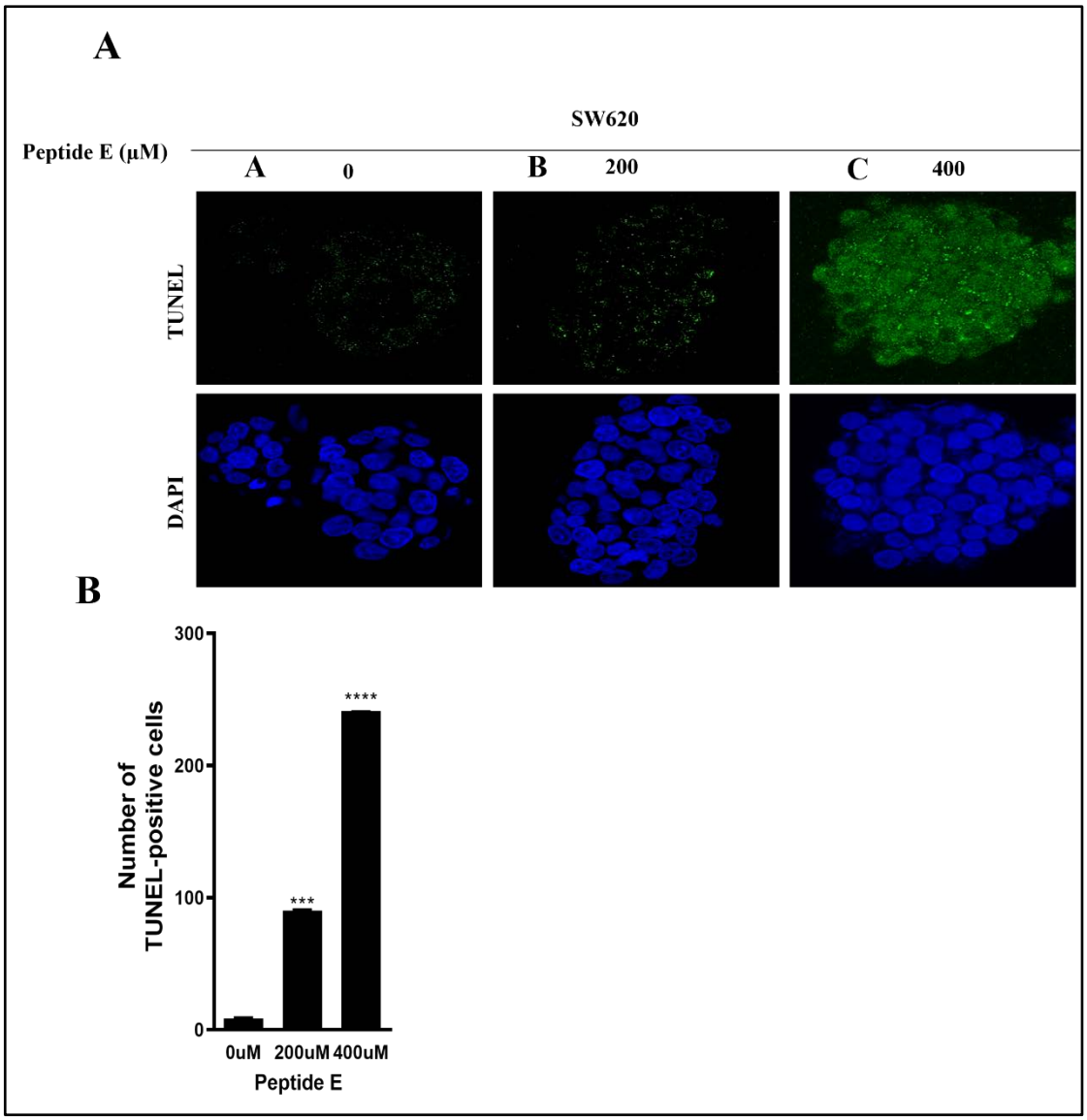

Figure 8b. TUNEL assay was performed in SW620 cells after treatment with different doses 0 (control), 200, 400 µM of peptide E for 24 h. (A) Top panel shows representative confocal images, and (B) the lower panel shows the quantification of TUNEL positive cells using Image J software. Error bar represents mean± SD.

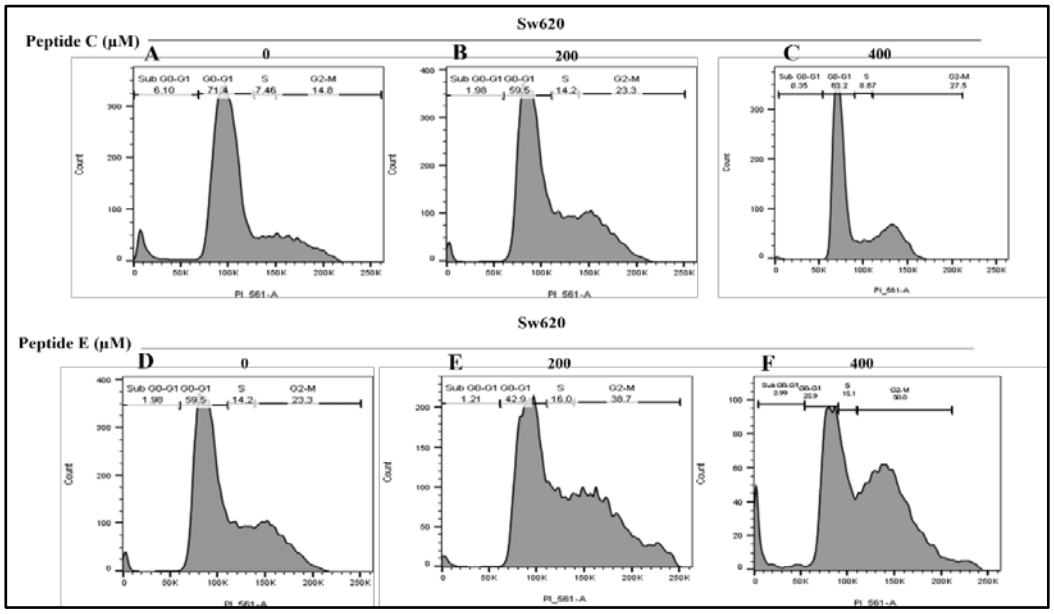

Figure 9. Cell cycle analysis using propidium iodide (PI) staining and flow cytometry. SW620 cells were treated for 24 h with (A, D) only media (B) 200 µM of peptide C; (C) 400µM of peptide C (E) 200µM of peptide E (F) 400µM of peptide E.

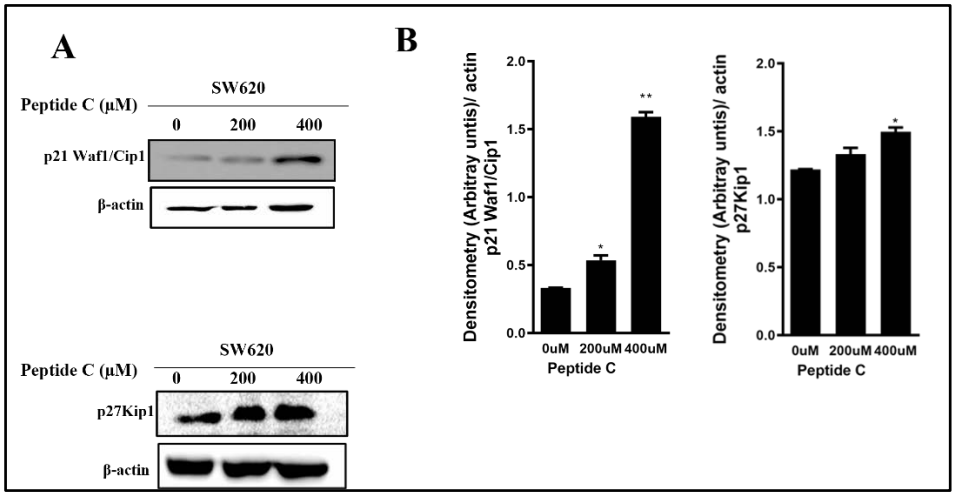

*Figure 10a. Effect of peptide C on cell cycle regulators as examined by Western blot analysis. (A) Expression of p21and Expression of p27 (B) Densitometry analysis of the western blots also showed Peptide C significantly increased the expression of p21 and p27. mean± SD. (n = 3) *p < 0.05, **p < 0.01.*

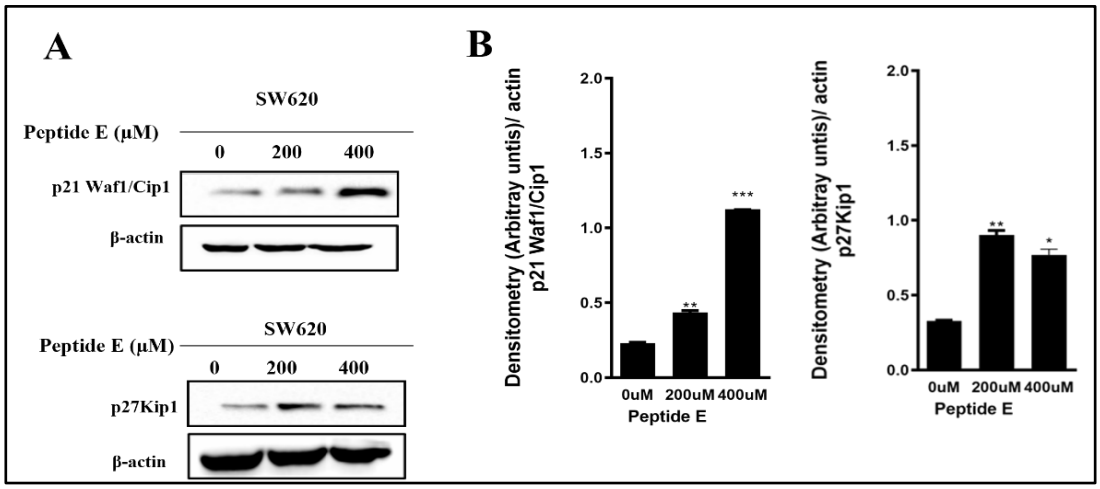

*Figure 10b. Effect of peptide E on cell cycle regulators as examined by Western blot analysis. (A) Expression of p21and Expression of p27 (B) Densitometry analysis of the western blots also showed Peptide E significantly increased the expression of p21 and p27. mean± SD. (n = 3) *p < 0.05, **p < 0.01.*

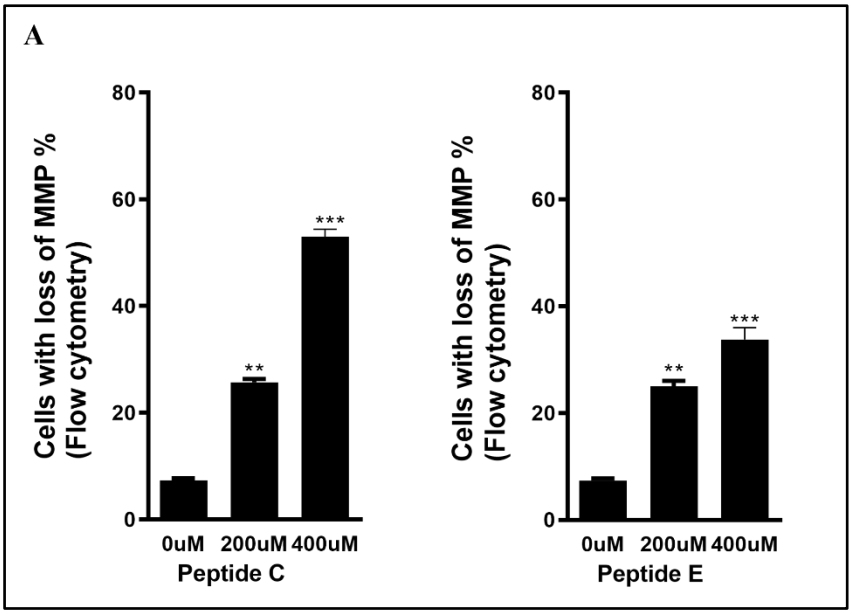

Figure 11. Peptides C and E treatment cause a loss of MMP in SW620 cells. SW620 cells were treated with increasing doses of peptide C and E for 24 h. After JC1 staining, cells were analyzed by flow cytometry.

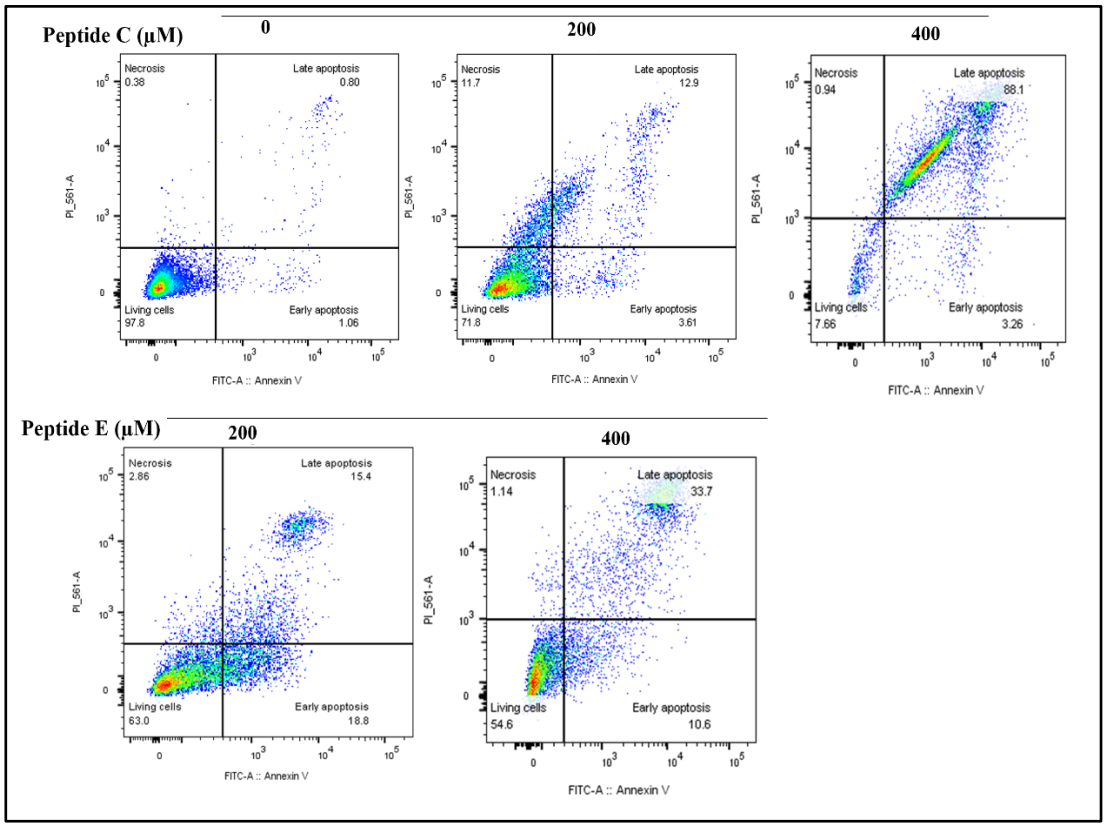

Figure 12: peptide C and E induced apoptosis in SW620 cells. (A, B, C, D, E) SW620 cells were treated with 0 (control), 200 and 400µM of peptide for 24 h and stained with annexin-V-FITC, PI and analyzed using flow cytometry.

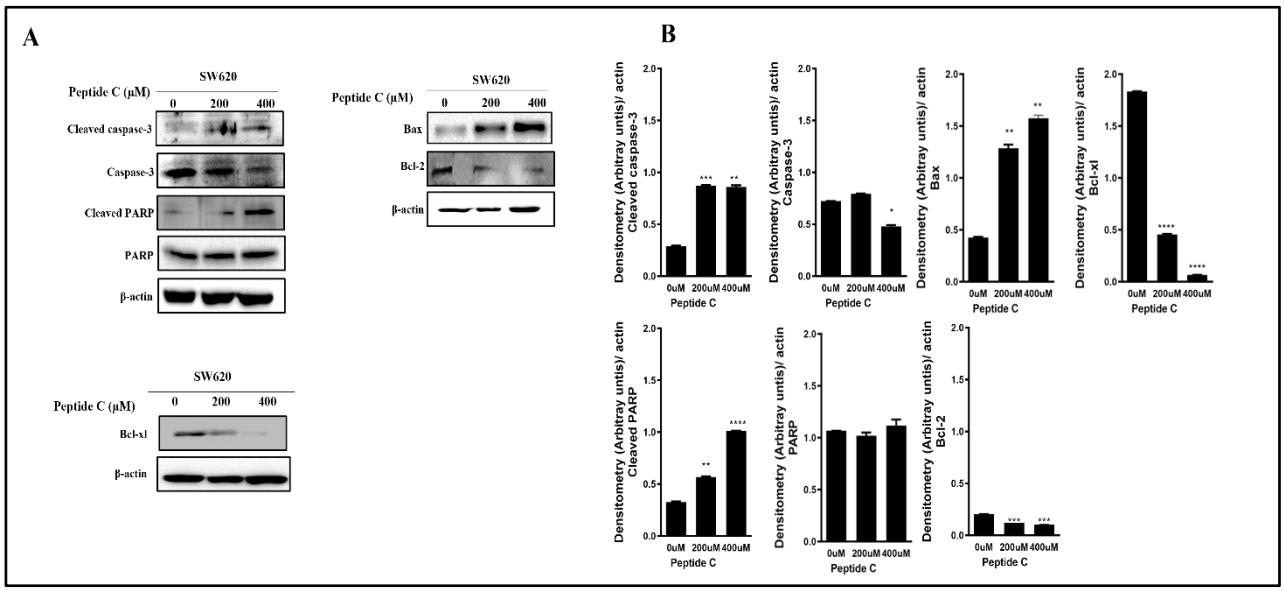

Figure 13a. Peptide C induced apoptosis in SW620 cells by increasing the expression of proapoptotic markers (cleaved caspase-3, cleaved PARP, Bax) while decreasing the expression of antiapoptotic markers (Bcl-XL, Bcl2) as shown in Figure (A).
*(B) Densitometry analysis using Image J software as shown in figure B mean± SD. (n = 3) *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.*

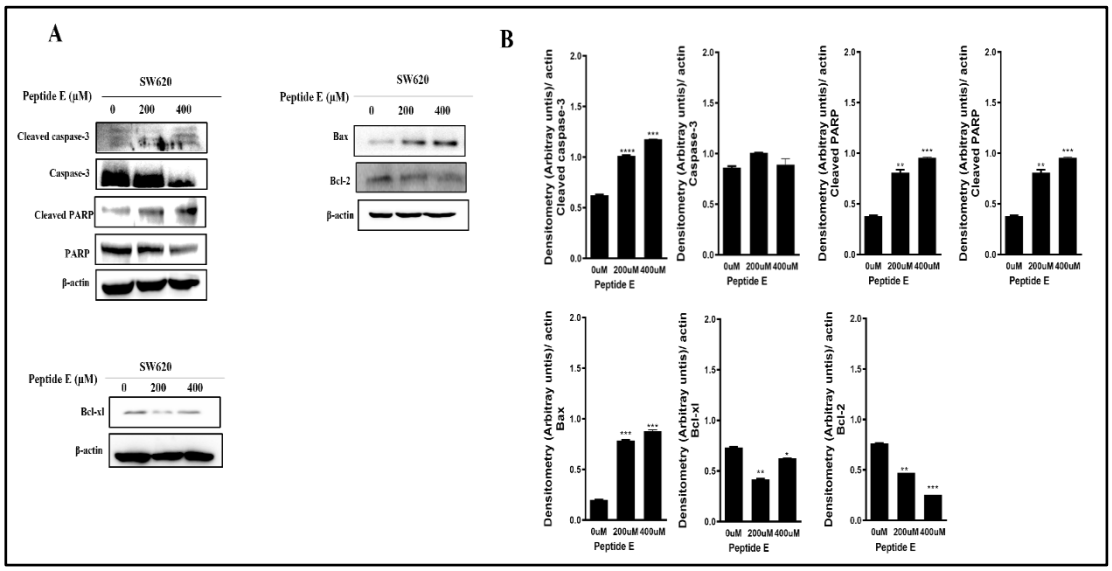

*Figure 13b. Peptide E induced apoptosis in SW620 cells by increasing the expression of proapoptotic markers (cleaved caspase-3, cleaved PARP, Bax) while decreasing the expression of antiapoptotic markers (Bcl-XL, Bcl2) as shown in Figure (A). (B)Densitometry analysis using Image J software as shown in figure B. mean± SD (n = 3) *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001*

5.3. BSAMPs inhibited activation of NF-kB, STAT3, Akt, EGFR, β-catenin and c-Myc signaling pathways

NF-kB, STAT3, Akt, EGFR, β catenin and c-Myc signaling pathways play an important role in cell proliferation, inflammation, apoptosis, and survival in many cancers, including CRC. Here, we wanted to investigate whether peptides C and E inhibit activation of NF-kB, STAT3, Akt, EGFR, β-catenin and c-Myc signaling

molecules as they directly affect cell survival and inflammation. Thus, the protein expression levels of p-NF-kB, NF-kB, IkB-α, STAT3, p-STAT3, Akt, p-Akt, EGFR, p-EGFR, β-catenin and c-Myc were detected by western blotting after treatment of cells with increasing concentrations of peptide C and E. The results indicated that peptides C and E decreased the phosphorylation of NF-kB, STAT3, Akt, and EGFR but not total NF-kB, STAT3, EGFR, and Akt levels compared with the control group in a dose-dependent manner as shown in Figure 14 a, 15a, 14b, 15b. A decrease in β-catenin and c-Myc expression levels was also decreased (Figure 14 a, 15a, 14b, 15b).

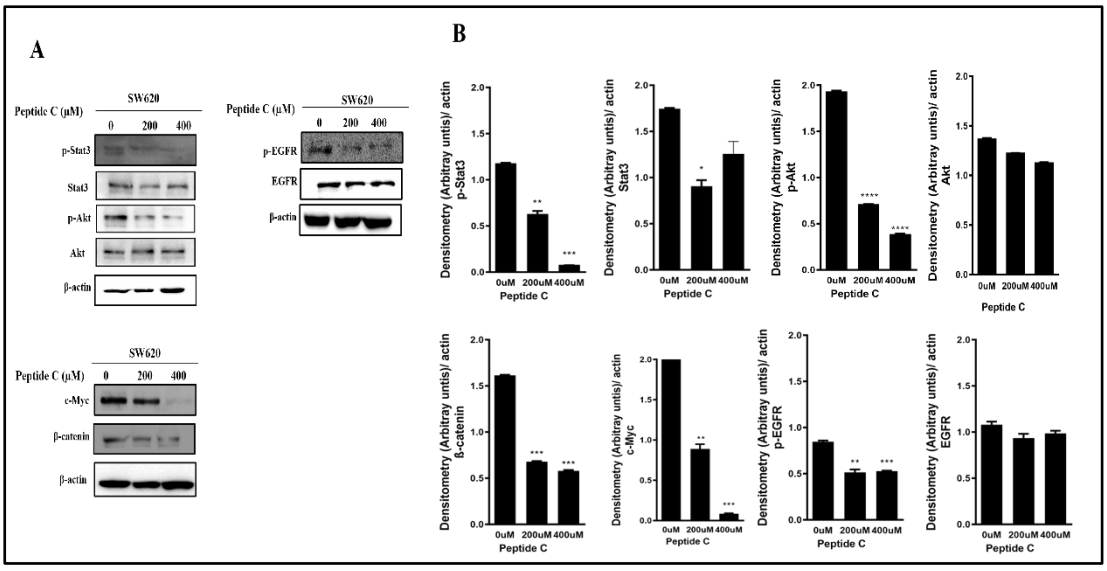

*Figure 14a. Effect of peptide C on cell proliferation and survival in SW620 cells using western blot analysis. (A). Peptide C downregulated the expression of p-STAT3, p-Akt, p-EGFR, β-catenin, and c-Myc without affecting the total protein expression levels (B). Densitometry analysis using Image J software. mean± SD (n = 3) *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001*

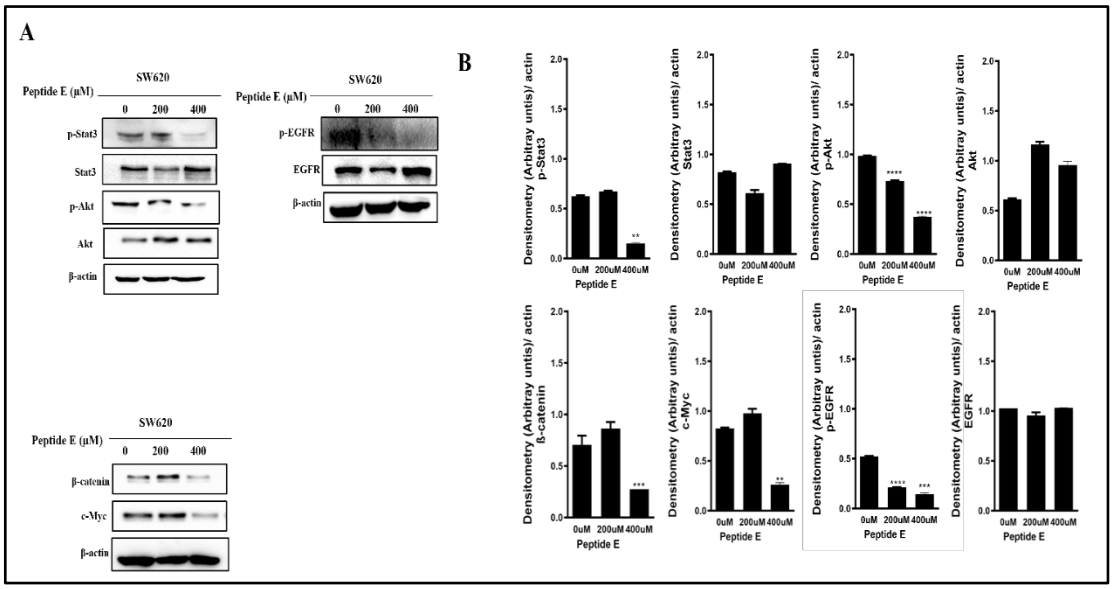

*Figure 14b. (A) Peptide E downregulated the expression of p-STAT3, p-Akt, p-EGFR, β-catenin and c-Myc without affecting the total protein expression levels (B) Densitometry analysis using Image J software. mean± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001.*

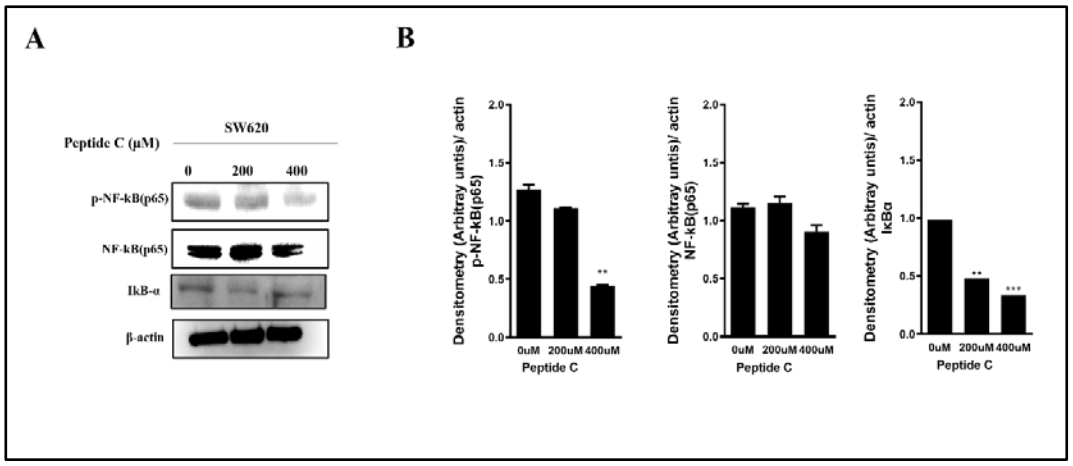

*Figure 15a. Effect of peptide C on pro-inflammatory markers as examined by Western blot analysis. (A) Peptide C downregulated the expression of p-NF-KB (p65) and IKB-α without affecting the total protein expression levels of NF-KB (B) Densitometry analysis using Image J software. mean± SD. (n = 3) **p < 0.01, ***p < 0.001.*

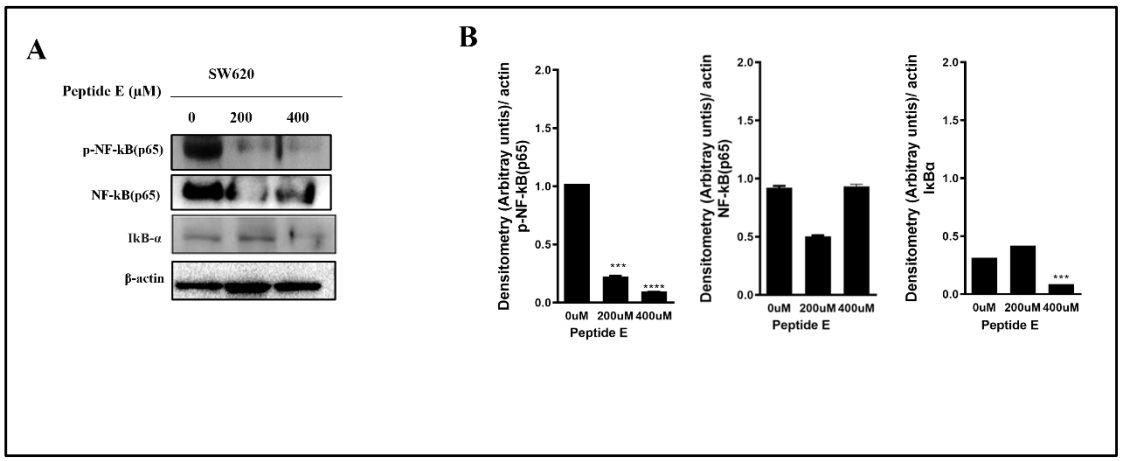

*Figure 15b. Effect of peptide E on pro-inflammatory markers as examined by Western blot analysis. (A) Peptide C downregulated the expression of p-NF-KB (p65) and IKB-α without affecting the total protein expression levels of NF-KB (B) Densitometry analysis using Image J software. mean± SD. (n = 3) **p < 0.01, ***p < 0.001.*

6. DISCUSSION

CRC is one of the most frequent malignancies in humans and a leading cause of cancer-related death. CRC's global burden by 2030 is anticipated to rise by 60% to over 2.2 million new cases and 1.1 million deaths (M. Arnold et al., 2017). Surgery is still the first line of treatment, especially for patients in the early stages of the disease. However, over half of all colorectal cancer cases relapse, and the tumor frequently metastasizes following surgery, which could be due to a failure to detect metastases or failure to completely remove the tumor. The five-year survival rates of CRC patients improved dramatically when surgery is paired with radiotherapy and chemotherapy; however, the toxicity and side effects of these therapies are a worry for increasing overall patient survival quality. Another major concern is the drug resistance followed by chemotherapy (Van der Jeught, Xu, Li, Lu, & Ji, 2018). As a result, innovative, more efficacious, and safer treatments are needed in clinical settings to improve CRC care. AMPs have recently received much interest as a unique therapeutic option for treating a variety of malignancies, as they target tumor cells while causing minimal toxicity to normal tissues (Deslouches & Di, 2017; Mader & Hoskin, 2006; Raileanu, Popescu, & Bacalum, 2020). These peptides are essential components of the host's innate immune system and are secreted by almost every organism (bacteria, fungi, invertebrates, vertebrates, and plants) in response to varied infections and stressful circumstances (Jafari et al., 2022; Otvos, 2017). Because cancer cells have higher amounts of negatively charged phosphatidylserine on their surfaces than normal cells, cationic amphipathic peptides could be a good source of anticancer medicines that are both selective and resistant to current resistance mechanisms.

In our study, we explored the antiproliferative, anti-inflammatory, and proapoptotic role of AMPs designated as BSAMPs as these are engineered synthetic

derivatives. Two BSAMPs have been developed so far, in collaboration with the research group of the Co-Supervisor, and selected to be used in this study, which we referred to as peptide C (Sequence: GVLCCGYRCCSKWGWCGTTK) and peptide E (Sequence: CWWMTRRAWR) throughout the thesis for easy nomenclature. These peptides have not been reported earlier for any anticancer activity, and thus our study is novel. Human colorectal cancer cell line SW620 was used as a research model to study the effect of selected peptides (C and E) on cell proliferation, inflammation, and apoptosis. SW620 is highly metastatic cell line with mutant p53 isolated from colorectal cancer patient.CCD841 was used as a non-transformed control epithelial cell line with wild type p53 and isolated from normal colon. The antiproliferative effect of peptide C and E on SW620 cells was studied using CellTiter- Glo viability assay as described in materials and methods. Our results showed that both the peptides C and E inhibited cell proliferation in SW620 cells in a dose-dependent manner. Among the different concentrations tested (0, 100, 200, and 400μM), an increasing trend in growth inhibition was observed as the dose was increased with more significant inhibition at 400 μM. Our results are in concordance with the studies which have shown that AMPs, ACPs and bioactive peptides inhibit cell growth in human CRC cells and in other cancer cells (Fan et al., 2022; L. Su et al., 2010; L. Y. Su, Shi, Yan, Xi, & Su, 2015). The inhibition of clonogenic cell survival by peptides C and E, significant at 400 μM also supports the antiproliferative effect of these peptides. The ability of a single cell to multiply indefinitely, thereby keeping its reproductive ability to create a large colony or clone, is determined by the clonogenic cell survival, and its inhibition is one of the essential mechanisms to induce apoptosis (Munshi, Hobbs, & Meyn, 2005).

As apoptosis is one of the important mechanisms to inhibit to induce cancer cell death and inhibit cell growth, so the next aim was to assess the apoptotic effect of

peptide C and E on SW620 cells. To achieve this goal, we performed multiple assays such as Annexin V/PI, cell cycle, TUNEL, mitochondrial membrane potential (MMP), and assessment of proapoptotic markers to confirm the results. We found that both the peptides C and E induced apoptosis by including cell cycle, DNA damage, loss of MMP, inhibiting antiapoptotic proteins but increasing proapoptotic markers. The significant inhibition was obtained at 400 µM. It is well known that the caspase family of proteins plays an important role in apoptosis (McIlwain, Berger, & Mak, 2013). It is also documented that caspase-3 targets PARP for cleavage and results in increased degradation of microsomal DNA, causing apoptosis (Chaitanya, Steven, & Babu, 2010). Our results also showed increased expression of cleaved caspase -3 and PARP with both the peptides C and E, significant at 400 µM. Members of the Bcl-2 family have been shown to play an important function in maintaining mitochondrial membrane integrity (Breckenridge & Xue, 2004; Harris & Thompson, 2000). The Bcl-2 family consists of anti-apoptotic (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic (Bax, Bak, PUMA, Noxa, and Bim) proteins which need to be well regulated for cellular homeostasis and any imbalance causes failure in apoptosis resulting in increased cell migration, invasion, metastasis and resistance to chemotherapy (Um, 2016). In the current study, we found that treating SW620 cells with peptides C and E increased Bax expression while decreasing Bcl-2 expression in a concentration-dependent manner with significant effect at 400 µM, as well as causing significant alterations in mitochondrial membrane potential. Our findings align with those of earlier published studies (Huang, 2000; Kazi et al., 2011; Raghav, Verma, & Gangenahalli, 2012; X. Zhang et al., 2021).

STAT3 and EGFR signaling inhibition decrease cancer cell growth, indicating their role in tumor cell survival and proliferation, suggesting a better therapeutic option

(Buettner, Mora, & Jove, 2002; Khan et al., 2019; C. Zhang et al., 2018). STAT3 is also a downstream effector of activated EGFR, which causes translocation of phosphorylated STAT3 to the nucleus to regulate transcription of genes involved in cell proliferation, migration, invasion, and apoptosis resistance (C. Zhang et al., 2018). Our data showed that both peptides C and E inhibited activation of STAT3 and EGFR in a concentration-dependent manner with a significant effect at 400µM. Another signaling pathway that is aberrantly activated in CRC is the Wnt/β-catenin signaling which is required for intestinal homeostasis under normal physiological conditions. Mutations in tumor suppressor Adenomatous Polyposis Coli (APC) cause aberrant activation of Wnt/β-catenin signaling, resulting in increased transcription of oncogenes such as c-Myc and CyclinD-1, thereby contributing to tumor progression (Shang, Hua, & Hu, 2017).

In our study, we found decreased expression of β-catenin and c-Myc with the treatment of peptides C and E in SW620 cells in a concentration-dependent manner with significant effect at 400 µM. As inflammation, apoptosis, and tumor growth are tightly linked so next we wanted to investigate the effect of peptides C and E on one of the important pro-inflammatory markers, NF-kB, which is critical in the inflammatory response and has been shown to be involved in the development of colitis-associated CRC (Greten et al., 2004). It has also been shown that aberrant activation of NF-kB contributes to increased cell proliferation, migration, invasion, and drug resistance (Hassanzadeh, 2011). Our data clearly showed suppression of NF-kB activation in SW620 cells with both peptides C and E in a concentration-dependent manner, but a higher effect was seen at 400µM.

In future directions, there is a need to study the multifaceted role of BSAMPs in cancer development and progression. More specifically, the role of BSAMPs in

inflammation, proliferation, migration, and invasion requires more investigation. BASMPS also need to be tested in additional colorectal cancer cells and more detailed experiments in normal colon cell lines. Finally, performing in vivo assays using zebrafish and mice as translational models can help explore the therapeutic efficacy of BSAMPs in CRC and other types of cancer

In conclusion, our results demonstrate that BSAMPs (peptides C and E) could serve as potential therapeutic agents for CRC treatment because they can effectively inhibit tumor growth and induce apoptosis. BSAMPs modulate the proliferative, inflammatory, and apoptotic signaling pathways, according to our *in vitro* studies. These findings add to our knowledge of the molecular mechanisms behind BSAMP anticancer action, paving the way for developing more effective and safer anticancer peptides for the treatment of CRC.

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