2. Review of literature

2.1 Literature survey

Cancer remains a major cause of death affecting millions of people and is caused by the growth and spreading of abnormal cells in an uncontrolled manner \(^{(110)}\). The worldwide statistics reveal that the most commonly diagnosed cancers are lung, breast and colorectal \(^{(110,111)}\).

Colorectal cancer (CRC) ranks second of all gastrointestinal malignant tumors and it is one of the leading causes of cancer related deaths worldwide. It ranks third in males and second in females leading causes of cancer related death worldwide \(^{(112)}\).

Population based time trend studies show a rising trend in the incidence of CRC in India \(^{(113)}\). In the past five years, the incidence of colon cancer is reported to be high in Asia \(^{(114)}\). Colon cancer is a major cause of mortality in the Western world \(^{(115)}\).

The management of cancer currently suffers several issues. Cancer treatment strategies include radiation therapy, chemotherapy and a combination of these, chemoradiotherapy, all of which exert cytotoxicity on cancer cells \(^{(116,117)}\). Colorectal cancer is often curable by surgery, but the prognosis for patients with metastatic disease remains poor. In the majority of metastatic patients, the standard treatment remains palliative chemotherapy \(^{(118)}\).

Fluorouracil (FU) based therapy has been the main treatment for metastatic CRC for the last 40 years. Major progress has been made by the introduction of regimens containing new cytotoxic drugs such as irinotecan or oxaliplatin. The combinations commonly used, such as FOLFIRI (leucovorin, FU and irinotecan) and leucovorin, FU and oxaliplatin can reach an objective response rate of approximately 50%. However, these new combinations
remain inactive in about half of the patients and in addition, resistance to treatment appears in almost all patients who initially were responders\(^{86,87}\).

Topoisomerase I inhibitor irinotecan (CPT-11) are used as a second-line chemotherapeutic agent for patients who have failed to respond to previous 5-FU-based chemotherapy, but the survival remains poor for patients with metastatic colorectal carcinoma\(^{119}\).

Chemotherapeutic drugs that are widely used in cancer treatment have the serious drawback of nonspecific toxicity because these agents target any rapidly dividing cell without discriminating between healthy and malignant cells\(^{120}\).

Naturally occurring Antimicrobial Peptides (AMPs) represent one of the first evolved and successful forms of chemical defense of eukaryotic cells against bacteria, protozoa, fungi and viruses\(^{121}\). AMPs are generally between 12 to 50 amino acids. These peptides include two or more positively charged residues provided by arginine, lysine or, in acidic environments, histidine, and a large proportion (generally >50%) of hydrophobic residues. AMPs have been found in every species that has been tested, including bacteria, fungi, plants, and animals.\(^{93}\).

Most AMPs kill both Gram-positive and Gram-negative bacteria, while a significant number of these bactericidal peptides have been shown to have anticancer and antiviral activities\(^{93}\). The use of cytolytic peptides in cancer treatment has the advantage and they are not affected by the multidrug resistance phenotype\(^ {122}\).

Lu et al., demonstrated that F11-322 (PFR peptide), a nine amino acid-residue peptide fragment derived from human lactoferricin, possesses potent cytotoxicity. It has antitumor activity both in \textit{in vitro} and \textit{in vivo} models of leukemia and characterize the novel biological action of PFR peptide via necrosis induction in leukemia cells\(^ {123}\).
Han et al., observed that pardaxin, isolated from the marine fish species *Pardachirus marmoratus* is a potential marine drug for adjuvant chemotherapy for human oral squamous cell carcinoma (OSCC) and oral cancer. It substantially alleviated carcinogenesis in the 7,12-dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch model by lowering prostaglandin E\(_2\) levels\(^{(124)}\).

Zhang et al., showed that Bovine myeloid antimicrobial peptide 28 (BMAP-28), significantly inhibited the proliferation of the human thyroid cancer TT cells *in vitro* and prevented the tumor growth in the TT-xenograft mouse model\(^{(125)}\).

Zhao et al., observed that HPRP-A2, a synthetic 15-mer cationic peptides showed strong anticancer activity to gastric cell lines BGC-823 and SGC-7901 and low toxicity against human red blood cells. HPRP-A2 synergized strongly with doxorubicin (DOX) to enhance the efficacy of killing gastric tumor cells *in vitro*\(^{(126)}\).

Mechkarska et al., reported that Pseudhymenochirin-1Pb (Ps-1Pb) and pseudhymenochirin-2Pa (Ps-2Pa) isolated from skin secretions of the frog *Pseudhymenochirus merlini* (Pipidae) are cytotoxic against different tumor cells, had cytokine-mediated immunomodulatory properties and broad-spectrum of antimicrobial activity\(^{(127)}\).

Huang et al., reported that pardaxin inhibits proliferation and induces apoptosis of human cervical carcinoma HeLa cell line, by activation of JNK signalling, together with the release of AIF and cytochrome-C from mitochondria\(^{(128)}\).

Gu et al., demonstrated that GLI13-8, a synthetic peptide showed cytotoxicity towards three human carcinoma cells (HepG2, SGC7901 and A375) in a dose-dependent manner and no toxicity towards the normal human fibroblasts (MRC-5). GLI13-8, kill HepG2 cells through the disruption of the cell membrane by forming transmembrane pores and the induction of apoptosis in HepG2 cells through mitochondria-dependent pathway\(^{(129)}\).
Ren et al., showed that FK-16, a fragment of LL-37, exhibits a better anticancer activity than the full-length peptide. FK-16 induces AIF/EndoG-dependent apoptosis and autophagic cell death via the common p53-Bax/Bcl-2 cascade in colon cancer cells (130).

Wang et al., observed that Temporin-1CEa identified in methanol extracts of the skins of the Asian frog Rana erythraea and the European hybrid frog Rana esculenta triggers a rapid cytotoxicity in breast cancer Bcap-37, cells through membrane-destruction and intracellular mechanisms involving mitochondria (131).

Slaninová et al., evaluated the selective toxicity of AMP (melectin, lasioglossins, halictines and macropin) from venom on two normal cell lines (human umbilical vein endothelial cells (HUVEC) and rat intestinal epithelial cells (IEC) and on three cancer cell lines (HeLa S3, CRC SW 480 and CCRF-CEM) (132).

Wang et al., demonstrated that temporin-1CEa, an amphiphilic α-helical cationic peptide had cancer cell selectivity towards twelve human carcinoma cell lines and relatively lower cytotoxicity on normal human umbilical vein smooth muscle cells (HUVSMCs) (133).

Hsu et al., reported that chrysophsin-1 displayed antitumor activity against human fibrosarcoma (HT-1080), histiocytic lymphoma (U937), epithelial carcinoma (HeLa) cells and modulates the inflammatory response by significant TNF-α suppression in RAW264.7 cells (134).

Chang et al., demonstrated that Tilapia hepcidin (TH)1-5TH1-5 inhibited the proliferation of tumor cells and induced an inflammatory response in HeLa cells, but not in HT1080 cells (135).

Zhang et al., showed that analog of AMP Polybia-MPI, with thioamide bond substitution, significantly improved anticancer activity and suppress the growth of sarcoma xenograft tumors in mice (136).
Lu and Chen, reported that SK84 glycine-rich AMP isolated from *Drosophila virilis* had specific inhibitory effects on the proliferation of several cancer cell lines (Human leukemia THP-1, liver cancer HepG2, and breast cancer MCF-7 cells), but no hemolytic activity (137).

Ceron *et al.*, observed that Cecropin A is able to induce apoptosis in promyelocytic cell line, HL-60 through a signalling mechanism mediated by ROS, but independently of caspase activation (138).

Fadnes *et al.*, indicated that heparin sulfate on the surface of the tumor cells (FEMX and HT-29 cells) sequesters antimicrobial peptide (Lactoferricin B and KW5) away from the phospholipid bilayer and thereby impede their ability to induce cytolysis (139).

Wu *et al.*, demonstrated that CB1a, a cecropin-derived peptide, showed high cytotoxic activity against leukemia and stomach carcinoma cells but low toxicity against non-cancer cells. The net positive charge of the peptide (+12) proved to be important for its activity (140).

Suttmann *et al.*, showed that Cecropin A and B have significant inhibition of malignant cell proliferation against bladder cancer cell lines by direct cancer cell lysis and target cell membrane disruption (141).

Maher *et al.*, observed that Melittin isolated from the venom of *Apis mellifera* exhibits necrotic cytotoxicity in gastrointestinal cells which is attenuated by cholesterol (142).

Furlong *et al.*, investigated the role of ceramide in LfcinB-induced apoptosis in CCRF-CEM and Jurkat T-cell acute lymphoblastic leukemia cell lines. Manipulation of cellular ceramide levels in combination with LfcinB enhanced the ability of LfcinB to trigger apoptosis in both Jurkat and CCRF-CEM cells (143).
Araya et al., showed that Lys 49 phospholipase A2 homologues of snake venoms exhibited antitumor activity against murine tumor cell lines (B16 melanoma, EMT6-mammary carcinoma, S-180 sarcoma, P3X myeloma, End endothelial cells) and tumor mass reduction of 36% was observed in murine model of subcutaneous solid tumor growth of EMT6 mammary carcinoma (144).

McKeown et al., reported that Human neutrophil peptides (HNPs)1 are α-defensins from the azurophilic granules of neutrophils were found to have a cytotoxic effect on several different types of human and mouse tumor cells, including Raji human B-lymphoma cells, human oral squamous carcinoma cells, and MOT mouse terato carcinoma cells (145).

Eliassen et al., demonstrated that LfcinB exhibits in vitro cytotoxic activity against different types of mouse and human cancer cell lines including leukemia cells, fibrosarcoma cells, various carcinomas, and neuroblastoma cells. Non-transformed fibroblasts were not substantially affected by LfcinB (146).

Shi et al., showed that Tachyplesin I inhibits the growth of cancer cells through a non-cytolytic mechanism. Tachyplesin I treatment of SMMC-7721 human hepatoma cell and BGC-823 human gastric adenocarcinoma cell cultures leads to a decrease in the proliferative capacity of these cancer cells (147).

Mader et al., showed that Bovine lactoferricin (LfcinB) selectively induced apoptosis in human leukemia and carcinoma cell lines but did not affect the untransformed cells (148).

Papo et al., explored the cytotoxic activity of modified 15-amino acid diastereomeric amphipathic peptide designated DK6L9 against human prostate cancer cell line (149).

Okumura et al., demonstrated that A 27-mer peptide of the C-terminal domain [hCAP18 (109–135)] of hCAP-18 which corresponds to amino acid residues 6–32 of LL-37, was found to induce apoptosis in a human oral squamous carcinoma cell line, SAS-H1 cells
via a mechanism involving mitochondrial depolarisation without any detectable activation of caspase-3 (150).

Kim et al., observed that Gaegurin 5 and Gaegurin 6 isolated from the skin of a Korean frog *Rana rugosa* had selective cytotoxic activity against neoplastic cells (151).

Hui et al., demonstrated that the combination of cecropin A and the conventional chemotherapeutic agents 5-fluorouracil and cytarabine, at certain doses, showed synergistic cytotoxic effect on CCRF-SB human lymphoblastic leukemia cells (152).

Srisailam et al., explored that cecropin B1 analogue, which possesses two amphipathic α helices, shows potent cytotoxic activity against several human leukemia cell lines at peptide concentrations that do not lyse normal fibroblasts or erythrocytes (153).

Chernysh, et al., observed that alloferon 1 and alloferon 2 isolated from the blood of an insect, the blow fly *Calliphora vicina* (Diptera) had antiviral and antitumoral capabilities (154).

**Magainin II**

**Antimicrobial and Antiviral activity**

Yüksel et al., reported that establishment of the dual functional membranes (Magainin II. And epidermal growth factor) approach suggests a promising strategy for wound dressings, vascular grafts and dental membranes as well as catheters and fixation devices (155).

Anupa et al., demonstrated that administration of Ala (8,13,18)-magainin II amide causes shift in the balance of immune-inflammatory responses involving downstream pathways of TLRs in cytrophoblast from placental villi (156).
Yüksel et al., reported that both Gram-negative Escherichia coli and Gram-positive Staphylococcus aureus were inhibited by Magainin II (Mag II), was covalently immobilized on poly (lactide-co-glycolide) (PLGA) and PLGA/gelatin electrospun fibrous membranes (157).

Speck et al., 2014 suggest that two cyclic AMPs (c-WWW, c-WFW) and a helical magainin II amide were suitable alternative for conventional antibiotics in liquid boar sperm preservation (158).

The synthetic hybrids designed from antimicrobial peptides cecropin A, LL-37 and magainin II, have antimicrobial activity against bacterial species such as Bacillus anthracis, Burkholderia cepacia, Francisella tularensis LVS and Yersinia seudotuberculosis (159).

Ryu et al., observed the antifungal and anti-inflammatory action of cecropin A(1-8)-Magainin2(1-12) hybrid peptide analog P5 against Malassezia furfur infection in Human Keratinocytes (160).

Liu et al., explored that conjugation of magainin II enhances the cytotoxicity of magainin II in mice bearing MCF-7 tumor grafts (161).

Thwaite et al., showed that magainin II exhibited significant bactericidal activity against Burkholderia cepacia complex genomovars in a human bronchial epithelial cell line (BEAS-2B) (162).

Kulkarni, et al., showed that alpha defensin, magainin and cathelicidin-type antimicrobial peptides (AMPs) induced apoptotic death of leishmania results from calcium-dependent, caspase-independent mitochondrial toxicity (163).

Cirioni et al., showed that magainin II and cecropin A, a combined treatment exerted strong antimicrobial activity and achieved a significant reduction in plasma endotoxin and TNF-alpha concentrations in three rat models of pseudomonas aeruginosa strains (164).
Kuzina et al., have reported that magainin-2 is active against the plant pathogenic bacterium Xylella fastidiosa. Plate growth inhibition assays showed that magainin 2, was toxic to all X. fastidiosa strains\(^{(165)}\).

Cirioni et al., reported that in vitro and in vivo study of magainin II combined with vancomycin exhibited efficacy against Staphylococcus aureus with intermediate resistance to glycopeptides\(^{(166)}\).

Pexiganan, a magaininII analogue combined with betalactams showed to be the most effective for sepsis treatment\(^{(167)}\).

Albiol et al., showed that Magainins I and II exhibited inhibitory action against herpes simplex virus type 1 (HSV-1) and 2 (HSV-2) but inactive against the arenavirus Junin virus (JV)\(^{(168)}\).

Giacometti et al., reported the therapeutic efficacy of MSI-78 in different intra-abdominal sepsis rat models. It decreases endotoxin and TNF-alpha plasma concentrations and reduces lethality\(^{(169)}\).

Expression of MSI-99, synthetic analog of magainin II (MII) in transgenic tomato enhances resistance to bacterial speck disease\(^{(170)}\).

The synthetic magainin derivatives MSI-751 and MSI-774 exhibit antimicrobial activity against oral pathogens by disruption of cell membrane integrity\(^{(171)}\).

Transgenic tobacco expressing MSI-99, a substitution analog of magainin, have been shown to enhance disease resistance against Sclerotinia sclerotiorum, Alternaria alternata and Botrytis cinerea. Transgenic banana plants show resistance to Fusarium oxysporumf. sp. cubense and Mycosphaerella musicola\(^{(172)}\).
Kwak et al., reported that a hybrid peptide derived from cecropin A and magainin-2, termed P1, inhibits the differentiation of osteoclasts in various osteoclast culture systems. Its inhibitory effect is mediated through the inhibition of NF-kappaB and JNK activation induced by the osteoclastogenic cytokine, receptor activator of NF-kappaB ligand (RANKL)\(^{(173)}\).

Cirioni et al., showed that intraperitoneal administration of magainin-1, magainin-2, and Magainin-2 amide can reduce bacterial growth and plasma endotoxin and TNF-alpha concentrations in a rat model of Gram-negative septic shock\(^{(174)}\).

Gosh et al., reported that intravaginal administration of an anti-microbial agent, Ala\(^{8,13,18}\)-magainin II amide, during blastocyst implantation inhibits pregnancy establishment in a dose-related manner in the rhesus monkey (Macaca mulatta)\(^{(175)}\).

Mystkowska et al., demonstrated that Magainin-2 amide is embryotoxic and that embryotoxicity is enhanced by cyclodextrin, albumin, hydrogen peroxide and acidification in murine embryos\(^{(176)}\).

Li et al., reported that transgenic tobacco plants expressing the engineered Magainin analog peptide Myp30 showed a significantly increased resistance against the oomycete, Peronospora tabacina (Adam) in vitro and the growth of a bacterial pathogen Erwinia carotovora subsp. carotovora (Jones)\(^{(177)}\).

Dhawan et al., demonstrated that intravaginal administration of (Ala\(^{8,13,18}\))-magainin II amide is a potential anti-implantation strategy for intercepting pregnancy\(^{(178)}\).

Magainin A arrests sperm motility and 1mg of Magainin A administered intravaginally blocks conception in rabbits\(^{(179)}\).
Magainin II combined with beta-lactam antibiotics have antimicrobial activity against multidrug-resistant nosocomial isolates of Acinetobacter baumannii\(^\text{180}\).

**Anticancer activity**

The antimicrobial peptide, apidaecin 1b, magainin 2 and buforin II in conjugation with photosensitisers showed increased efficiency of photodynamic therapy in cancer cells\(^\text{181}\).

Anghel *et al.*, showed the cytotoxic effect of magainin II on the MDA-MB-231 (breast adenocarcinoma) and M14K (human mesothelioma) in the concentration dependent manner\(^\text{182}\).

Liu *et al.*, showed that conjugation of MG2 to N-terminus of the cell penetrating peptide (Antp) can significantly enhance its antitumor activity and the fusion of CAP to Antp might be an alternative for cancer-targeted therapy\(^\text{183}\).

Liu *et al.*, studied the conjugation of magainin to bombesin against cancer cell line. The bombesin conjugation enhances the cytotoxicity of magainin II in cancer cells through improved binding\(^\text{184}\).

Henrik *et al.*, reported that magainin II shows a synergistic effect with cecropin peptides (Cecropin A and B) inhibit bladder cancer cell proliferation and viability in a dose dependent fashion\(^\text{185}\).

Lehmann *et al.*, showed that magainin II peptide exerts cytotoxic and antiproliferative efficacy by pore formation in bladder cancer cells but has no effect on normal murine or human fibroblasts\(^\text{186}\).

Cruz-Chamorro *et al.*, revealed that magainin, but not nisin, produced a loss of cell viability in HL-60 cells, a minor increase of hemolysis and promotion of cell death by
magainin occurs via cytochrome-C release accompanied by a substantial increase of proteasome activity \(^{(187)}\).

Shin et al., showed that the alpha-helical antibiotic peptide P18, designed from cecropin A (1-8) magainin 2 (1-12) hybrid, strong bactericidal and tumericidal activity without inducing hemolysis \(^{(188)}\).

Scott et al., \(^{(189)}\) Soballe et al., \(^{(190)}\) showed that Magainin II isolated from the skin of the African frog *Xenopus laevis*, shows broad antimicrobial spectrum, anti-endotoxin and anticancer activity.

Baker et al., demonstrated that magainin peptides have been shown to improve survival of animals with ascites-producing tumors in *in vivo* \(^{(191)}\).

Ohsaki et al., showed that magainins analogues such as Magainin A and Magainin G inhibit the growth of human small cell lung cancer (SCLC) cell lines and most importantly, these cancer cell lines are not affected by the *mdrl* gene, which is responsible for developing multidrug resistance \(^{(192)}\).

Magainin 2 and its more potent synthetic analogues (magainins A, B, and G) cause the rapid lysis of both hematopoietic and solid tumor cell lines at concentrations that are 5–10 fold lower than magainin concentrations that are lytic for normal human peripheral blood lymphocytes or neutrophils \(^{(102)}\).

**Insilico analysis**

In our previous study on *in silico* analysis, the interaction between peptides such as Aurein 1.2, Aurein 2.4, Alloferon, Magainin, Cecropin and Citropin 1.2.5 with colon cancerous protein Transforming growth factor beta receptor II (TGFβRII) was extensively
studied by using Hex 4.2 Software (193). Hex is an interactive molecular graphics program for calculating and displaying feasible docking modes of protein pairs and DNA molecules (194). A cancerous protein TGFβR II structure was retrieved from PDB database. The anticancerous peptide (AMP) sequences were retrieved from antimicrobial peptide database. AMP structures were modelled using peptide tertiary prediction server. The interactions between magainin and TGFβRII had high Energy minimization (E-min) value of -482.3. Docking study revealed that magainin peptides could be used against colon cancer since they showed higher E-min value for TGFβR II. Therefore it was of interest to evaluate the efficacy of magainin II as an anticancer agent on colon cancer cell lines (Figure 2.1).

**Figure 2.1 Interaction of TGFβRII with Magainin II by HEX4.2 software**

Colorectal cancer (CRC) resistance to fluoropyrimidines and other inhibitors of thymidylate synthase (TS) is a serious clinical problem often associated with increased intracellular levels of TS. Since the tumour suppressor gene p53, which is mutated in 50% of CRC, regulates the expression of several genes, it may modulate TS activity, and changes in the status of p53 might be responsible for chemo resistance (195).
Mutations in p53 have been related to chemo resistance \(^{(196)}\). p53 status plays an important role in the determination of 5-fluorouracil sensitivity in colon cancer. Wild type (WT) for p53 showed improved response to 5-fluorouracil and prolonged survival in patients with tumours \(^{(197)}\). The cells carrying mutant p53 in the NCI cell line panel were less sensitive to 5-FU \(^{(198)}\). Peters \textit{et al.}, observed that the panel of 14 colon cancer cell lines with a p53 mutation was found to be resistant to 5-FU \(^{(84)}\).

Human colorectal cancer cell lines are an important, commonly used preclinical model system for studying this disease, and have provided essential insights into tumor, molecular and cell biology. Cell lines are the fundamental tool used in the discovery of new antitumor compounds and for the discovery of drug sensitivity, resistance and toxicity biomarkers, with molecular markers of response to conventional chemotherapies and targeted agents showing clinical utility in patients \(^{(199–204)}\).

\textbf{2.2 Need and Scope of the study}

Conventional chemotherapeutic agents leucovorin, 5-fluorouracil(5-FU) and irinotecan are alkylating agents and antimetabolites that display little or no selectivity against normal mammalian cells, which results in severe side effects \(^{(205)}\). Moreover cancer cells can develop resistance to conventional chemotherapy agents by cellular changes through multimechanisms \(^{(88)}\). Development of chemotherapeutic agents that can be used for effective and safe treatment has gained interest. Antimicrobial peptide recently have received attention as alternative chemotherapeutic agents that overcome the limitations of current drugs, such as selective cytotoxicity for cancer cells, ability to bypass the multidrug-resistance mechanism and additive effects in combination therapy \(^{(100,122,206)}\).

**Lacunae**
An in depth literature search has shown that very few studies in India are focused on the use of magainin II on colon cancer cell lines. The effect of this magainin II in both wild and mutant p53 colon cancer cells has not been studied.

2.3 Hypothesis, Aim and Objectives

Hypothesis

Magainin II has anticancerous activity and have advantages over the conventional chemotherapeutic drugs such as low toxicity, unaffected by chemo resistance and effective against colon cancer.

Aim

To evaluate the anticancerous activity of magainin II on colon cancer cell lines.

Objectives

In order to experimentally validate our in silico analysis of magainin II the objectives of the present study are
- To evaluate the anticancer activity of magainin II in comparison with conventional chemotherapeutic drug, 5-fluorouracil on mutant p53 HT-29, COLO 205, COLO 320 DM, HCT-15 and wild p53 HCT116 colon cancer cell lines.

- To study the extent of apoptosis in colon cancer cell lines treated with 5-FU and magainin II.

- To find out the molecular pathway of magainin II on colon cancer cells through antiapoptotic or proapoptotic gene and protein expression.

- To determine the telomerase activity of magainin II on colon cancer cell lines.