Study the molecular mechanism of a novel colon cancer therapy by microbial protease mediated apoptosis

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "**Study the molecular mechanism of a novel colon cancer therapy by microbial protease mediated apoptosis**" Submitted by Sri. **Dwiprohi Kar** who got his name registered on **09th December 2016** For the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Amit Pal, Scientist G, ICMR-NICED, Kolkata** and that neither this thesis nor any part of it has been submitted for either any degree or any other academic award anywhere before.

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DEDICATED TO MY PARENTS

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ABBREVIATIONS

Δ	Delta
µg	Microgram
µg/ml	Microgram/milliliter
µl	Microliter
µM	Micromole
AC	Adenylate cyclase
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
BCIP	5-bromo 4-chloro 3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
BoNT	Botulinum neurotoxin
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CFU	Colony forming unit
ChIP	Chromatin Immunoprecipitation
CIN	Chromosomal instability
cm	centimeter
CPE	Clostridium perfringes enterotoxin
CRC	Colorectal cancer
Ct	Cycle threshold
Da	Dalton
DAPI	4’,6-diamidino-2-phenylindole
DCC	deleted in colon cancer

ABBREVIATIONS

DCF	Dichlorodihydrofluorescein
DCFDA	Dichlorodihydrofluorescein diacetate
DCFH	Dichlorofluorescin
DE-52	Diethylaminoethyl cellulose
DT	Diphtheria toxin
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FAP	Familial adenomatous polyposis
FASTA	Fast All
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony stimulating factor
g	Gram
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPCR	G-protein coupled receptor
h	Hour
HAP	Hemagglutinin protease
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ABBREVIATIONS

HB-EGF	Heparin-binding epidermal growth factor
Tf	Transferrin
IL	Interleukin
ITS	Insulin-Transferrin-Selenium
kDa	Kilo Dalton
kV	Kilovolt
MAPK	Mitogen-activated protein kinase
mCRC	Metastatic colorectal cancer
$MgCl_2$	Magnesium chloride
mM	millimole
MMP	Matrix metalloprotease
MSI	Microsatellite instability
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
nm	nanometer
ºC	Degree Celsius
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PAR	Protease activated receptor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PKC	Protein kinase C

ABBREVIATIONS

pM	Pico mole
PMSF	Phenylmethylsulfonyl fluoride
PrtV	Protease of *Vibrio cholerae*
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Real-Time quantitative PCR
SDS	Sodium dodecyl sulfate
TCBS	Thiosulfate-citrate-bile salts agar
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
TRIS	tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine
TSB	Tris buffer saline
UPEC	Uropathogenic *Escherichia coli*
VEGF	Vascular epithelial growth factor
V	Volts
V. cholerae	*Vibrio cholerae*

CHAPTER 1

INTRODUCTION

INTRODUCTION

Colon cancer is a health hazard, specifically in developing countries, where early diagnosis systems are lacking and mortality rates continue to rise [1,2]. Metastases to bone, lung, liver, and the central nervous system represent the prime complication of treatment and the major cause of death [3, 4]. The recent discovery of new factors involved in colon cancer progression in vitro are difficult to translate into diagnostic tools to accurately identify patients at high risk of metastasis. To improve the treatment and survival of these patients, a better molecular understanding of the early mechanisms leading to metastasis is required [3, 4].

Conventional therapies are accompanied by severe dose-limiting side effects. Cancer cells divide more quickly than healthy cells and chemotherapy drugs target these cells. However, in the process, they also fast-growing healthy cells leading to side effects. Natural products play an important role as a source of effective anticancer agents and 60% of currently used anticancer agents are derived from natural sources like plants, marine organisms, and micro-organisms [5, 6]. Several proteases and several toxins have been reported to have anti-tumor activities [7-10].

Bacterial toxins have been tested for cancer treatment. Bacterial toxins are cytotoxic and can alter cellular processes that control proliferation, apoptosis, and differentiation. These alterations are associated with carcinogenesis and may either stimulate cellular aberrations or inhibit normal cell controls. Bacterial toxins *per se* or in synergy with anticancer drugs or irradiation could possibly enhance the efficacy of cancer treatment [11]. *Clostridium perfringens* type A strain, the causative agent of gastroenteritis, produces Clostridium perfringens enterotoxin (CPE) [12, 13]. Studies have shown that purified CPE exerts an acute cytotoxic effect on pancreatic cancer cells and leads to tumor necrosis and inhibition of tumor growth *in vivo* [14, 15]. Adenylate Cyclase (AC) toxin from *Bordetella pertussis* showed extensive apoptosis with increasing dose of AC toxin in lung cancer cells [16]. Proteases have fundamental roles in biological processes and are associated with a wide variety of pathological conditions, including cancer. Proteases have long been associated with cancer progression because of their ability to degrade extracellular matrices, which facilitates invasion and metastasis. Intracellular and extracellular proteases can function as signaling molecules in various cellular processes that are essential for cancer biology [17]. These protease regulated processes include proliferation,

adhesion, migration, differentiation, autophagy, apoptosis, and evasion of the immune system. There have been very few reports on the use of bacterial proteases in tumor regression. A protease isolated from the culture supernatant of a Gram-negative bacteria *Serratia marcescens* kums 3958 showed very potent antitumor activity when injected into tumors in BALB/c mice [8]. Pathogenic *Vibrio cholerae* produces a plethora of proteases among which hemagglutinin protease (HAP) is noteworthy in virulence and pathogenesis [18]. Colorectal carcinoma is the third most commonly diagnosed cancer and the third leading cause of cancer-related deaths in both males and females in developing countries [1, 2], and the fourth dominant cause of cancer mortality worldwide. In our earlier studies, we reported the apoptotic attributes of HAP in breast cancer cells [7, 19]. In the present study, we aspire to show the apoptotic response of microbial proteases in colon cancer cells along with its roles in tumor regression in mice models. We are targeting our focus on microbial protease-mediated therapy of colon cancer cells. It will open new paths to microbial protease-mediated cancer therapy.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Colorectal cancer

Colorectal cancer is caused by the abnormal growth of epithelial cells which form the lining of the colon or rectum. These small growths (termed polyps) are often benign, although some have the potential to develop and become cancerous. It is estimated that up to two-thirds of colorectal polyps are pre-malignant and associated with a risk of colorectal cancer [20]. Screening and awareness can reduce the mortality of colorectal cancer by detecting and removing polyps before they become cancerous, or by discovering the cancer at an earlier stage, where treatment has a higher success rate [21]. However, there are often no initial symptoms and the cancer may already have spread to other parts of the body by the time the patient is diagnosed.

2.1.1 Causes and risk factors

There are several risk factors that may increase the chance of an individual developing colorectal cancer.

- Family history: A person's risk doubles if a direct relative has previously had the disease. There is an even greater risk if more than one relative has had colorectal cancer [22].
- Genetics: Individuals with inherited disorders such as familial adenomatous polyposis (FAP), where an individual is prone to polyp formation, have a higher risk of developing colorectal cancer [22].
- Colorectal polyps or inflammatory bowel disease: A history of polyps or inflammatory bowel disease, where the bowel is inflamed for many years, increases the risk of colorectal cancer [23].
- Age: Although a person can develop colorectal cancer at any age, the risk increases greatly with age. Over 90% of colorectal cancer cases are diagnosed in patients over the age of 50 [23].
- Lifestyle: A sedentary lifestyle is associated with a higher risk of colorectal cancer. Studies have also linked obesity, lack of exercise, smoking, and excessive alcohol consumption to a greater risk of colorectal cancer [23].

2.1.2 Symptoms and diagnosis

Early diagnosis of colorectal cancer has the potential to improve survival rates; however early symptoms (such as abdominal pain) may be confused with other diseases [24], meaning many patients have advanced disease when diagnosed [25]. Almost 85% of patients referred to the hospital have one or more of the following high-risk symptoms [26]:

- Rectal bleeding
- A mass in the abdomen or rectum
- Change in bowel habit
- Perianal symptoms, such as abscesses or lesions

As the cancer becomes more advanced, other symptoms can develop. For example, excessive bleeding from the colon can cause anemia, which leaves the patient feeling breathless and tired. If the cancer begins to obstruct the colon, further symptoms include bloating, constipation and vomiting [27].

Methods of diagnosis vary from country to country but typically if a patient presents high-risk symptoms to their doctor they will be given a physical examination. If this raises any concerns, a number of additional tests may be performed [28].

- Colonoscopy – the entire length of the colon is viewed using a colonoscope.
- Sigmoidoscopy – a small tube (sigmoidoscope) is used to view the lower colon.
- Double-contrast barium enema – x-rays of the colon and rectum. Barium lines the colon allowing an outline to be viewed in x-rays [28].

A biopsy, where sample tissue is removed during a colonoscopy or Sigmoidoscopy, is required to confirm the diagnosis of colorectal cancer and determine how advanced the disease is (staging) [28].

2.1.3 Epidemiology-Incidence & mortality

Colorectal cancer is diagnosed in over 1.2 million people globally each year; it is the second most common cancer in women and the third most common cancer in men.

The disease is responsible for approximately 609,000 deaths each year (8% of all cancer deaths) [29], making it the fourth leading cause of death after lung, stomach, and liver cancers.

2.2. An overview of our current understanding of mechanisms leading to human colorectal cancer

The vast majority of malignant colorectal cancer arise out of benign adenomatous polyps over a course of several decades, so the peak incidence of colorectal carcinoma occurs between the ages of 60-80 years. The morphology of colorectal cancer is directly influenced by the molecular pathways that are disrupted. The "Vogelstein model" [30] was a conceptual breakthrough in understanding the molecular etiology of colorectal cancer. It described the sequential accumulation of mutations in four genes that correlated with histological features that develop during colorectal cancer progression, i.e. inactivating mutations in the Adenomatous Polyposis Coli (APC) gene, *K-RAS* gene activating mutations, inactivating mutations in chromosome 18 which later was identified as the *DCC* gene ("deleted in colon cancer") and SMAD family members, and finally inactivating mutations of the *TP53* gene [30]. Depending on the molecular origins and clinical behavior, colorectal cancers are divided into two groups; the ascending and descending colon cancer [31]. The ascending and descending colon cancer have been summarized below.

2.2.1 Descending colon/rectal cancer

Approximately 55% of colorectal cancers occur in the descending colon and rectum. These tumors are typically initiated through the mechanism of chromosomal instability (CIN) [31]. In this case cancer polyps are developed in descending colon.

2.2.2 Ascending colon cancers

For the 20% of colon cancers that originate in the ascending colon, cancer initiation is usually associated with microsatellite instability (MSI) [31]. Despite that, a greater

understanding of the molecular basis of colorectal cancer has been achieved with the development of current gene-identification techniques these research results have failed to make substantial improvement in outcomes in colorectal cancer patients. Thus, more promising strategies for drug delivery systems to achieve better outcomes are needed.

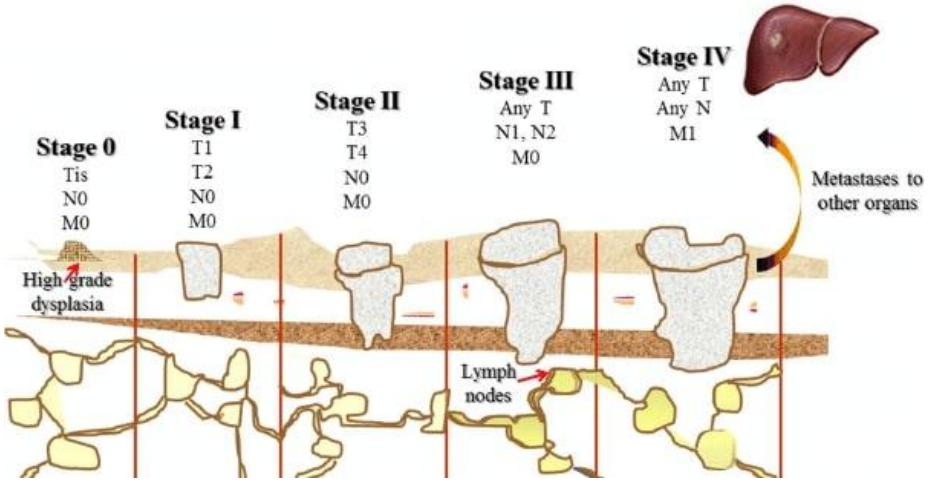

Figure1. Classification of colorectal cancers (American joint Commission of Cancer): Tis- carcinoma in situ: intraepithelial or invasion of lamina propria; T1- tumor invades submucosa; T2- tumor invades muscularis propria; T3- tumor invades through muscularis propria into subserosa or into nonperitonealized pericolic or perirectal tissues; T4- tumor penetrates the surface of the visceral peritoneum or tumor directly invades or is histologically adherent to other organs or structures; N0- no regional lymph node metastasis; N1- metastasis in one to three regional lymph nodes; N2- metastasis in four or more regional lymph nodes; M0- no distant metastasis; M1- distant metastasis [American joint commission on cancer].

2.3 The current treatment regimen of colorectal cancer and its drawbacks

Classification of cancer by anatomic disease extent is one of the most important factors for prognosis and therapeutic decision. Based on the current staging systems (Figure-1), colorectal cancer can be treated by surgery, chemotherapy, radiation, immunotherapy or palliative care [32]. Surgical resection offers high cure rates for colorectal cancer in early stages, of which the success rates are 90% and 75% for

Stage I and II colorectal cancer respectively [33]. Fortunately, no additional treatment is required for patients with stage I colorectal cancer following surgical procedure as they have little benefit from additional treatment with low recurrence rate (about 3%). However, adjuvant chemotherapy is recommended for all individuals with stage III colorectal cancer, which have a higher risk of relapse at about 60% [34]. Chemotherapy can be added as post-surgery adjuvant, pre-surgery neo-adjuvant or as primary therapy to inhibit tumor cell growth, induce cell apoptosis or decrease metastasis opportunity [35]. Although there has been a remarkable advance in chemotherapy for colorectal cancer patients in the past two decades, stage IV disease is usually incurable [36]. The best remedy for patients with metastatic colorectal cancer (mCRC) is to improve the quality of life by improving systemic treatments [37].

At present, several drugs are available for the management of colorectal cancer. 5-Fluorouracil (5-FU)/ Leucovorin is the first-line treatment, and the most common chemotherapy for metastatic colorectal cancer by inhibiting thymidylate synthase [38, 39]. An extensive body of data shows that Fluoropyrimidines, Irinotecan, and Oxaliplatin have emerged as cornerstones of chemotherapy for colorectal cancer. However, these pharmaceutical therapeutic regimens are usually accompanied by severe mucositis, myelosuppression, cumulative neurosensory toxicity, and hematological adverse reactions due to nonspecific distribution into intestinal mucosa, bone marrow, liver, and other healthy tissues [40-42].

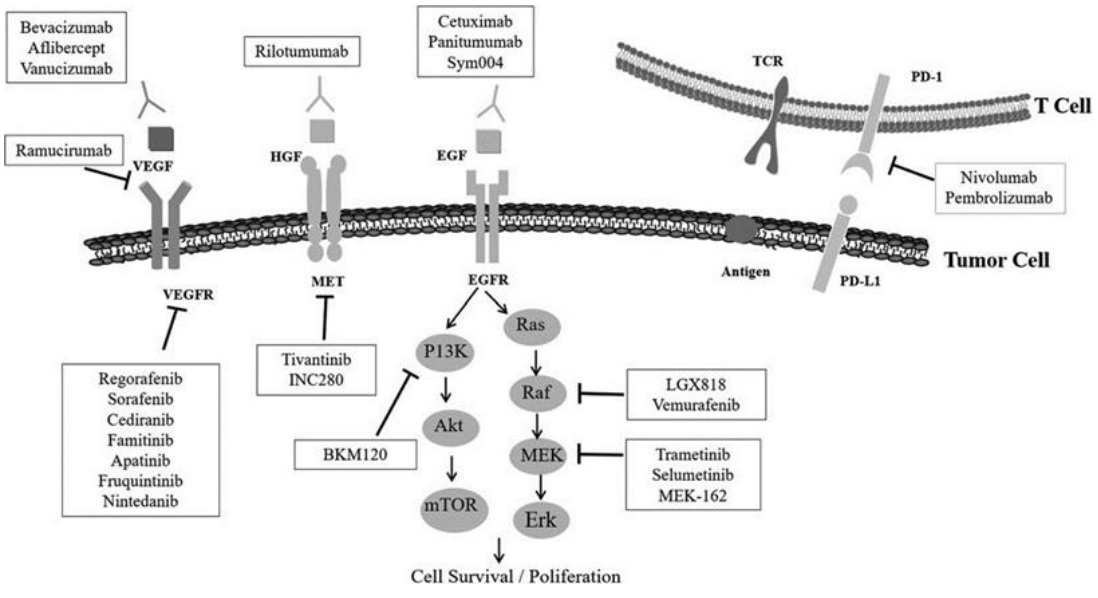

Figure2. Signaling cascades of targeted drugs/immune checkpoint inhibitors undergoing clinical trials in metastatic colorectal cancer therapy [Geng *et al*, 2017].

2.4 Bacterial toxins in cancer therapy: A novel experimental strategy

In the past decade, the major advance in the clinical management of mCRC is the introduction of biologic agents. A majority of biologic agents are also called targeted drugs because they aim at signaling cascades involved in regulating tumor growth, drug resistance, metastatic spread, angiogenesis, and apoptosis [44]. Effectiveness of this therapy depends on the targeted delivery of therapeutics at the right site and minimizing the off-target side effects. With the clinical use of targeted agents, the median overall survival (OS) has improved up to more than 30 months [45, 46].

2.4.1 Bacterial toxins in cancer treatment

Bacterial toxins have to some extent already been tested for cancer treatment. Bacterial toxins can kill cells or at reduced levels alter cellular processes that control proliferation, apoptosis and differentiation. Cell-cycle inhibitors, such as cytolethal distending toxins (CDTs) and the cycle inhibiting factor (Cif), block mitosis. CDTs are found in several species of Gram-negative bacteria, including *Campylobacter jejuni* and *S. typhi* while Cif is found in enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *E. coli*. The anti-tumor effect of toxins is probably with reduced side-effects compared to traditional tumor treatment. Bacterial toxins per se or when combined with anti-cancer drugs or irradiation could therefore possibly increase the efficacy of cancer treatment [11].

2.4.2 Bacterial toxins binding to tumor surface antigens

Diphtheria toxin (DT) binds to the surface of cells expressing the heparin-binding epidermal growth factor like growth factor (HB-EGF) precursor. DT-HB-EGF complex is internalized after endocytosis via clathrin-vesicles. The internalized active toxin, called DT fragment A, catalytically ribosylates elongation factor-2 (EF-2)

leading to inhibition of protein synthesis with subsequent cell lysis and/or induction of apoptosis [47-50], makes it a potential candidate for targeted cancer therapy.

Clostridium perfringens type A strain, the causative agent of gastroenteritis, produces Clostridium perfringes enterotoxin (CPE). The C terminal domain of CPE is responsible for high affinity binding to the CPE receptor (CPE-R) and the N-terminal is assumed to be essential for cytotoxicity [12, 13]. Studies have shown that purified CPE exerts an acute cytotoxic effect on pancreatic cancer cells and led to tumor necrosis and inhibition of tumor growth in vivo. It is being investigated for colon, breast, and gastric cancers. Moreover, before evaluating CPE for systemic cancer therapy, its long-term efficacy and lack of toxicity in vivo need to be demonstrated [51-53].

A more recent study has demonstrated for the first time that botulinum neurotoxin (BoNT) briefly opens tumor vessels, allowing more effective destruction of cancer cells by radiotherapy and chemotherapy. It has been proposed that BoNTs act by their effect on tumor microenvironment rather than by direct cytotoxic effect on tumor cells [54].

Some bacterial toxins (Alfa-toxin from *Staphylococcus aureus*, AC-toxin from *Bordetella pertussis*, Shiga like toxins, and Cholera toxin) are presently being studied on mesothelioma cells (P31) and small lung cancer cells (U-1690). Preliminary results with AC-toxin showed increased cytotoxicity with increasing dose in both cell lines, along with marked increase in apoptosis. However, cholera toxin did not induce apoptosis [16].

2.4.3 Bacterial toxins conjugated to ligands

Protein toxins such as Pseudomonas exotoxin, diphtheria toxin, and ricin may be useful in cancer therapy because they are among the most potent cell-killing agents. Although they are very lethal, yet for therapeutic efficacy, those toxins need to be targeted to specific sites on the surface of cancer cells. This process is accomplished by eliminating binding to toxin receptors by conjugating the toxins to cell-binding proteins such as monoclonal antibodies or growth factors. These conjugates bind and

kill cancer cells selectively thus sparing normal cells, which do not bind the conjugates. A wide variety of DT ligands such as IL-3, IL-4, granulocyte colony stimulating factor (G-CSF), transferrin (Tf), EGF, and vascular endothelial growth factor (VEGF) have been studied for targeting tumors [48]. The transferrin-DT conjugate (Tf-CRM 107) and DT-EGF have reached the stage of clinical trials in patients with brain tumors and metastatic carcinomas respectively [55]. Similarly, a large variety of antibodies and ligands to surface antigens overexpressed in different tumors have been conjugated to PE. Important ones tested in clinical trials are IL-4, IL-13, monoclonal antibody- recognizing a carbohydrate antigen Lewis Y, reacting with metastatic adenocarcinoma cells (Mab B3), and transforming growth factor (TGF-α) [56].

Secreted microbial proteases have long been associated with cancer prognosis because of their ability to degrade extracellular matrices, which facilitates invasion and metastasis. Intracellular and extracellular proteases can function as signaling molecules in various cellular processes that are essential for cancer biology [17]. These protease regulated processes include proliferation, adhesion, migration, differentiation, autophagy, apoptosis, and evasion of the immune system. In the context of the different effects of proteases in cancer biology, the discovery of the Protease-Activated Receptors (PARs) in the early 90s is mention-worthy, which are reported to transduce several downstream cellular processes in response to their ligands (proteases).

2.5. *Vibrio cholerae* and its proteases

Vibrio cholerae are facultative anaerobic, monotrichous, asporogenous and motile, curved or straight, Gram-negative rod-shaped bacteria of 1.4-2.6 μm in length and were first isolated by the Italian anatomist Filippo Pacini in 1854. They grow in alkaline conditions but are inhibited if pH drops to 6 or below [57].

There are more than 208 serotypes of *V. cholerae* that have been reported according to their lipopolysaccharide composition and structure. Amongst them only O1 and O139 cause pandemic cholera. O1 serotype can be further classified on the basis of

their physiological properties such as polymyxin resistance, mannose sensitivity, hemagglutinin, hemolysin, and phage infection [58], into classical and El Tor biotype.

V. cholerae express genes that code for protein directly or indirectly involved in the virulence of bacteria. During infection, *V. cholerae* secretes Cholera Toxin (CT) which causes profuse watery diarrhea. Cholera toxin activates the adenyl cyclase enzyme in the intestinal mucosa leading to the increased level of intracellular cAMP and secretion of H_2O, Na^+, K^+, Cl^- and HCO^{3-} into the lumen of the small intestine. The cholera toxin is composed of five binding subunits of 11,500 Da molecular weight and a single active subunit (A1) of molecular weight 23,500 Da and a bridging piece (A2) of 5,500 Da which actually link A1 to 5B subunit.

V. cholerae contains both major virulence factor such as cholera toxin (CT) and toxin coregulated pili (TCP) responsible for profuse watery diarrhea and bacterial colonization. The bacteria also secrete other protein factors called associated accessory factors (VAAF).

The genus Vibrio consists of many pathogenic species that include *V.cholerae, V. parahemolyticus, V.vulnificus, V.mimicus, and V.fluialis*. In addition to toxins and hemolysin produced by vibrios, protease is also recognized as one of the pathogenic factors in some Vibrio species. The protease in vibrios are divided into two main groups- the zinc metalloproteases and the serine proteases. *V.cholerae* and *V.vulnificus* secrete proteases belonging to the thermolysin family of zinc-metalloproteases and are immunologically cross-reactive with each other.

2.5.1 Hemagglutinin protease

V.cholera O1, the causative agent of epidemic cholera secretes a 32 kDa zinc-containing protein hemagglutinin protease (HAP) encoded by the hapA gene that plays an important role in the pathogenesis of ctx-negative *V.cholerae* non-O1, non-O139 strains. El Tor biotypes of *V.cholerae* strains produce HAP at higher levels compared to classical biotypes. The secreted metalloprotease HA/P is encoded by the hapA gene. Mutants lacking a functional hapA gene show very little protease activity, suggesting that HA/P is responsible for the major proteolytic activity exerted by

V.cholerae. The mucin degrading ability of this protease helps vibrios to overcome the protective mucus barrier of the intestine, enhancing the access of bacteria towards the gastrointestinal epithelia and consequently, promoting bacterial colonization. HA/P nicks the A-subunit of Cholera toxin [59, 60] and digests proteins such as mucin, fibronectin, lactoferrin, and secretory immunoglobulin A, that may participate in host defense against cholera [61]. HAP can also hydrolyze mucin to enhance the detachment of *V.cholerae* from cultured epithelial cells [62].

2.5.2 Serine protease

Partial purification of proteases from CHA6.8ΔprtV strain have shown the presence of a 59-kDa trypsin-like serine protease encoded by the VC1649 gene. This serine protease was also found to induce a hemorrhagic response in the rabbit ileal loop models [63].

2.5.3 PrtV protease

The PrtV protease of *V.cholerae* was identified in 1997 by Oigerman et al., as a 102 kDa metalloprotease. The gene encoding PrtV is located on chromosome II within a tentative pathogenicity island. The close proximity of the prtV gene to the hlyA gene suggested that the former gene product might be capable of damaging host cells or proteolytically activating VCC (Vibrio cholera cytolysin), also called haemolysin A or El Tor haemolysin, encoded by the hlyA gene, which is a highly conserved genetic element in *V.cholerae,* independent of biotypes or serogroups. The PrtV protease belongs to a M6 metalloprotease family which displays a HEXXHXXGXXD motif. The PrtV is synthesized as a 102 kDa protein, but undergoes several N- and C-terminal processing steps during *V.cholerae* envelope translocation and prolonged incubation. Purified PrtV protease forms of 81 or 73 kDa are stable in presence of calcium ions. Removal of calcium results in further rapid auto proteolysis [64]. The PrtV can degrade extracellular matrix components, fibrinogens and fibronectin and also modulates the inflammatory response in human intestinal epithelial cells by *V.cholerae* cytolysin [65].

2.6 Cancer regression by proteases

Over the years, proteases have been associated with a wide variety of pathological conditions, including cancer. Their ability to degrade extracellular matrices has been shown to facilitate and to some extent regulate invasion, metastasis, proliferation, adhesion, migration, differentiation, autophagy, apoptosis, and evasion of the immune system. Amongst the very few reports of the use of bacterial proteases in tumor regression, a protease isolated from culture supernatants of a Gram negative bacteria *Serratia marcescens* kums 3958 is noteworthy. The protease showed very potent antitumor activity when injected into Meth-A or RL1 tumors in BALB/c mice at 30 μg per tumor [8]. Moreover, several serine proteases such as trypsin and elastase have been reported to aid in the therapy of certain melanoma by activation of oncolytic Influenza A viruses to target tumors [66].

In a previous study, we have shown that HAP secreted by *V.cholerae* has anticancer properties [7, 19]. It induced cytotoxicity in cancer cell lines (Breast adenocarcinoma derived Elrich Ascites Carcinoma) by activating the intrinsic pathway of apoptosis. It also inhibited tumor cell proliferation in a breast cancer adenocarcinoma model. Treatment of HAP was found to significantly improve the lifespan of tumor-bearing mice. These results clearly demonstrate the role of HAP in cancer regulation via apoptosis [7, 19].

2.7 Mechanism of Protease mediated anticancer therapy

The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development, and chemical-induced cell death. Inappropriate apoptosis (either too little or too much) is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders, and many types of cancers. The ability to modulate the fate of a cell is recognized for its immense therapeutic potential.

2.7.1 Mechanism of apoptosis

The mechanisms culminating in apoptosis are highly complex and sophisticated, involving energy-dependent cascades of molecular events (Figure 3). To date, predominantly two apoptotic pathways have been reported, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, recent evidence has shown that the two pathways and their molecules are in fact associated and can influence each other [67]. There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cell. The extrinsic, intrinsic and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and eventual phagocytosis. The granzyme A pathway activates a parallel, caspase independent cell death pathway via single stranded DNA damage [68].

2.7.2 Extrinsic pathway

The sequence of events that define the extrinsic phase of apoptosis are best characterized with the FasL/FasR and TNF-α/TNFR1 models. In these models, there is clustering of receptors and binding with the homologous trimeric ligand. Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP [69-71]. FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 [72].

Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP which binds to FADD and caspase-8, rendering the ineffective [73, 74]. Another potential

apoptotic regulator is Toso, which has been reported to block Fas-induced apoptosis in T-cells via inhibition of caspase-8 processing [75].

2.7.3 Intrinsic pathway

The intrinsic signaling pathway that initiates apoptosis involves a diverse array of non-receptor-mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act either in a positive or negative manner. Negative signals imply the absence of certain growth factors, hormones, and cytokines that can lead to the failure of suppression of death programs, thereby triggering apoptosis. In other words, the withdrawal of factors leads to loss of apoptotic suppression and subsequent activation of apoptosis. Other positive stimuli include radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All these stimuli cause changes in the inner mitochondrial membrane leading to the opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential, and release of cytochrome c, Smac/DIABLO, and the serine protease HltrA2/Omi [76-78]. These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds and activates apoptotic protease activating factor-1 (Apaf-1) as well as procaspase-9, forming an “apoptosome” [79, 80]. The clustering of procaspase-9 leads to caspase9 activation, resulting in the activation of caspase-3 from precursor procaspase-3 gradually inducing the intrinsic pathway of apoptosis.

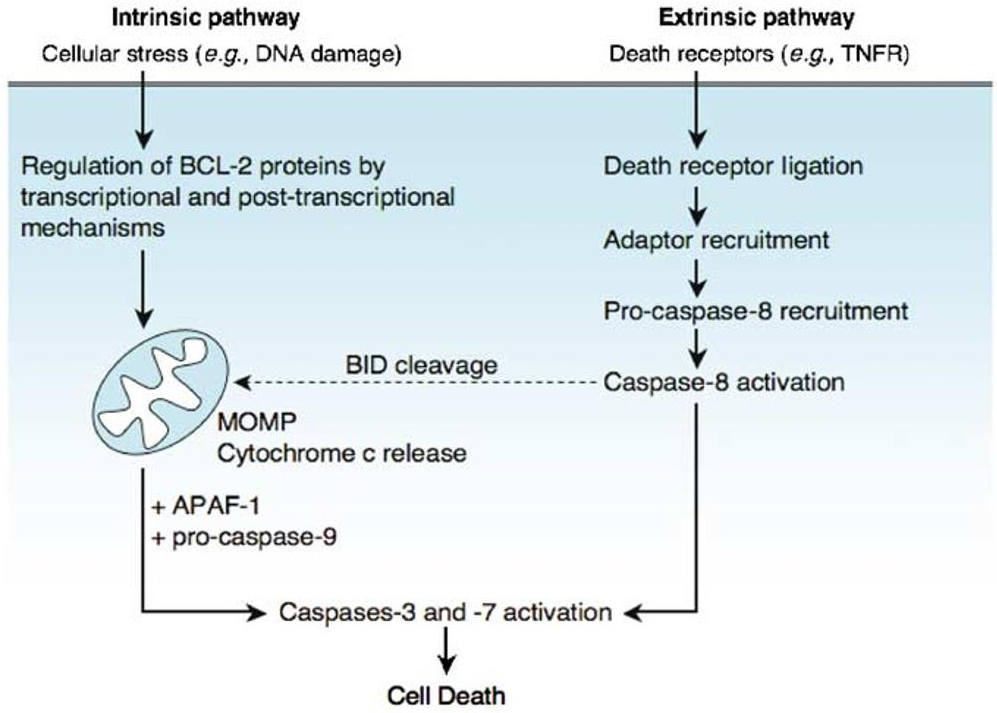

Figure 3. Schematic representation of apoptotic events [Chipuk *et al*, 2006].

2.8 Apoptosis and protease activated receptors

As mentioned earlier, bacterial proteases have been reported to be associated extensively with different pathological conditions, including cancer. Considerable work on the intricate effects of proteases over the extra and intracellular ambiance has led to the discovery of a number of targets and receptors. Protease-activated receptors (PARs), a subfamily of G protein-coupled receptors (GPCRs), are mention-worthy amongst such receptors. A class A category GPCR, PARs currently comprise four members, namely PAR1, PAR2, PAR3, and PAR4 [82, 83]. While PAR1, PAR3, and PAR4 serve as targets of the coagulation factor thrombin, PAR2 usually harbors another serine protease trypsin as its ligand [84]. All the PARs though structurally similar to all GPCRs, manifest a very unique mechanism of activation. While the bulk of GPCRs are activated reversibly by hydrophilic ligands, the PARs are exclusively activated by endogenous proteases [85-87]. Serine proteases cleave the extracellular amino termini to expose a motif termed the “tethered ligand”, which in turn interacts with the extracellular domains of the receptor resulting in an irreversible activation

leading to specific downstream signaling [84]. Since their discovery, the PARs have been associated with multiple normal and disease states, namely the cardiovascular, musculoskeletal, gastrointestinal, respiratory, and central nervous system, as well as several cancers. Where PAR1, in particular, is observed to promote tumor cell invasion [88-90], and epithelial cell malignancies [91], simultaneously various reports suggest the upregulation of PARs and their potential activating proteases in tumor tissues including prostate and colon cancer as well as malignant melanoma [92-95]. Therefore, based on the studies from diverse disease models and their respective roles, numerous agonists and antagonists are in trial to determine their therapeutic potential [96].

Proteinase-activated receptor 1 (PAR1) is the earliest discovered member of a unique class of G-protein-coupled signaling receptors activated via cleavage of their extracellular amino terminus by proteinases such as thrombin, trypsin, and tryptase [97, 98]. Three additional human PARs have been identified since the cloning of PAR1: PAR-2, activated by trypsin and mast cell tryptase, PAR-3 by thrombin, and PAR-4 by thrombin, trypsin, and neutrophil cathepsin G [99]. Thrombin was originally identified as a key mediator of the coagulation cascade and a potent activator of platelet aggregation. It is now well established that thrombin is a physiological activator of PAR1, 3, and 4, and PAR activation is involved with platelet aggregation, vasodilation and vasoconstriction, increased vascular permeability, granulocyte chemotaxis, calcium dependent chloride secretion in intestinal epithelial cells, and other inflammatory events of the gastrointestinal tract [100-103].

Increasing evidence suggest thrombin to be pro- or anti-apoptotic in various cell types [104, 105]. The influence of PAR1 activation on cell death has been most thoroughly examined in neurons and astrocytes. Vaughn *et al.* demonstrated that low concentrations of thrombin or thrombin receptor activating peptide (TRAP) protect rat primary neurons and astrocytes from cell death induced by hypoglycemia or oxidative stress, whereas higher concentrations lead to cell death in both cell types under normal conditions [106].

The potential of thrombin as a growth factor was further reported in non-neuronal cell types, including vascular and airway smooth muscle cells [107, 108], fibroblasts [109, 110], and several tumor cells [111, 112]. Zain *et al.* [113] further reported that low concentrations of thrombin (0.1 to 0.5 U/ml) enhanced tumor cell growth, whereas higher levels (0.5 to 1 U/ml) led to caspase-dependent apoptosis in those cells. The effect was reported to be mediated by PAR1. In addition, PAR1 expression increases in breast carcinoma biopsies, and PAR1 inhibition via antisense cDNA prevents the invasion of metastatic breast cancer cells in culture through a basement membrane [114].

2.9 Targeting protease-activated receptors: Their potential therapeutics

The association between blood coagulation, with respect to venous thrombosis and cancer development, was first described in the nineteenth century by Drs. Trousseau and Bouiillaud [115]. According to existing data, coagulants, serine proteases, and matrix metalloproteases (MMPs) facilitate tumor cell metastasis by modulating a number of host vascular cell responses as well as by acting directly in tumor cells themselves. Since the 1970s, hormone-like effects of proteases in target tissues have been recognized, e.g., insulin-like effects of pepsin or chymotrypsin as well as mitogen actions of thrombin and trypsin at the cell membrane [116, 117]. Collectively, the thrombin-activated receptors have come to be referred to as Protease-Activated receptors (PARs). In the late twentieth century, pioneering work identified the presence of the G-protein-coupled thrombin receptor at the surface of cancer cells in solid tumors [118]. The predominant activators of PARs in cancer cells are thrombin, MMPs, trypsin, TF, FVIIa, FXa, and their ternary complex TF/FVIIa/FXa [119-121]. The expressions of PAR is implicated in the development of several types of human malignant cancers and correlate directly with the degree of invasiveness exhibited by both primary and metastatic tumors [122, 123].

2.9.1 Mechanism of activation

PARs are transmembrane G-protein-coupled receptors (GPCRs) [124, 125]. The mechanism of PAR activation is most thoroughly investigated with PAR1 [126, 127]. The predominant activator of PAR1, thrombin binds to the receptor N-terminus $LDPR^{41}$-S^{42} sequence and cleaves the R^{41}-S^{42} peptide bond [128]. The new, unmasked sequence generated as such acts as a tethered ligand that binds intramolecularly to residues $^{42}SFLLRN^{47}$ in the conserved region of the second loop of the receptor to induce transmembrane signaling [129]. The sequence of the tethered ligand is distinct and characteristic for each of the PARs.

2.9.2 G protein-mediated signaling by PARs

Protease-activated receptors similar to other GPCRs, signal via a variety of G proteins, including G_q, G_i, $G_{12/13}$ but not directly by G_s [130, 131]. For G-protein mediated signaling, the receptor acts as a ligand-triggered guanine nucleotide exchange factor, stimulating the exchange of GTP for GDP in the G_α subunit of the heterotrimeric G protein oligomer. This exchange enables the 'release' of the G_α subunit from its tight binding to the $G_{\beta\gamma}$ dimer subunit. Each of the G protein moieties (G_α-GTP and $G_{\beta\gamma}$) are then independently able to interact with downstream signaling effectors like phospholipase C (G_q) or ion channels ($G_{\beta\gamma}$). This 'dual effector' signaling, resulting in principle from the same PAR-activated G protein heterodimer ($G_qG_{\beta\gamma}$), can converge for complex downstream signaling, leading to NF-κB activation and Intracellular adhesion molecule-1 (ICAM-1) transcription by the recruiting parallel G_q/protein kinase C (PKC) and G_i/phosphatidylinositol 3-kinase (PI3K) pathways that converge [132, 133]. Alternatively, activated PARs have also been shown to mediate MAPK signaling via a $G_{12/13}$-triggered 'biased signaling' process, bypassing a G_q-mediated calcium signaling event [134]. Such selective signaling depends not only on the agonist per se [e.g. thrombin, neutrophil elastase, MMP1 or activated protein C (APC) for PAR1] but also upon the localized membrane topology. For instance, triggering of PAR1 localized in the caveolae by APC can signal a set of downstream effectors that are different from those regulated when thrombin activates PAR1 in a non-caveolar region [130].

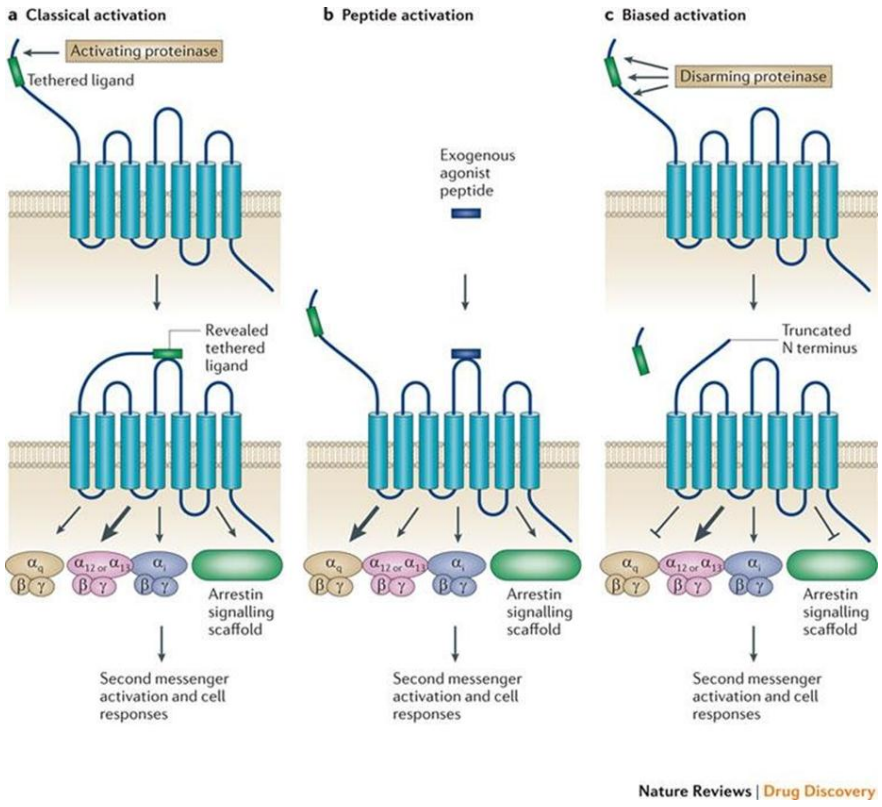

Figure 4 depicts the activation of proteinase-activated receptors (PARs) by proteolytic cleavage and the revealing of the tethered ligand to stimulate signaling (part a), the activation of PARs by exogenous application of synthetic agonist peptides in the absence of proteolytic unmasking of the tethered ligand (part b), and the disarming of signaling through PARs by proteolytic cleavage downstream of the receptor activating site to truncate the tethered ligand and make it unavailable for activating proteinases (part c). Such processing of PARs can, in addition to disarming some signaling pathways, simultaneously activate other signaling pathways. Disarmed receptors may sometimes be retained on the cell surface and be available for activation by synthetic agonist peptides [Nature Reviews, Drug Discovery].

2.9.3 Thrombin/PAR1 in cancer cells

Thrombin can elicit a signaling response via direct interaction with PAR1 present on tumor cells [131-134]. In vitro studies with various cancer cell lines showed a correlation between overexpression of PAR1 in cancer cells and greater invasiveness and development of distant metastases [131, 133, 135-143]. Moreover, in patients

with lung, gastric, or breast cancer, PAR1 expression was an independent, unfavorable prognostic factor in terms of overall survival, while in prostate cancer patients, it turned out to be a prognostic factor for local recurrence [135, 136, reviewed in 144]. Decreased expression of PAR1 was associated with reduced invasiveness of cancer cells [145].

PAR1 expression has been confirmed in melanoma, breast, lungs, esophageal, gastric, colon, prostate, pancreatic, liver, ovarian, endometrial, and head and neck cancers [135, 138, 139, 149, reviewed in 144, 150, 151]. The cellular effect induced by PAR1 depends on the concentration of agonist, such that low concentrations of thrombin (less than 3 nM) stimulate cancer cell proliferation and tumor growth, while high thrombin levels lead to apoptosis [152]. Most cellular effects are triggered via long-lasting activation of second messengers ERK1/2. However, multiple intracellular signaling pathways may be implicated in thrombin/PAR1 activation [153, 154].

2.9.4 Apoptosis, proliferation, migration, and invasion

In murine models of benign tumors, PAR1 activation results in tumor growth and invasion by silencing proapoptotic genes [143]. However, in epithelial cancers and melanoma cells thrombin-mediated PAR1 activation triggers prosurvival pathways [143, 155-159]. Overexpression and activation of PAR1 in nonmetastatic melanoma cell lines stimulate the Akt/PKB signaling pathway, leading to a decrease in Bim and Bax expression, as well as cleaved caspase-3 and caspase-9 levels. Inhibition of PAR1 activity also decreased tumor growth during in vivo experiments, confirming apoptosis-related effects [155].

In numerous cancers, the response to thrombin-induced PAR1 activation increases cell proliferation, as well as motility and migration in Matrigel barrier assays [147,156,158,160]. In Hep3B liver carcinoma cells, PAR1 and PAR-4 activate common promigratory signaling pathways via activation of the receptor tyrosine kinases Met, PDGFR, and ROS kinase, as well as the inactivation of the protein tyrosine phosphatase, PTP1B [161]. In nasopharyngeal cancer, thrombin-induced PAR1 activation leads to increased expression of MMP-2 and MMP-9, which are

closely associated with tumor metastasis they can degrade the extracellular matrix and disrupt the basement membrane [138,162].

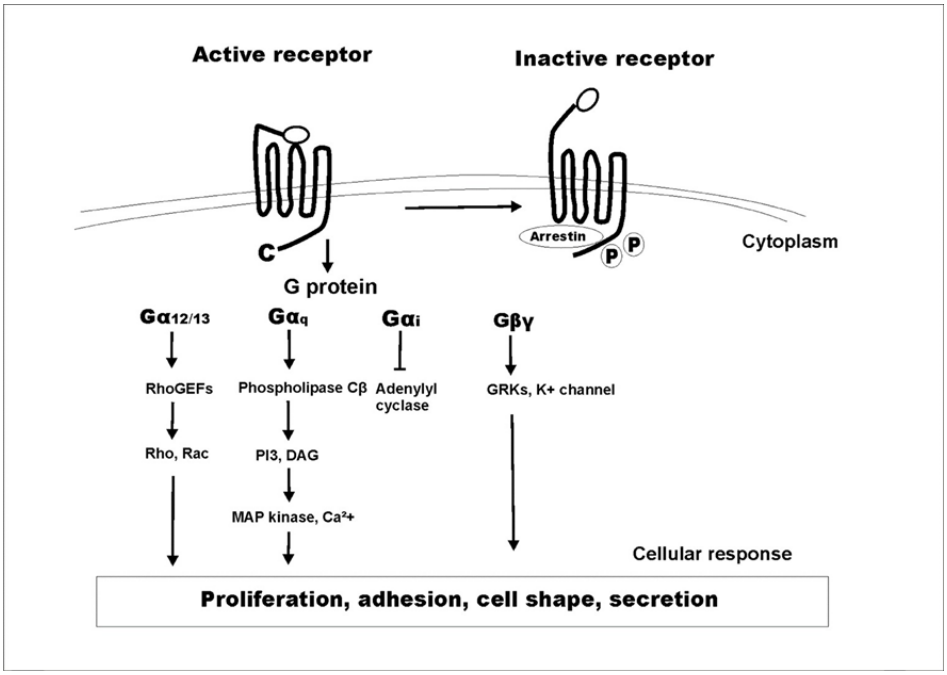

Figure 5. Protease-activated receptor 1 (PAR1) activation and signaling [Wojtukiewicz *et al*, 2015].

2.9.5 PARs and colon cancer

In a large number of cancers, stimulation of PARs leads to transactivation of epidermal growth factor receptor (EGFR) contributing to cancer development and progression. With respect to colon cancer progression, this mechanism includes PAR-2 mediated EGFR transactivation and a subsequent increase of cyclooxygenase (COX-2) expression in colonic epithelial cancer cells [164]. Moreover, PAR1 and PAR-2 induce migratory and proliferative effects in colon cancer cells that involve transactivation of the EGFR and activation of p42/p44 MAPK signaling pathways [165-167]. Likewise, activation of PAR1 by thrombin induces persistent EGFR and ErbB-2 transactivation, sustained p42/p44 MAPK signaling, and invasion in breast cancer cells [168]. The EGFR transactivation by PAR1 or PAR-2 leads to COX-2 expression [169], enhanced cell proliferation in colon carcinoma cells [164,166], and cell migration in renal carcinoma cells [162] is dependent on matrix metalloprotease (MMP) activity. Hence, pharmacologic targeting with PAR1/PAR-2 and EGFR antagonists may be supplemented with MMP and possibly COX-2 inhibitors.

PARs are unique to the G protein-coupled receptor family in their lack of any physiologically soluble ligands. In contrast to classical receptors, PARs are activated by N-terminal proteolytic cleavage and removal of specific peptides, which in turn serve as tethered ligands that interact with extracellular loop domains to initiate receptor signaling. Therefore, owing to the importance of PARs in various pathophysiological conditions (including cancer) and their complexity of regulation by specific cleavage modes (activation or inactivation), various short synthetic peptide agonists and antagonists are devised to mimic the PAR-tethered ligand resulting in either induction or prevention of the cleavage-dependent signaling, guiding to therapy [170]. To date, nearly 50 GPCR peptide drugs have been approved predominantly for metabolic diseases or oncology, and more than 10 potentially first-in-class peptide therapeutics are in the pipeline. Examples include the glucagon-like peptide 1 (GLP-1) receptor agonists for the treatment of type 2 diabetes mellitus.

2.10 The era of anticancer peptides as novel candidates for cancer therapy

Traditional anticancer chemotherapeutics and antitumor monoclonal antibodies have failed to achieve their desired levels of selective toxicity, owing to their large size and nonspecific uptake by the liver and reticuloendothelial system resulting in life-threatening, dose-limiting toxicity to the liver and bone marrow [171,172]. Moreover, steady constrain from both public and regulatory authorities for lower healthcare costs and better anticancer drug efficacy, quality, and safety respectively have shifted the focus of major pharmaceutical R&Ds to the discovery of tumor-related peptides to constitute more cost effective and selective anticancer drugs in the future [173,174]. Peptides are polypeptide chains of 5 to 50 amino acids or 5000 Da in molecular weight, usually manufactured by chemical or enzymatic synthesis, recombinant DNA biotechnology, cell-free expression, and transgenic animal or plant species [172,173]. With a smaller size, better tissue penetration, less immunogenicity, and lower production costs with more activity per unit mass, peptides offer advantages over recombinant antibodies and proteins at the biological level [175,176]. Examples include the Ra-V, which triggers mitochondrial apoptosis by mediating the loss of mitochondrial membrane potential leading to cytochrome-c release and gradual

activation of the caspase apoptotic pathway in human breast cancer cells [177], and the *Bacillus subtilis* lipopeptide, reported to inhibit the growth of K562 myelogenous leukemia cells and induce apoptosis by generating a ROS burst, gradually resulting in the intrinsic pathway of apoptosis [178]. Thus such, and many other peptides have evolved as promising therapeutic agents in the treatment of cancer [179].

2.11 Future aspects of Microbial proteases and peptides as a targeted therapy of colon cancer

The modern conventional treatment modalities for cancer are restricted to chemotherapy, radiation, and surgery. While surgical recurrences are common, chemotherapy is plagued with the inability of precise drug delivery thus leading to severe side effects. Drug resistance, altered bio distribution, biotransformation, and drug clearance are other common obstacles [180]. To tackle such problems, targeted chemotherapy and drug delivery systems were developed. The discovery of several proteases and peptide receptors along with certain tumor-related peptides hold promise as both effective and selective anti-cancer drugs [172,173,181]. Owing to their small size, ease of synthesis, tumor penetrating ability, and good biocompatibility, peptides have garnered considerable attention as potential candidates for targeted cancer therapy throughout the decade [179,182]. The present-day treatment for colorectal cancer is confined predominantly to surgery followed by adjuvant chemotherapy, and very rarely resorting to radiation, the outcome widely depends on tumor-specific molecular features, tumor location, and patient characteristics [183-185]. With little success, the bulk of patients experience both acute and chronic side-effects [Colorectal Cancer Facts & Figures 2020-2022, American Cancer Society]. Such grave observations have urged active research in the development of targeted therapy of colorectal cancer, namely the Lon protease secreted from the uropathogenic *Escherichia coli* (UPEC) was shown to degrade c-MYC and delay tumor progression and increase survival of MYC dependent mice colon cancer models [186], and arginine-rich hexapeptide “RRKRRR” that inhibited both growth and metastasis of human colon carcinoma cells (HM7) in a nude mice xenograft model [187].

REVIEW OF LITERATURE

Colorectal cancer is the third most common cancer in men and second most common in females, accounting for 8% of all cancer-related deaths, making it the fourth most recurrent cause of death due to cancer [Consensus Document for Management of Colorectal cancer, ICMR, 2014]. Estimated cases rise exponentially every year. Although prevalent in developed countries [188], studies demonstrate an immediate association of colorectal cancer with lifestyle choices such as diet, physical inactivity, smoking, and alcohol [189], along with certain metabolic disorders like diabetes (Type II) and obesity. Thereby, putting developing countries such as ours at substantial risk. Thus, research for early screening and effective targeted therapy of colorectal cancer demands immediate attention. Peptide vaccines have shown promising results in immunological as well as clinical responses and such immunotherapy are believed to be the future of cancer treatment, where several have undergone phase I and II clinical trials with optimistic outcomes, candidates such as Mucin-1 (MUC-1, Stimuvax) [190, 191], carcinoembryonic antigen [192,193], and Ras oncoprotein peptide [194-196] have undergone phase III trials.

CHAPTER 3

OBJECTIVE OF THE STUDY

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Many species of pathogenic bacteria produce cell-surface or secreted proteases as they rely on proteolysis for a variety of purposes during the infection process [197]. These proteases have a high potential to enhance bacterial pathogenesis through the degradation of critical host proteins and by mimicking the activity of host regulatory proteases that control important zymogen systems [198]. Pathogenic *Vibrio cholerae* produce a plethora of proteases among which hemagglutinin protease (HAP) is noteworthy in virulence and pathogenesis [18]. Previous studies have shown proteases to be associated with cancer prognosis because of their ability to degrade extracellular matrices, which facilitate invasion and metastasis [199]. On the contrary, recent studies show such proteases to play anti-cancer roles depending on different stimuli [8,7,19]. Colorectal carcinoma is the third most commonly diagnosed cancer and the third leading cause of cancer deaths in both males and females in developing countries [1,2]. Recently, HAP has been reported to induce apoptosis in breast cancer cells [7,19]. In our present study, we intend to demonstrate the apoptotic response of HAP in colon cancer cells along with its role in tumor regression in mice models.

1) Purification of bacterial proteases from *Vibrio cholerae*.
2) Study the preventive role of bacterial protease on colon cancer progression and tumor formation in both in vivo and in vitro animal models.
3) Study the mechanism of bacterial protease-induce induced apoptosis in colon cancer cells.
4) Study the bacterial protease-induced major signaling pathways and their role in colorectal cancer therapy.

CHAPTER 4

MATERIALS AND METHODS

4.1 Reagents

All bacteriological media Tryptic soy broth (TSB), Thiosulfate-citrate-bile salts-sucrose agar (TCBS) and Bacto-agar were obtained from Difco Laboratories, USA. Dulbecco's Modified Eagle (DMEM) medium and RPMI-1640 were purchased from Gibco, USA and Invitrogen respectively. Streptomycin and Penicillin were purchased from Sigma Chemical Company, USA. Bovine serum albumin was purchased from Bio-Rad, USA. Sodium bicarbonate was purchased from SRL, India. DE52 (Diethylaminoethyl cellulose) was purchased from Whatman, Kent, England and Sephadex G-100 was purchased from MP Biomedicals. Acrylamide, N, N'-ethylene bis acrylamide, and Sodium dodecyl sulphate, were purchased from Sigma Chemical Company, USA. Coomassie brilliant blue G, Glycerol, Methanol, β-Mercaptoethanol, Glacial acetic acid, Glycine and ammonium sulphate were purchased from SRL, India. N,N,N',N.-Tetramethylethylendiamin (TEMED) was purchased from E. Merck, India. Agarose and TRIS (Hydroxymethyl) Aminomethane (Tris buffer) were purchased from Sigma Chemical Company, USA. All DNA and protein markers were obtained from Himedia, India. Trizol was purchased from Invitrogen, USA. SYBR green, Ethidium bromide, Tween-20 and Bradford reagent were purchased from Bio-Rad, USA. Glucose was purchased from E. Merck, India. Trichloroacetic acid and dialysis tubing were purchased from Himedia, India. EDTA and Azocasein were purchased from MP Biochemicals, USA. PAR 1 inhibitor (ML161) was purchased from Sigma Chemical Company and inhibitors of p38 (SB203580) and NFκB (MG132) were obtained from Calbiochem, USA. All antibodies (anti Bax, anti Bcl-2, anti p53, anti Caspase 3, anti Caspase 9, anti Caspase 8, anti p38, anti p-p38, anti p65, anti p50, anti PAR1, anti PKC-zeta, anti p-PKC zeta, anti SP1, anti Ap2, anti Actin and anti Tubulin) were purchased from Santa Cruz Biotechnology, USA

4.2 Animals

Swiss Albino mice between 22-25 g obtained from the animal facility of National Institute of Cholera and Enteric Diseases, Kolkata, India, were used for *in vivo* studies like Survival kinetics assays. Mice were maintained as per the principles and guidelines of the ethical committee for animal care of National Institute of Cholera

and Enteric Diseases. The experimental designs were approved by Institutional Animal Ethics Committee (License No: PRO/125/April 2016- March 2019), NICED, Kolkata, India.

4.3 Bacterial strain

V. cholerae O1 El Tor strain C6709 was kindly provided by Dr. Rupak Bhadra, Indian Institute of Chemical Biology (IICB), Kolkata, India. *V. cholerae* cells were routinely grown in Tryptic soy broth (TSB) (Difco, USA) at 37^{o}C with shaking. *V.cholerae* strains were selected on TCBS agar plate, forming yellow colonies. Bacterial strains and oligonucleotides used in this study are listed in Table 1. Antibiotic- Streptomycin was used at 100 µg/ml concentration. Bacterial cells were maintained at -70^{o}C in TSB containing 20% sterile glycerol.

4.4 Purification of HAP from C6709 strain

4.4.1 Media and culture conditions

Purification of HAP was performed from *V. cholerae* O1 strain C6709. C6709 strain was inoculated into 5 ml of sterile Tryptic soy broth (TSB) (Difco, USA) pre-culture supplemented with µg/ml streptomycin and grown at 37^{o}C shaker incubator. 100 µl of log-phase culture was inoculated in 2 liter TSB, incubated for 18 H at 37^{o}C under agitation at 120 rpm in an orbital shaker (OSI503, Firstek Scientific). Cells were harvested at 10,000 rpm for 20 mins at 4^{o}C in an SS34 rotor (Sorvall, USA). Cell free supernatant was filtered through 0.22 µm pore sized cellulose acetate membrane (Millipore, USA) to remove residual cells.

4.4.2 Ammonium sulphate precipitation and dialysis

The proteins present in the cell free culture supernatant was concentrated with 60% ammonium sulphate (Merck, Germany) at 4 ^{o}C, centrifuged at 10000 rpm, dissolved in 20 mM Tris-HCL (pH-7.4) buffer and were dialyzed against same buffer at 8 h

intervals. Dialysis tubing (Himedia, India), retaining protein of molecular weight of 10 kDa or greater, was used throughout the experiment.

4.4.3 Anion exchange chromatography (DE 52)

The DE-52 column was packed and equilibrated with 20 mM Tris-HCL buffer (pH-7.4). The column was connected in a Biologic Duo Flow Chromatographic System (Bio-Rad, USA). The concentrated crude proteins were loaded onto the DE-52 and non-binding (NB) fraction was eluted with 20 mM Tris-HCL. Protein peaks from each elution fraction were pooled, dialyzed, concentrated and tested for protease activity.

4.4.4 Gel filtration chromatography (Sephadex G-100)

The G-100 matrix was swollen in 20 mm Tris-HCL buffer (pH-7.4) overnight. Frequent changes of buffer were done to remove any impurities present. The column was packed, equilibrated with the same buffer and connected to a Biologic Duo Flow Chromatographic System (Bio-Rad, USA). The non-binding fraction showed maximum protease activity and was further loaded onto Sephadex G-100 chromatographic column. Proteins were eluted with Tris-HCL buffer. Each peak was collected, pooled, concentrated and protease activity was measured and subjected to SDS-PAGE.

4.4.5 Estimation of Protein concentration

The concentration of proteins in the eluted fractions were estimated by method of Bradford (1976) using bovine serum albumin (BSA) as standard. Protein estimation was done using Bio-Rad microassay procedure in microtiter plates. Different concentrations of BSA were diluted in buffer to make the final volume of 160 µl in a microtiter plate. Test samples of volumes 10 µl were also diluted in buffer similar to BSA dilutions. 40 µl of the Bradford dye reagent was added to each well, mixed

thoroughly and incubated at RT for 5 mins. Absorbance was taken at 595 nm in a microplate spectrophotometer (Bio-Rad, USA). Using the OD values of BSA samples a standard curve was prepared and relative protein concentrations were measured from the standard curve.

4.4.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

10 µg of purified protein obtained from G-100 column chromatography was denatured in sample buffer containing 10% glycerol, 0.05% bromophenol blue, 2% SDS, 5% 2-marcaptoethanol and 10 mM Tris-HCL buffer, pH 6.8 and resolved in a 10% polyacrylamide gel with a discontinuous buffer system at a constant 60V for the stacking gel and 120V for the resolving gel. Proteins with known molecular weights (Bio-Rad, USA) were used as molecular weight markers. Gels were fixed with methanol and glacial acetic acid and stained with Coomassie blue.

4.4.6.1 Preparation of reagents for SDS-PAGE

i. Solution A (30% Acrylamide stock solution)
   Acrylamide 29.2 gram
   N, N'-ethylene bis acrylamide 0.8 gram
ii. Solution B (1.5 M Tris-HCl buffer pH-8.8)
   1.5 M Tris hydroxymethylaminomethane (Tris)
   0.4% Sodium dodecyl sulphate
iii. Solution C (0.5 M Tris-HCl buffer pH-6.8)
   0.5 M Tris hydroxymethylaminomethane (Tris)
   0.4% Sodium dodecyl sulphate
iv. Solution D (Ammonium persulphate APS)
   10% APS is prepared in distilled water.
v. TEMED (N,N,N',N'-tetramethylene-ethylenediamine)
   Used as supplied by manufacturer.

4.4.6.2 Preparation of Resolving and Stacking Gel

A 10% resolving gel and 4.5% stacking gel were prepared using the different reagents in volumes as given in the table below.

	Resolving Gel			Stacking Gel	
	15%	12.5%	10%	7.5%	4.5%
Solution A	4.5 ml	3.75 ml	3.0 ml	2.25 ml	0.45 ml
Solution B	2.25 ml	2.25 ml	2.25 ml	2.25 ml	--
Solution C	--	--	--	--	0.75 ml
10% APS	0.04 ml	0.04 ml	0.04 ml	0.04 ml	0.01 ml
TEMED	0.005 ml	0.005 ml	0.005 ml	0.005 ml	0.005 ml
Water (dd)	2.25 ml	3.0 ml	3.75 ml	4.5 ml	1.8 ml

4.4.6.3 Electrophoresis buffer

Electrophoresis buffer was prepared by dissolving 14.4 g glycine, 3 g Tris (hydroxymethyl) Aminomethane and 1g Sodium dodecyl sulphate (SDS) in 1000 ml distilled water. pH was adjusted to 8.3.

4.4.6.4 Preparation of Sample buffer

5X Sample buffer was prepared by mixing 60 mM Tris-HCl (pH 6.8) with 2% SDS, 5% Mercaptoethanol, 25% glycerol and 0.1% bromophenol blue.

4.4.6.5 Preparation of Sample

Proteins (5-40 μg) were mixed with 1X sample buffer and boiled for 5-10 mins in a boiling water bath. The samples were then loaded onto the wells of the gel.

4.4.6.6 Procedure of Electrophoresis

Electrophoresis was carried out on a vertical slab gel apparatus (Bio-Rad, USA) at constant room temperature (25^{o}C) using constant voltage of 80 volts while the proteins are in the stacking gel and increased to 120 volts once the proteins reached the resolving gel.

4.4.6.7 Fixing, Staining and destaining of gel

After electrophoresis the gel was fixed in 10% methanol and glacial acetic acid for 30 mins and stained with 0.1% Coomassie brilliant blue dissolved in 45% methanol and glacial acetic acid for 30 mins with gentle shaking. The stained gel was subsequently destained with 10% methanol and glacial acetic acid.

4.4.7 Protease estimation assay

4.4.4.1 Azocasein assay

Protease content of pooled fractions were estimated by azocasein assay. Casein was chosen as substrate to assay proteolytic activity by the method of Charney and Tomarelli (1947) with minor modifications. The substrate-enzyme mixture was incubated at 37^{o}C for 1 hr. and reaction was terminated by adding 10% Trichloroacetic acid (TCA) and kept on ice for 30 mins, the precipitated protein was removed by centrifugation and the supernatant was transferred to a clean tube containing 0.5 M NaOH. Absorbance was measured at 440 nm.

4.5 Synthesis of Pro-apoptotic peptide

The pro-apoptotic peptide “PFISED”, and the jumbled peptide “FEPIDS” were commercially synthesized and purchased from Eurogentec, Belgium. 95% HPLC grade purified peptide was used for the study.

4.6 Cell culture

Human and mice colon cancer cells, HT29 and CT26 respectively were used in this study. Both HT29 and CT26 cells were grown in 25 ml cell culture flask (BD biosciences), containing Dulbecco's Modified Eagle (DMEM) medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) along with penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml) (Sigma, USA) at 37^{o}C in a CO_2 incubator (Heraeus, Germany). Cells with 70 to 80% confluence were seeded in a 6 well culture plates (BD Biosciences) at a concentration of approximately 10^5 cells/well. Cells were permitted to reach 70 to 80% confluence by allowing them to grow for 24 h in a CO_2 incubator. CT26 cells were kindly provided by Dr. Rathindranath Baral, Chittaranjan National Cancer Institute (CNCI), Kolkata, India.

4.6.1 Maintenance and preparation of CT26 from mice peritoneal cavity

CT26 cells were kindly provided by Dr. Rathindranath Baral, Chittaranjan National Cancer Institute (CNCI), Kolkata, India. CT26 cells were maintained in Swiss albino mice through serial intraperitoneal (I.P) inoculation at 10-day intervals. 10^6 number of viable CT26 cells were implanted into the peritoneal cavity of each mouse (22-25 g body weight) and allowed to multiply.

For *in vitro* studies CT26 cells were isolated from the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells were incubated at 37^{o}C for 2h. The cells of the macrophage lineage adhered to the cell culture flasks. The non-adherent cell population were collected gently and washed with PBS. More than 98% of this separated cell population was morphologically characterized as CT26.

For *in vivo* studies 10^6 number of viable CT26 cells were implanted into the peritoneal cavity of each mouse (22-25 g body weight) and allowed to multiply, followed by proposed weekly treatment protocol starting from a week after the inoculation.

4.6.2 Explant culture of normal colon tissue

Normal colon tissue was harvested from normal Swiss Albino mice. Tissues were sectioned into approximately 1 mm × 1 mm slices. These tissue sections were randomized and cultured in 48-well flat bottom plates in presence of DMEM medium supplemented with 10% fetal bovine serum, 1X Insulin-Transferrin-Selenium (ITS) and 1X penicillin and streptomycin. Tissue slices were either treated with 1.0 μg/ml of HAP for 1h or kept untreated. Both treated and untreated tissue samples were used for cDNA preparation for real time PCR of PARs, as described by Majumder *et al*.

4.6.3 Treatment protocol

Purified HAP from C6709 were diluted to a final concentration of 1.0 μg/ml. Cells were incubated overnight in serum free media before HAP treatment. The exhaust media was aspirated from 6 well plate/25 ml cell culture flasks containing HT29/CT26 cells grown to confluence, washed with PBS, complete media along with HAP was added and grown at 37^{o}C in a CO_2 incubator for 1h to 2h (according to protocol requirement). Similar protocols were followed with the antimicrobial peptide 'PFISED', where the final concentration was 100 μM and incubation for 16 h.

4.7 *In vivo* studies

4.7.1 Survival kinetics assay

Survival kinetics was studied by implantation of 10^6 number of CT26 cells into the peritoneal cavity of Swiss albino mice (22-25 g body weight) and allowed to multiply. Animals were divided into three different groups of 10 animals each, in (i) normal set (non-tumor-bearing); (ii) tumor-bearing set; and (iii) HAP-treated tumor bearing set where 1.0 μg HAP (or 100 μM peptide) was injected intraperitoneally on the day after

the inoculation of CT26 cells and injected once in a week. Life span of each group of mice was evaluated by measuring the percentage of survival rate in each group at 10 days interval by using a formula:

$$\frac{Number\ of\ live\ animals\ in\ a\ group}{number\ of\ initial\ animals\ in\ that\ group} \times 100$$

Each experiment was replicated thrice.

4.8 Flow cytometry assay

Flow cytometry analysis was performed as described by Pal *et al*. HT29 and CT26 cells (10^6 cells) were incubated with HAP from *V.cholerae* strains C6709 at concentrations 1.0 μg/ml for 2h (or 100 μM peptide for 16 h) or pre-incubated with 0.5 μM PAR1 inhibitor (ML161) or 3.0 μM NFκB inhibitor (MG132), or 10.0 μM p38 inhibitor (SB203580) for 30 min before HAP treatment. After HAP treatment cells were washed with PBS and stained with fluorescein isothiocyanate (FITC)-conjugated AnnexinV and propidium iodide (PI) (BD Pharmingen) according to manufacturer's instructions. Cells were stained with FITC-AnnexinV in AnnexinV binding buffer for 15 min followed by addition of PI and analyzed by the flow cytometry using Cell Quest Pro software (BD Biosciences). 10^4 cells were acquired for data analysis in each set of experiments. Values were expressed as mean $\pm$SD of mean of 3 different observations.

4.9 Immunobloting

4.9.1 Reagents for Immunobloting

Transfer buffer pH 8.3

Tris - 25 mM

Glycine - 192 mM

Methanol - 20%

SDS - 0.5%

Tris-buffer saline (TBS) pH 7.8

Tris - 20 mM

NaCl - 500 mM

Tween 20 Tris-buffer saline (TTBS)

TBS + 0.1% Tween 20

Blocking buffer

Bovine serum albumin 3% in TTBS

Antibody preparation

Primary antibody diluted in TTBS

Secondary antibody diluted in TBS

Substrate

BCIP - 3.75 mg in 100% Dimethylformamide (DMF)

NBT - 7.5 mg in 70% DMF

4.9.2 Preparation of Reagents

I. Solution A (30% Acrylamide stock solution)
   Acrylamide 29.2 gram
   N,N'-ethylene bis acrylamide 0.8 gram
II. Solution B (1.5 M Tris-HCl buffer pH-8.8)
III. Solution C (0.5 M Tris-HCl buffer pH-6.8)
IV. Solution D (Ammonium persulphate APS)
   10% APS in prepared in distilled water.
V. TEMED (N,N,N',N'-tetramethylene-ethylenediamine)
   Used as supplied by manufacturer.
VI. Double distilled water.

4.9.3 Procedure

Cell lysates were prepared by RIPA buffer from HAP treated (1.0 μg/ml of HAP for 1h or 100 μM peptide for 16 h) and untreated HT29 and CT26 cells. For inmmunoblot, 30 μg of cell lysate was loaded onto 10% SDS-PAGE electrophoresis and the separated proteins were transferred onto PVDF membrane (Bio-Rad, USA). The membrane was blocked at room temperature for 1h in 3% bovine serum albumin (Sigma-Aldrich, USA) PBS – Tween 20. After incubation, the membrane was washed and incubated overnight at 4^{o}C with specific rabbit/ mouse primary antibodies (1:1000 dilution in 3% [wt/vol] bovine serum albumin) (Santa Cruz, USA) to analyze the expression of PAR1, PKCζ, phos-PKCζ, p38, phos-p38, ERK, phos-ERK, JNK, phos-JNK, Sp1, tubulin and actin. The membranes were then washed and incubated with Alkaline Phosphate (AP) conjugated secondary antibody at room temperature (RT) for 1h. Finally, proteins were detected by AP substrate BCIP/NBT (Bio-Rad). For detection of Bax, Bcl2, cytochrome C, caspase 3, 8, 9 and p53 expression level was determined after 2h of HAP treatment.

4.9.4 Nuclear cytosolic fractionation

Nuclear cytosolic fractionation was performed as described by Rosner *et al*. HT29 cells were treated with 1.0 μg/ml of HAP for 1h. The HAP treated and untreated cells were then lysed with cytosolic extraction buffer (10 mM HEPES (pH 7.5), 0.1% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF and 0.5 mM DTT) to obtain cytosolic fractions. After centrifugation, pellets were then lysed with nuclear extraction buffer (20 mM HEPES (pH 7.5), 2.5% Triton X-100, 1.5 mM $MgCl_2$, 0.4 M NaCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF and 0.5 mM DTT) to obtain nuclear fractions. These fractions were utilized for Immunobloting to determine the nuclear translocation of Sp1.

4.10 Immunofluorescence

HAP treated (with 1.0 μg/ml of HAP for 1h) or peptide treated (with 100 μM peptide for 16 h) and untreated HT29 cells or cells pre-incubated with 0.5 μM PAR1 inhibitor (ML161) for 1h and then incubated with 1.0 μg/ml of HAP were fixed with 2% paraformaldehyde for 10 mins at room temperature followed by permeabilization with 0.1% Triton X-100 in 0.1% sodium citrate solution and blocked with 5% Bovine Serum Albumin. Cells were then incubated overnight with primary antibody (1:500) against PAR1, Sp1, AP-2, phos-p38, p65, and p50 at 4 ^{o}C. Cells were then incubated with FITC conjugated anti-rabbit Ig-G (1:500) or TRITC conjugated anti-mouse antibody (1:500) and kept in dark at 37^{o}C for 90 min. Cells were further washed with PBS and nuclei stained with DAPI as described by Kumagai *et al* and Ribble *et al*. Approximately 50 μl of 20% glycerol was placed on a clear glass slide. The glass cover slip was placed on it and viewed under confocal microscope (Model LSM 510 META, Zeiss, Germany) at 63X resolution.

4.10.1 Detection of ROS by DCFDA staining

The *in situ* ROS level was measured by oxidation of 2',7'-dichlorofluorescein diacetate (DCFDA) to highly fluorescent 2',7'-dicholoroflurescein (DCF). HT29 cells were treated with 1.0 μg/ml of HAP for 1h (or 100 μM of peptide for 16 h) or pre-incubated with either 0.5 μM PAR1 inhibitor (ML161) or 3 μM NFκB inhibitor (MG132), or 10.0 μM p38 inhibitor (SB203580) for 30 min and then treated with 1.0 μg/ml of HAP for 1h. Cells were then incubated with DCFDA for 20 mins at 37 ^{o}C. Cells were further washed with PBS and nuclei stained with DAPI. Approximately 50 μl of 20% glycerol was placed on a clear glass slide. The glass cover slip was placed on it and viewed under confocal microscope (Model LSM 510 META, Zeiss, Germany) at 63X resolution.

4.11 RNA extraction and cDNA preparation

Total cellular RNA was isolated from HAP treated (1.0 μg/ml of HAP) or peptide treated (100 μM of peptide) and untreated CT26, HT29 cells and normal mouse colon tissue by single-step method using Trizol reagent (Invitrogen, USA), followed by using RNA mini kit (Thermo Fisher Scientific, USA) according to manufacturer's protocol. RNA was quantified by spectrophotometric analysis and quality of the RNA samples determined by estimating the A260:A280 ratio.

4.12 Real-Time quantitative RT-PCR

cDNA was prepared from 2 μg of total RNA using the SUPERSCRIPTII First-strand synthesis system for subsequent analysis by Real-Time quantitative RT-PCR (Invitrogen, USA). The polynucleotide primer sequences for PARs 1-4 and GAPDH (internal control to normalize results) are listed in table-1. Real-Time quantitative RT-PCR was performed using SYBR green reagent (iQ SYBR green supermix, Bio-Rad). 25 μl of PCR mixture containing 10 pM of each primer, 12.5 μl of SYBR green reagent (Bio-Rad) and 2 μl of cDNA. The samples were placed in 96-well plate and sealed with optical sealing tape. PCR reactions were carried out using an iCycler iQ multicolor real time PCR detection system (Bio-Rad). The thermal cycling conditions used for Real Time PCR were: denaturation at 95 ^{o}C for 30 s, annealing either at 50 ^{o}C (for mouse specific primers of PARs) or at 55 ^{o}C (for human specific primers of PARs) for 30 s and extension at 60 ^{o}C for 30 s. Relative quantification was performed based on comparative cycle threshold (Ct) method as described by Kageyama *et al*. Briefly, the Ct of the target amplicon and the Ct of the internal control GAPDH were determined for each sample. The ΔCt value for each experimental sample was subtracted from the calibrator to obtain the ΔΔCt. The arithmetic calibrator $2^{-\Delta\Delta Ct}$ was used to calculate the amount of target relative to calibrator. The data were represented as percentage compared to control and derived by averaging the results obtained from three independent experiments.

Primer Name	5′ ← sequence → 3′
Mouse GAPDH (F′)	GACCCCACTAACATCAAAT
Mouse GAPDH (R′)	TGAGTTGTCATATTTCTCGT
Mouse PAR1 (F′)	CCCAGTGAAAATACATTTGA
Mouse PAR1 (R′)	AGGAGGGAGGCTTATATTTA
Mouse PAR2 (F′)	TCAATTACTTCCTCTCACTG
Mouse PAR2 (R′)	TTTTCTTCTCTGAGTGTTCA
Mouse PAR3 (F′)	CTACTACTACCACAATACCG
Mouse PAR3 (R′)	TGACAAAGTAAAGGAATGGA
Mouse PAR4 (F′)	AGACCCTTTCATCTACTACT
Mouse PAR4 (R′)	GAAGTGTAGAGGAGCAAAT
Human GAPDH (F′)	CGGAGTCAACGGATTTGGTCGTAT
Human GAPDH (R′)	AGCCTTCTCCATGGTGGTGAAGAC
Human PAR1 (F′)	GTAGTCAGCCTCCCACTAAAC
Human PAR1 (R′)	CACAGACACAAACAGCACATC
Human PAR2 (F′)	CCTCAGTGTGCAGAGGTATTG
Human PAR2 (R′)	AGCAGAATCAGCAGCCATATT
Human PAR3 (F′)	CCCATCATCCTTCCGATTCTAC
Human PAR3 (R′)	GTGGATGAGAGTCGTGTAACAG
Human PAR4 (F′)	GTCACTAGCAGAGGTCACTTTG
Human PAR4 (R′)	GCCTCTTAAAGTGCTGGGATTA

4.13 Chromatin Immunoprecipitation assay (ChIP)

The ChIP assay was performed as described by Majumder *et al.* HT29 cells were either treated with HAP from *V. cholerae* strains C6709 at concentrations 1.0 μg/ml for 1hr or kept untreated. These cells were used to cross link proteins with DNA by 2% formaldehyde treatment and the cross-linking reactions were stopped by 150 mM glycine for 4 mins. Cells were scraped and spin down at 13,000 rpm for 2 min, washed with PBS twice and pellet was frozen at -80 ^{o}C for 20 min. The pellet was resuspended in buffer C (20 mM HEPES pH 7.9, 2mM EDTA, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$ and 1 mM PMSF) after thawing it at 4 oC to lyse the cells.

Nuclei were pelleted at 13,000 rpm for 10 min and the pellet was resuspended in breaking buffer (1% SDS, 2% Triton X-100, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl). The resuspended pellet was then sonicated for two 10-s pulses in ice. Contents were then spin down. The debris was discarded and to the supernatant (nuclear extract), triton buffer (50 mM Tris-HCl pH 8.0, 1mM EDTA, 150 mM NaCl, 0.1% Triton X-100) was added. The nuclear extracts thus obtained were incubated with anti AP-2 and anti Sp1 antibodies respectively at 4 ^{o}C for overnight. Next day, the immune complexes were mixed with protein G agarose beads and incubated on a shaker at 4 ^{o}C for 6 hr. The immune complexes were then washed four times with triton buffer and twice with buffer D (10 mM Tris-HCl pH 8.0). Finally, the immune complexes were reverse cross-linked by adding SDS-NaCl-DTT buffer (62.5 mM Tris-HCl pH 6.8, 200 mM NaCl, 2% SDS, 10 mM DTT) and the complexes were incubated at 65 ^{o}C overnight. DNA was purified by ethanol precipitation and was used to amplify by Polymerase Chain Reaction using PAR1 upstream 276 bp sequence (Table-1), to study the interactions of Sp1 and AP-2 with the PAR1 promoter in response to HAP treatment. The PCR was subjected to an initial denaturation step (2 min at 96 ^{o}C), followed by 30 cycles of denaturation (1 min at 94 ^{o}C), annealing (1 min at 60 ^{o}C), and extension (1 min at 72 ^{o}C). The reaction was subjected to a final extension time of 5 min at 72 ^{o}C. PCR products were analyzed on a 1% agarose gel.

Primer Name	**5′ ← sequence → 3′**
Human PAR1 promoter (F′)	ACTTCTAGGCCCGGCAGTG
Human PAR1 promoter (R′)	GGTAAGATCAGGGTCCAAGC

4.14 Agarose gel Electrophoresis

Purified DNA obtained from polymerase chain reactions were mixed with 1x gel loading dye (Himedia, India) and loaded onto each wells of a 2% agarose gel in sample buffer containing 1x TBE buffer, pH 8.3. DNA were resolved with a continuous buffer system at an initial 80V and a latter constant 120V. DNA with

known molecular weights (Himedia, India) were used as molecular weight markers. After desired resolution, gels were observed in Gel documentation systems (Bio-Rad).

CHAPTER 5

RESULTS

5.1 Purification of HAP from culture supernatant of C6709

HAP was purified from culture supernatant of *V. cholerae* O1 El Tor strain C6709. The cell free and concentrated supernatant was loaded in DE-52 column. Non-binding fraction was pooled from DE-52 column that exhibited protease activity (Fig.1A). This pooled fraction was further purified by Sephadex G-100 column chromatography. The single peak obtained after purification in Sephadex G-100 column showed protease activity (Fig.1B) and a single 35 kDa band in 10% SDS-PAGE (Fig.1C). Ala-Gln-Ala-Thr-Gly-Thr-Gly-Pro-Gly-Gly-Asn-Gln-Lys-Thr-Gly sequence was determined as the first 15 amino acid sequences in the N-terminus of the 35-kDa protein band which revealed 100% homology with amino acid sequences of HAP analyzed with BLASTP and FASTA [207].

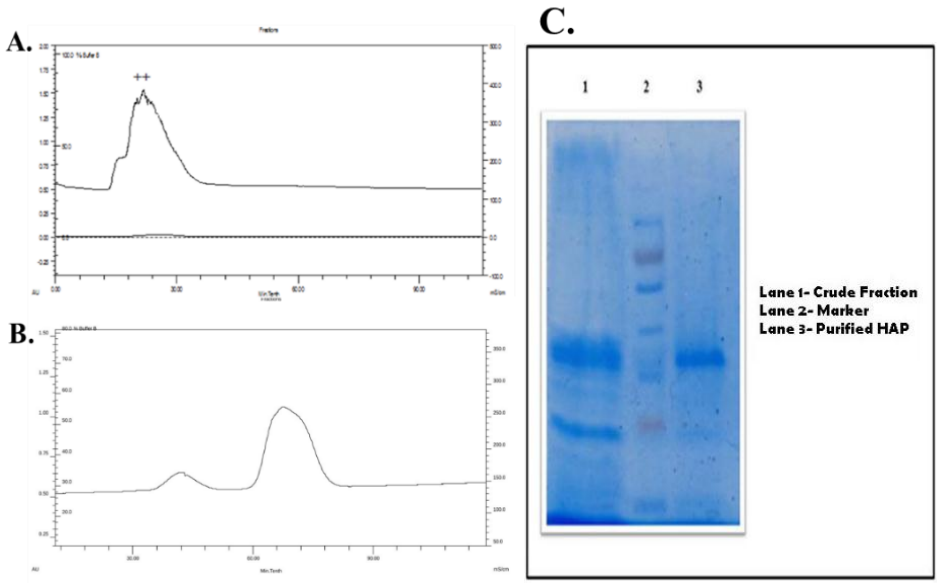


Fig.1. Chromatographic profiles of HAP purification from C6709 culture supernatant: A) Anion exchange chromatography on DE-52 column with ammonium sulphate precipitated protein. The non-binding fraction showed protease activity (++). B) Gel filtration chromatography on Sephadex G-100 column with the pooled, concentrated non-binding fractions from DE-52. C) 10% SDS-PAGE of the proteins from G-100 eluted fraction. The positions of the molecular weight markers are shown to the left of the gel and lane 1 shows 35 kDa of purified HAP.

5.2 Preventive role of HAP on colon cancer progression and tumor formation

5.2.1 Flow cytometric analysis of HAP mediated apoptosis in human and mice colon cancer cell lines

Purified HAP from culture supernatants of *V. cholerae* O1 El Tor strain C6709 showing protease activity, induced apoptosis was studied in human and mice colon cancer cells, HT29 and CT26 respectively. Our results showed that 1 and 1.5 µg/ml of HAP caused apoptosis in 71.70% and 68.40% of human (HT29) colon cancer cells (Fig.2 A,B). Similarly, in mice (CT26) colon cancer cells, 1 and 1.5 µg/ml of HAP triggered 63.40% and 85.50% of apoptosis respectively (Fig.2 C,D). In order to avoid any protease mediated toxicity within the cells we choose a standard dosage of 1 µg/ml of HAP in all our further studies.

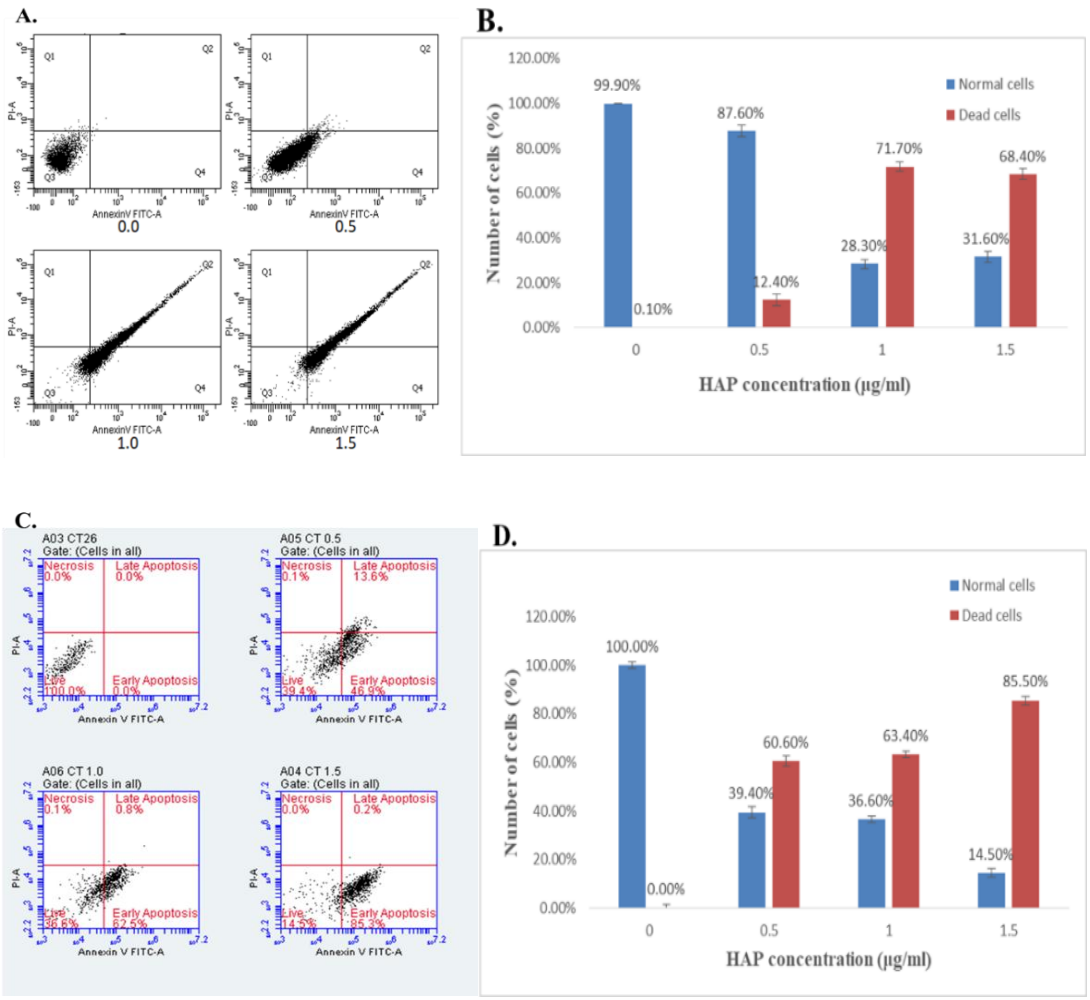


Fig.2. Dose dependent response of *V. cholerae* HAP in human and mice colon cancer cells: A) Human (HT29) colorectal cells showed apoptosis in response to HAP in a dose dependent manner, at 1 and 1.5 μg/ml dosage. B) Graphical representation of results in bar diagram. C) Mice (CT26) colorectal cells showed apoptosis in response to HAP in a similar dose dependent manner at dosage 1 and 1.5 μg/ml respectively. D) Graphical representation of the results in bar diagram.

5.2.2 Immunobloting analysis of HAP mediated activation of intrinsic pathway of apoptosis

The pathway of apoptotic response due to HAP was studied in human and mice colon cancer cells (HT29 & CT26). Cells were treated with 1 μg/ml of HAP and cell extracts were prepared and used for Immunobloting analysis. Results showed activation of pro-apoptotic markers Bax and p53. HAP treatment on both HT29 and CT26 cells resulted in over expression of Bax and p53 as compared to the control untreated cells. HAP treatment also resulted in the down regulation of Bcl-2, an anti-apoptotic protein. Further results showed activation of caspase 3, caspase 9 and significant increase in cytochrome C level in mitochondria free cytosolic fraction of HAP treated cells. Interestingly, no such change was observed in case of caspase 8 as a result of HAP treatment. Collectively these results suggested the activation of intrinsic apoptotic pathway (Fig.3 A,B). Thus our results suggest that HAP from *V.cholerae* induces intrinsic pathway of apoptosis in both human and mice colon cancer cells.

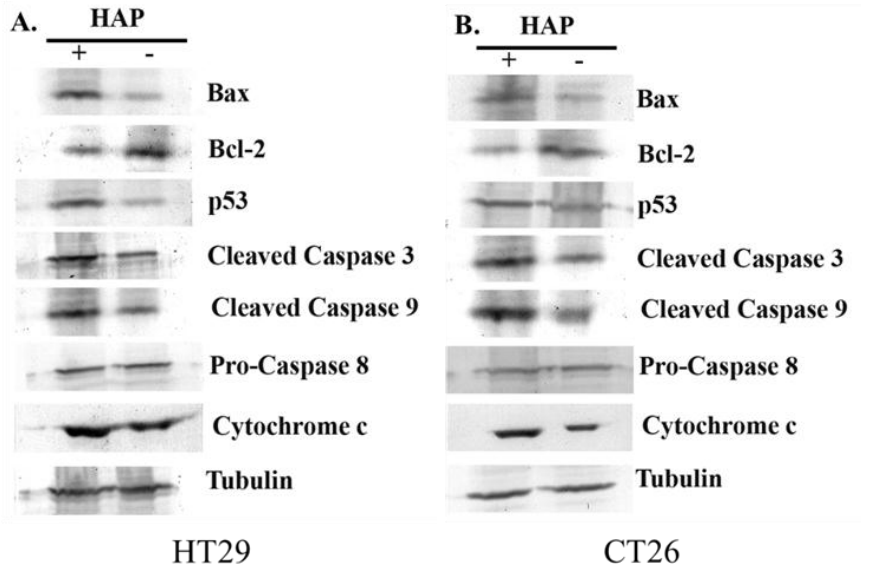


Fig.3. Immunoblot assays of major apoptotic markers in HAP treated HT29 and CT26 cells: A) Western blot analysis of HAP treated and untreated HT29 cells showed activation of intrinsic pathway of apoptosis. B) Western blot analysis of HAP treated and untreated CT26 cells showed similar activation of intrinsic pathway of apoptosis. Tubulin was used as control in both cases.

5.2.3 Mice survival kinetics assay

Tumor was induced intraperitoneally with 10^6 numbers of CT26 cells and survival kinetics was observed. After 7days of CT26 inoculation the mice were separated into groups of 10 animals. In the tumor control group the rate of survival of mice was 60% after 10 days and 0% after 20 days. In presence of 1 μg HAP injected at a weekly interval, the rate of survival was 40% after 30 days, 20% after 60 days and 0% after 100 days (Fig.4 A). Mice in the control group remained at 100% throughout the course of the assay.

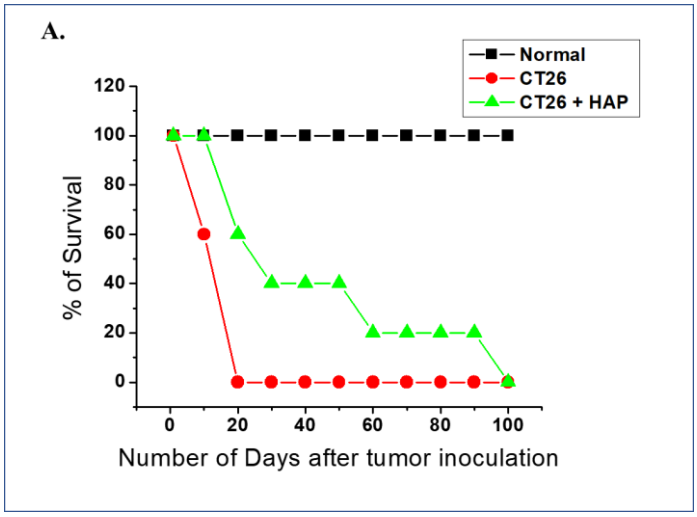


Fig.4. Survival kinetics assay on CT26 induced intraperitoneum mice model: A) Effect of HAP in CT26 induced intraperitoneum mice model showed significant increase in survival rate.

5.3 Mechanism of HAP induced apoptosis in colon cancer cells

5.3.1 HAP induced oxidative stress in colon cancer cells

To reveal the HAP induced apoptosis and antitumor pathway we investigated the level of ROS in HAP treated and untreated HT29 and CT26 cells by staining with DCFDA, a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative DCFH and is thereby trapped within the cells. In the presence of ROS, DCFH is oxidized to highly fluorescent DCF. HAP treatment for 2 h, significantly increased the ROS level in both HT29 and CT26 cells compared to untreated cells that could lead to cell death (Fig.5 A,B). Similar results were obtained in the case of iNOS, where a 2 h treatment of HAP was able to notably increase the expression of the same in CT26 cells, whereas no such change was observed in the case of normal mice colon tissues (Fig.5 C).

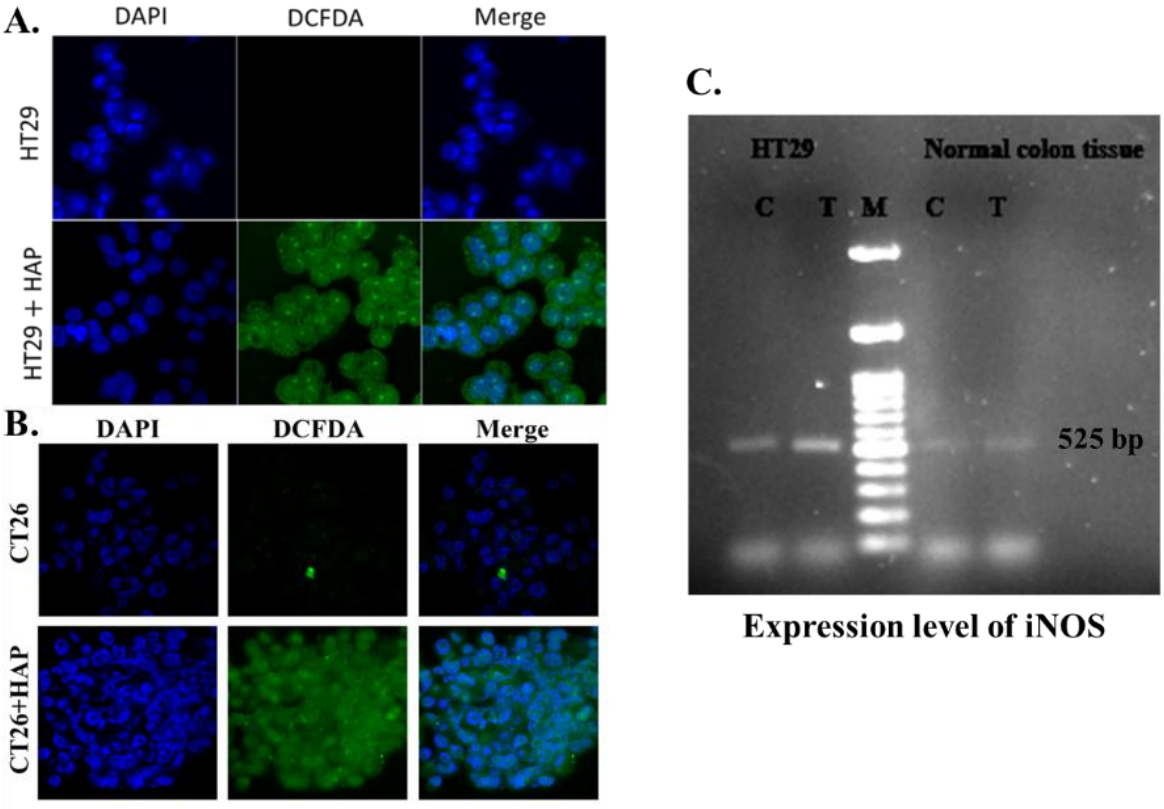


Fig.5. HAP induced oxidative stress was a major cause of HT29 and CT26 apoptosis: A-B) DCFDA staining observed under fluorescence showed that HAP increased the cellular ROS levels in both HT29 (5A) and CT26 (5B) cells. C) PCR followed by agarose gel electrophoresis showed that HAP induces overexpression of iNOS in CT26 cells but not in normal mice colon tissues.

5.3.2 HAP mediated activation of nuclear factor κB (NFκB) and MAP kinase pathways

HT29 cells were treated with 1 μg/ml of HAP for 1 h and cells were fixed for immunofluorescence. Results showed the level of phos-p38 was increased due to HAP treatment as observed by immunofluorescence (Fig.6 A). Further immunofluorescence analysis showed HAP treatment caused nuclear translocation of p65 (Fig.6 B) and p50 (Fig.6 C), indicating the activation of NFκB signaling.

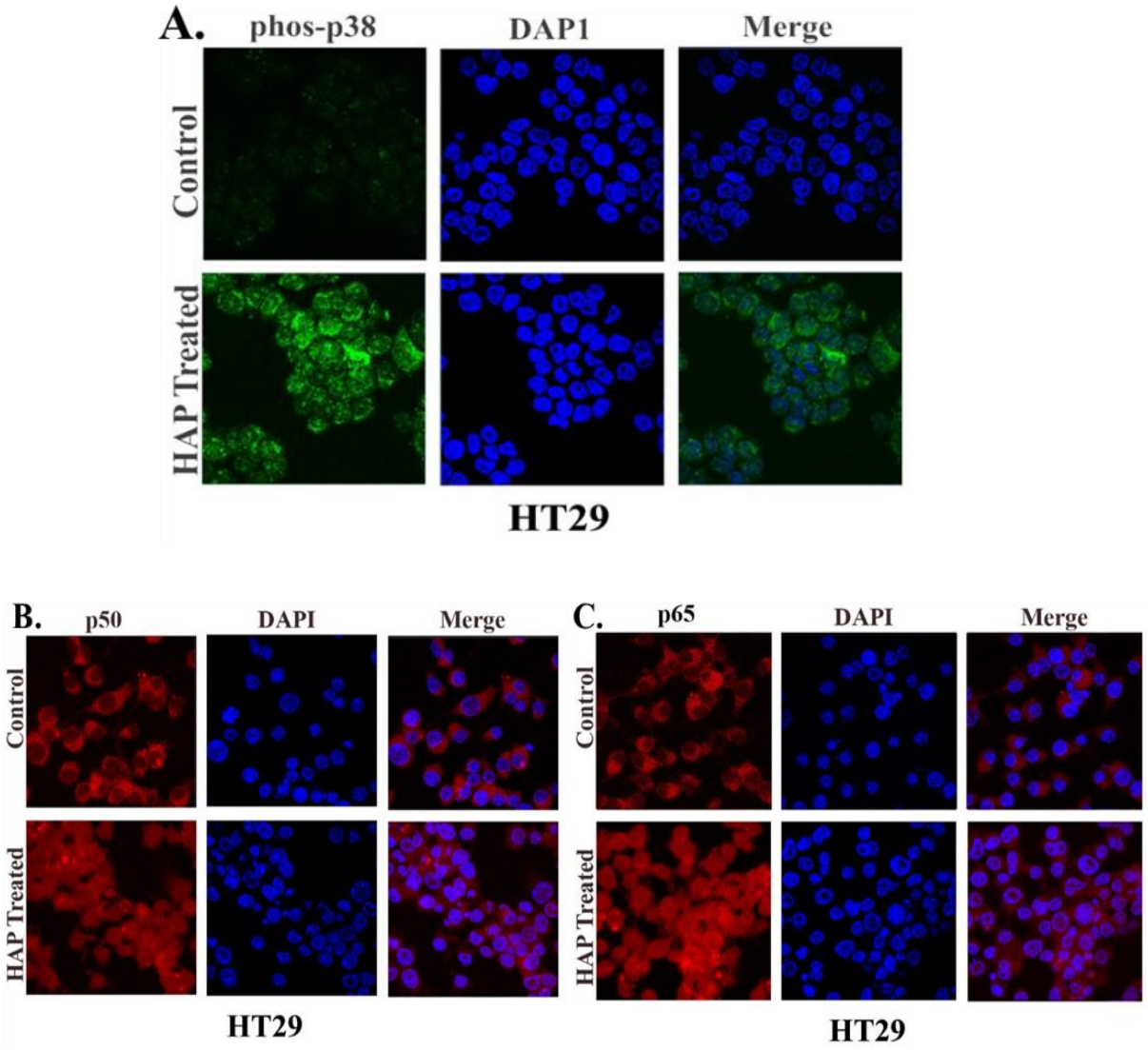


Fig.6. HAP induced NFκB and MAP kinase pathways in human colon cancer cells: A) Immunofluorescence showed HAP mediated phosphorylation of p38 and (B, C) nuclear translocation of p50 and p65 respectively.

5.4 HAP induced major signaling pathways

5.4.1 HAP mediated activation and over expression of PAR1

Human colon cancer cells (HT29), mice colon cancer cells (CT26), and normal mouse colon tissue (explants culture) were treated with 1 µg/ml of HAP for 1 h and cDNA was prepared from total RNA pool of HAP treated and untreated cells. Real Time

RESULTS

PCR revealed over expression of PAR1 in both CT26 (Fig.7 A) and HT29 (Fig.7 B), showing almost 16 fold over expression of PAR1 RNA compared to the other PARs (Fig.7 A,B). Interestingly there was no significant change in PARs expression level in normal mouse colon tissue (Fig.7 C). The Ct values revealed the level of PARs (PAR1-4) expression were always lower in normal mouse colon tissue when compared with the malignant cells. Moreover, in order to study the expression of PAR1 at the protein level, HT29 cells were treated with 1 µg/ml of HAP for 1 h and cells were either fixed for immunofluorescence or cell extracts were prepared and used for immunobloting analysis. CT26 and normal mouse colon tissue explants were also treated with 1 µg/ml of HAP for 1 h and cell extracts were prepared for immunobloting analysis. Immunofluorescence showed the level of PAR1 increased in response to HAP treatment in HT29 cells (Fig.7 D). Western blotting confirmed PAR1 over expression in both HT29 and CT26 cells as a result of HAP treatment whereas, PAR1 expression in normal colon tissues remained unaltered (Fig.7 E).

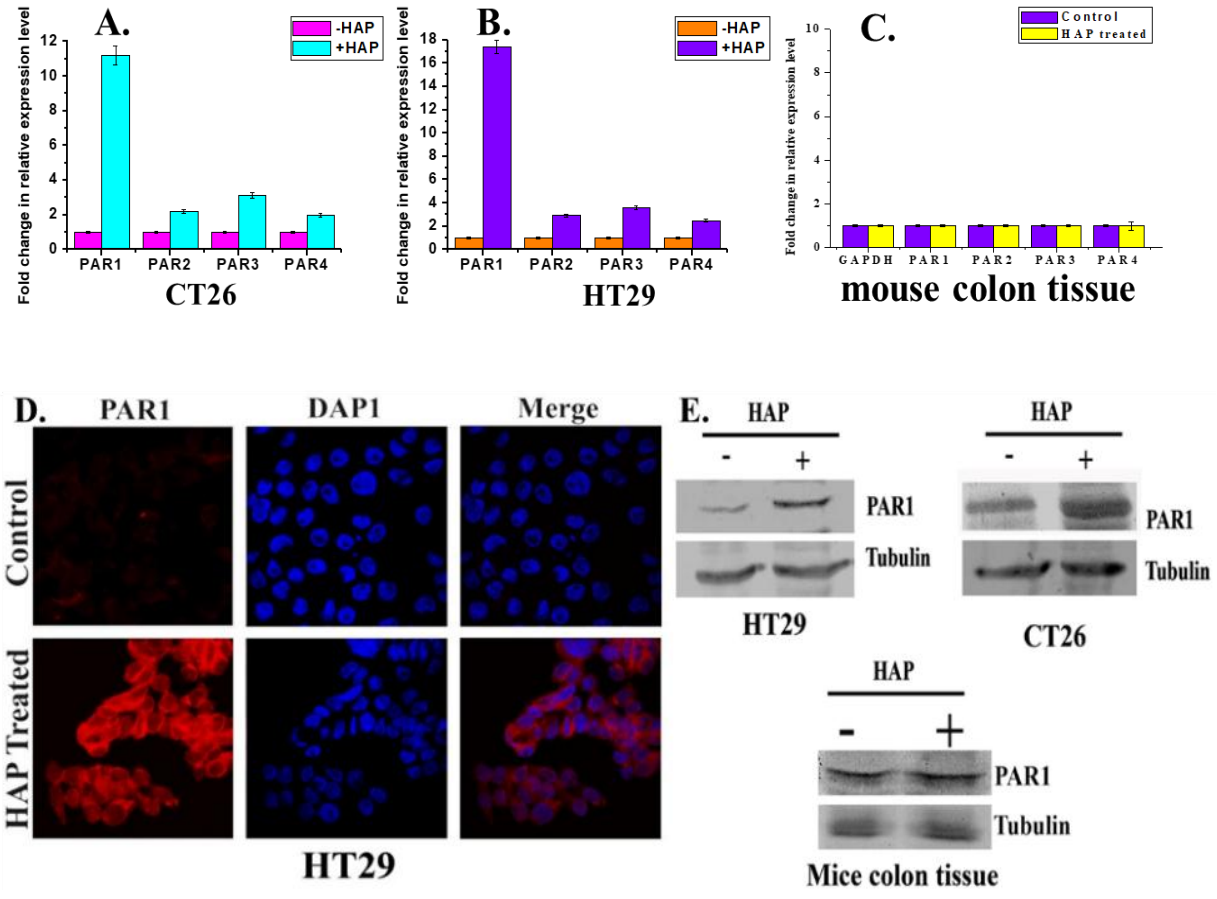


Fig.7. HAP induced PAR1 activation: A,B) Real Time PCR showed almost 16 fold over expression of PAR1 RNA compared to the other PARs in both HT29 and CT26 cells upon HAP treatment. C) There was no significant change in PARs expression level in normal mouse colon tissue in response to HAP treatment. D) Immunofluorescence revealed over expression of PAR1 in HT29 cells after HAP treatment. E) Western blot analysis further confirmed the HAP induced PAR1 over expression in both HT29 & CT26 cells but not in normal colon tissues.

5.4.2 HAP induced PAR1 mediated activation of NFκB and MAP kinase pathways

To investigate whether PAR1 activation induces both NFκB and MAP kinase pathways, HT29 cells were pre-incubated with PAR1 inhibitor for 30 min and then treated with 1 μg/ml of HAP. Immunofluorescences showed PAR1 inhibitor hampered the phosphorylation of p38 (Fig.8 A) and nuclear translocation of p65 (Fig.8 B) and p50 (Fig.8 C). After 30 min of pre-incubation with PAR1 inhibitor, phosphorylation of p38 was hindered but the total p38 concentration was not altered (Fig.8 A). These results showed that HAP mediated MAP kinase and NFκB pathways were inhibited by PAR1 inhibitor.

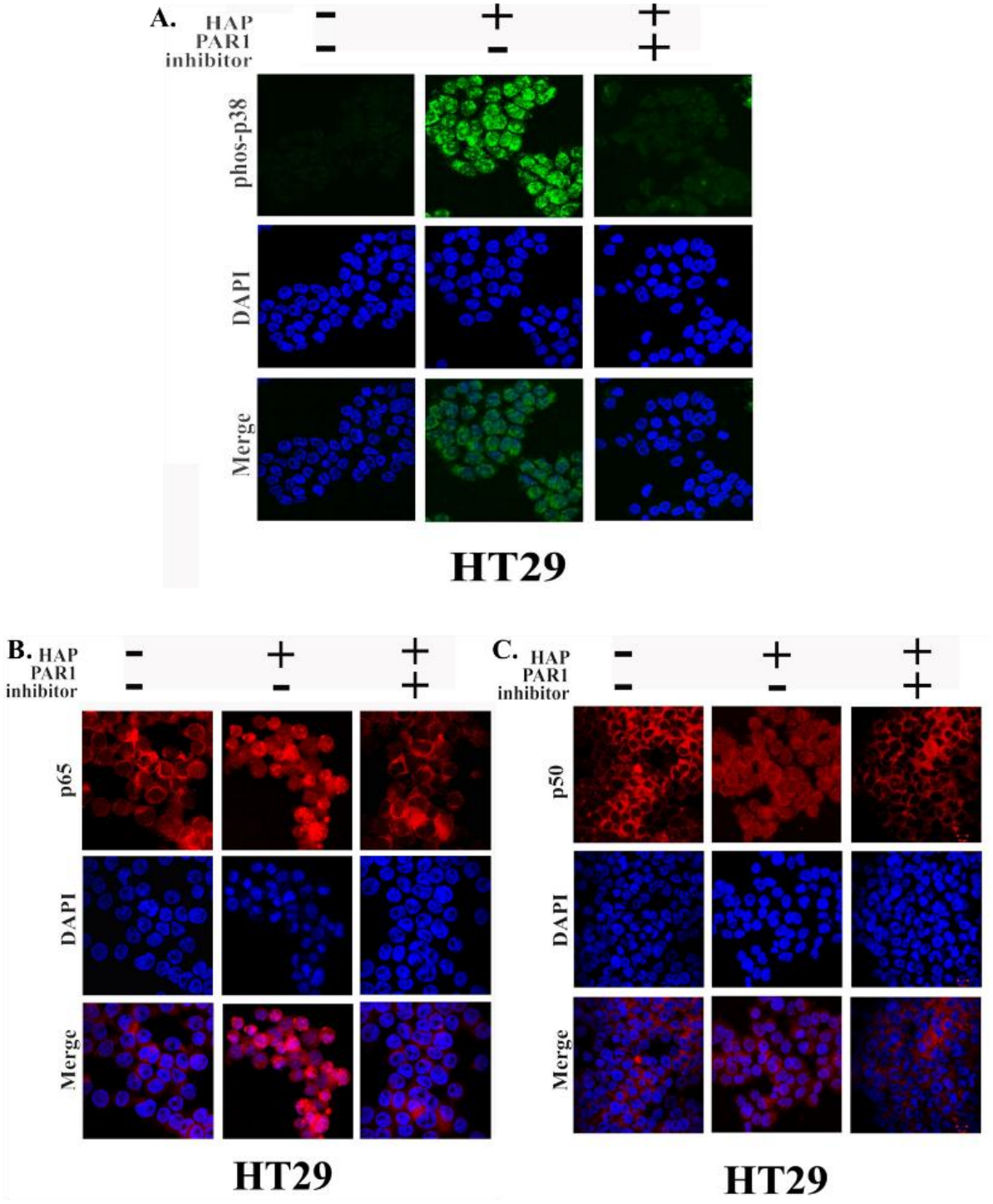


Fig.8. HAP induced NFκB and MAP kinase pathways were inhibited by PAR1 inhibitor in human colon cancer cells: A,B,C) Immunofluorescence assay revealed that HAP mediated p38 phosphorylation and nuclear translocation of P50 and p65 were inhibited by PAR1 inhibitor.

5.4.3 HAP induced PAR1 mediated activation of ROS levels

Our study have shown that HAP is a strong inducer of cellular ROS level. When we pre-incubated HT29 cells with PAR1 inhibitor the cellular ROS level decreased to its normal level. Furthermore, when we pre-incubated HT29 cells with either NFκB or p38 inhibitors the cellular ROS level decreased but even so higher than its normal levels. Interestingly when both inhibitors were used together the cellular ROS level declined to its normal level (Fig.9 A). These results showed HAP mediated PAR1 activation induce cellular ROS levels in and NFκB and MAP kinase dependent pathway, moreover both pathways can induce cellular ROS levels independently.

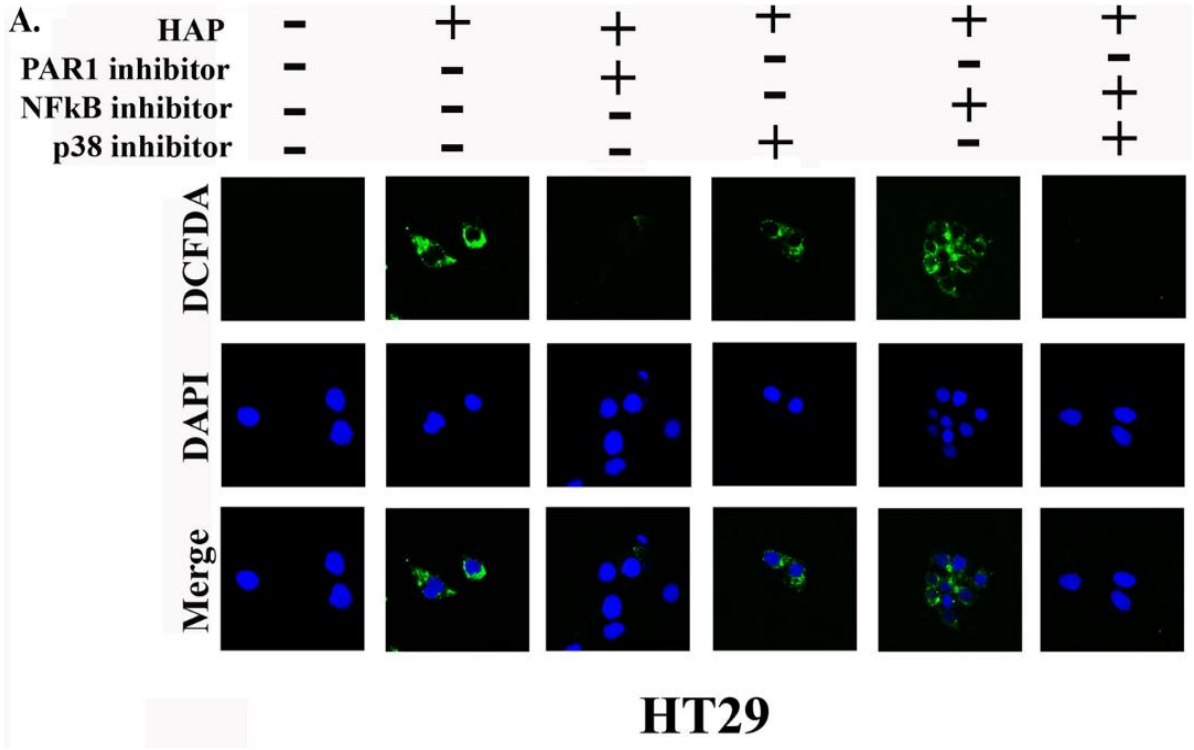
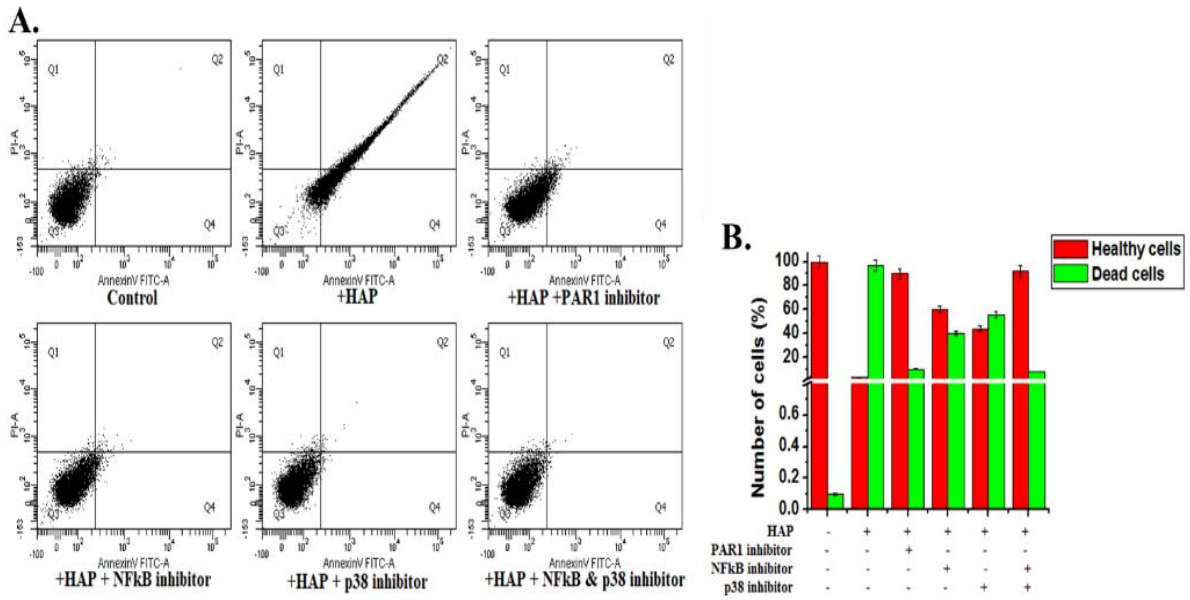


Fig.9 HAP induced cellular ROS by different signaling pathways:

A) HAP mediated cellular ROS generation was only partially blocked as a result of pre-incubation with NFκB and p38 inhibitors, but interestingly both inhibitors completely blocked HAP mediated ROS generation in a synergistic manner in HT29 cells. PAR1 inhibitor similarly blocked complete cellular ROS generation as a result of HAP treatment.

5.4.4 HAP mediated PAR1 activation leads to cellular apoptosis

We found HAP induced over expression of PAR1 and further prompted the activation of MAP kinase and NFκB pathways in a PAR1 dependent manner, in human colon cancer cells. To further examine whether these signaling pathways were responsible for HAP mediated cellular apoptosis, both HT29 and CT26 cells were first pre-incubated for 30 min with either NFκB inhibitor, p38 inhibitor or both, or PAR1 inhibitor followed by 1 μg/ml of HAP for 2 h. When HT29 cells were pre-incubated with 3 μM NFκB inhibitor only 40% cells showed apoptosis. When HT29 cells were pre-incubated by 10 μM p38 inhibitor only 50% cells showed apoptosis, but interestingly when both inhibitors were used together no apoptosis was observed (Fig.10 A). HT29 cells pre-incubated with PAR1 inhibitor showed no apoptosis, whereas HAP treated cells manifested cellular apoptosis more than 99.0% (Fig.10 A). Similar results were obtained in the case of CT26 cells (Fig.10 C). Bar diagram represents the percentage of cell death due to HAP treatment in presence and absence of mentioned inhibitors (Fig.10 B, D).



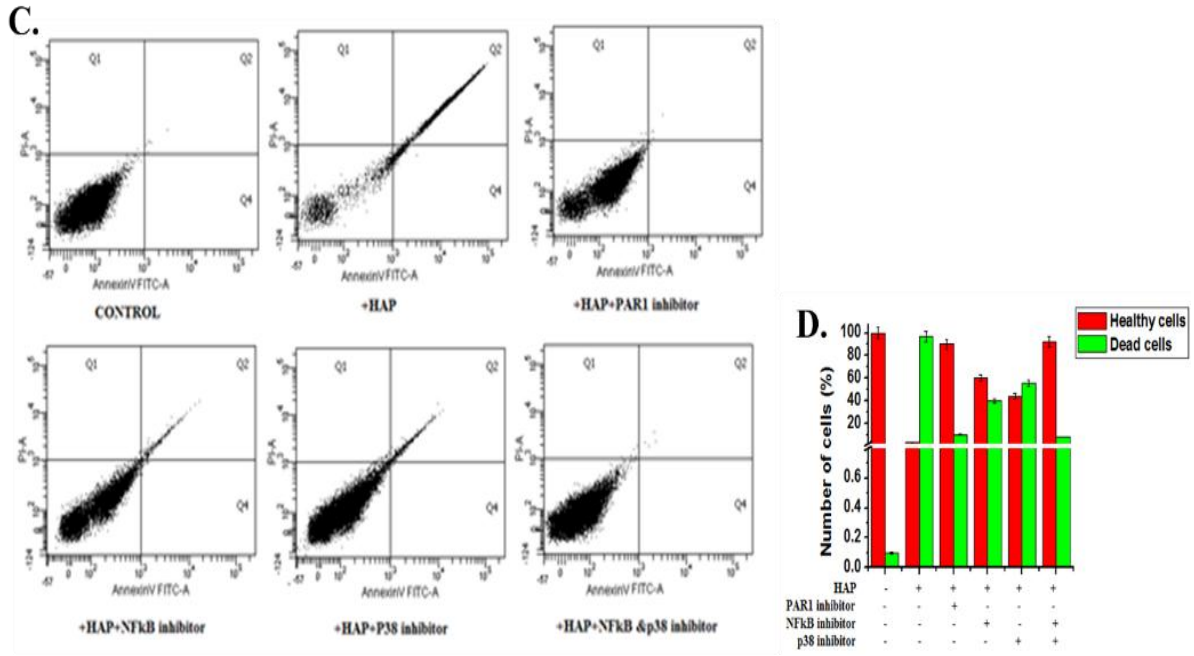


Fig.10. HAP mediated cellular apoptosis was analyzed by FACS in presence and absence of different inhibitors (NFκB, p38 and PAR1): A,B) Results showed partial inhibition of HAP mediated cellular apoptosis in presence of NFκB and p38 inhibitors, whereas both inhibitors synergistically could completely block cellular apoptosis in HT29 cells. C, D) Similar results were obtained in case of CT26 cells.

5.5 Mechanism of HAP mediated over expression of PAR1 in colon cancer cells

5.5.1 HAP mediated differential interaction of Sp1 and AP2 transcription factors with PAR1 promoter region

PAR1 expression is regulated by two transcription factors AP2 and Sp1. Both Ap2 and Sp1 bind differentially to the 5' regulatory regions of the PAR1 promoter in a mutually exclusive manner (Fig.11 A). To study the involvement of Sp1 and AP2 in regulation of PAR1, we investigated whether these proteins were associated with the PAR1 promoter region or not. For this purpose we performed ChIP assay. Chromatin fragments from HAP treated (1 μg/ml of HAP for 1 h) and untreated HT29 cells were

immunoprecipitated with an antibody of either Sp1 or AP2, and DNA from the immunoprecipitants were isolated. From this DNA, a 276-bp fragment of the PAR1 promoter region which is the binding site of Sp1 and AP2 was amplified by PCR. Results showed HAP treatment favored the binding of Sp1 instead of AP2 to regions upstream the PAR1 promoter (Fig.11 B). This result validates that HAP facilitates binding of Sp1 instead of AP2 with PAR1 promoter region.

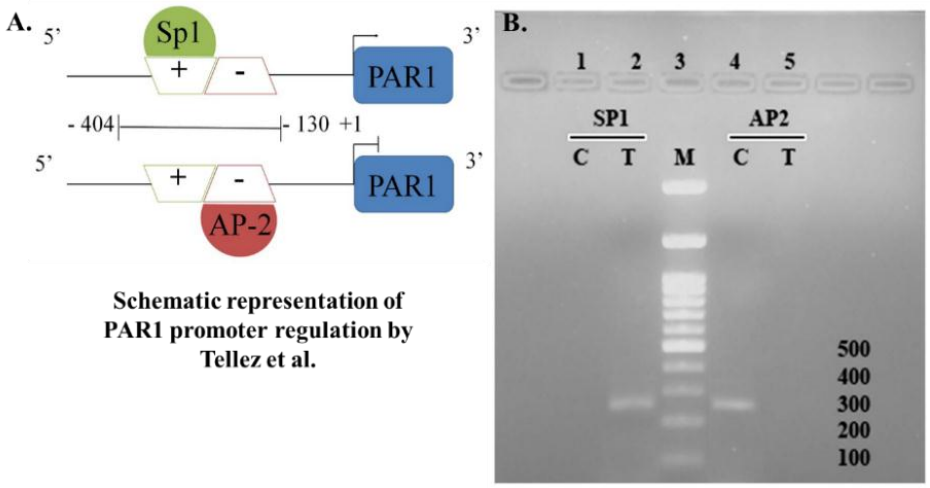


Fig.11. HAP facilitates binding of Sp1 instead of AP2 with PAR1 promoter region: A) Both AP2 and Sp1 bind differentially to the 5' regulatory regions of the PAR1 promoter in a mutually exclusive manner. B) ChIP assay demonstrated that HAP treatment facilitates binding of Sp1 instead of AP2 with PAR1 promoter region.

5.5.2 HAP facilitated over expression and activation of Sp1 in human colon cancer cells

HAP prompted binding of Sp1 over AP2 at upstream PAR1 regulatory elements. To investigate whether HAP bears any regulatory dominance over the expression of Sp1 or AP2 transcription factors, HT29 cells were treated with 1 µg/ml of HAP for 1 h and cells were fixed for immunofluorescence. Results showed that HAP treatment down regulated AP2 (Fig.12 A) while up regulated Sp1 (Fig.12 B). Therefore, HAP

treatment enhances the binding probability of Sp1 over AP2 with PAR1 promoter region that leads to over expression of PAR1.

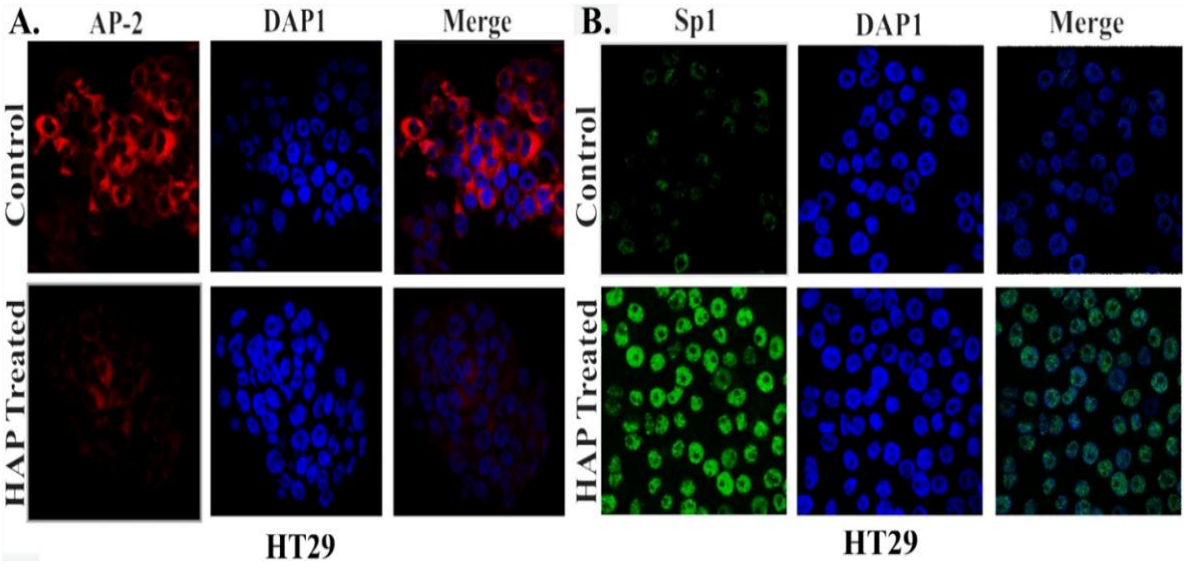


Fig.12. HAP mediates over expression and nuclear translocation of Sp1: A, B) Immunofluorescence revealed HAP treatment up regulated Sp1 levels, whereas AP2 levels were down regulated.

5.5.3 HAP induced activation and phosphorylation of protein kinase C-zeta (PKCζ) by phosphatidylinositol-3 kinase (PI3K)

It has long been reported that Protein kinase C zeta (PKCζ) is a crucial upstream signaling molecule in the yield of both SP1 and Ap2. Therefore, we examined whether PKCζ takes part in the HAP mediated overexpression of SP1. Further reports have shown that most of the PKC families of proteins are usually regulated by PI-3K. To further investigate whether HAP mediated phosphorylation of PKCζ is regulated by PI-3K, we pre-incubated HT29 cells with 6.0 nM of a PI-3K inhibitor, wortmanin for 30 min followed by 1 μg/ml of HAP for 1 h. Wortmanin treatment inhibited HAP mediated PKCζ phosphorylation and HAP mediated overexpression of Sp1 (Fig.13 A), furthermore treatment of wortmanin also was able to counteract the HAP mediated over expression of PAR1 (Fig.13 A). As a result, wortmanin treatment normalized the HAP mediated over expression of Sp1.

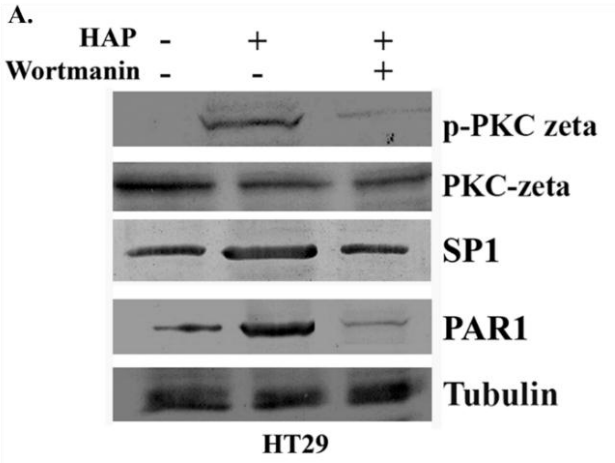


Fig.13. HAP mediated major signaling pathways leading to activation of Sp1: A) Wortmanin blocked the HAP mediated phosphorylation of PKCζ, as well as over expression of both Sp1 and PAR1.

5.5.4 Role of PI-3K in the HAP induced PAR1 mediated cellular apoptosis in colon cancer cells

In order to study the extensive role of PI-3K in the HAP induced PAR1 mediated cellular apoptosis in colon cancer cells, HT29 cells were pre-incubated with 6.0 nM wortmanin (PI-3K inhibitor), followed by 1 μg/ml of HAP for 2 h. Results showed cellular apoptosis was completely blocked as a result of wortmanin pre-incubation that indicate HAP mediated PI-3K activation is the major regulatory pathway of PAR1 mediated apoptosis (Fig.14 A,B).

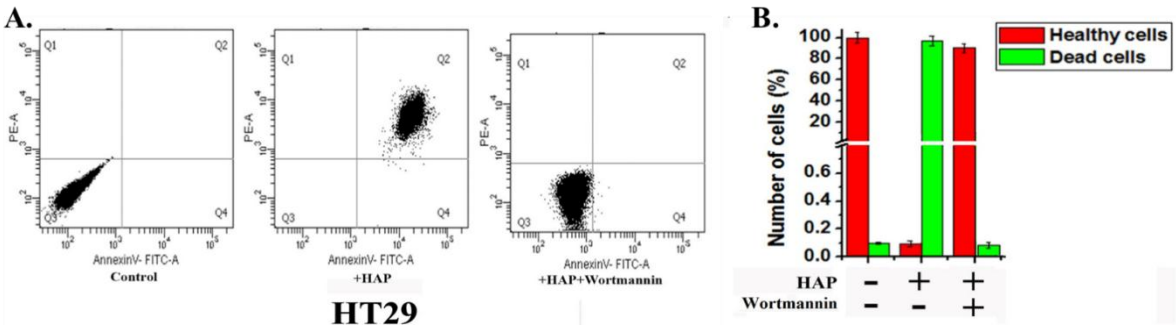


Fig.14. HAP induced PI-3K mediated cellular apoptosis: A) PI-3K inhibitor wortmanin blocked HAP induced cellular apoptosis in human colon cancer

cells. B) Bar diagram represents the percentage of cell death as a result of HAP treatment and pre-incubation with wortmanin followed by HAP treatment.

5.6 Mechanism of a novel pro-apoptotic peptide induced PAR1 mediated cellular apoptosis in colon cancer cells

5.6.1 Synthesis of the novel pro-apoptotic peptide 'PFISED'

Previous studies have shown that HAP cleaves PAR1 at a site different from its conventional ligand - thrombin. The cleaved N-terminal site of PAR1 interacts with a self transmembrane domain to activate its downstream signaling pathways. The new tethered ligand exposed by HAP (PFISED) by itself could be implemented as an activating peptide for PAR1. Therefore, the new N-terminal sequence of cleaved PAR1 'PFISED' can be used as a free peptide to initiate downstream signaling pathways similar to those observed previously as a result of HAP treatment.

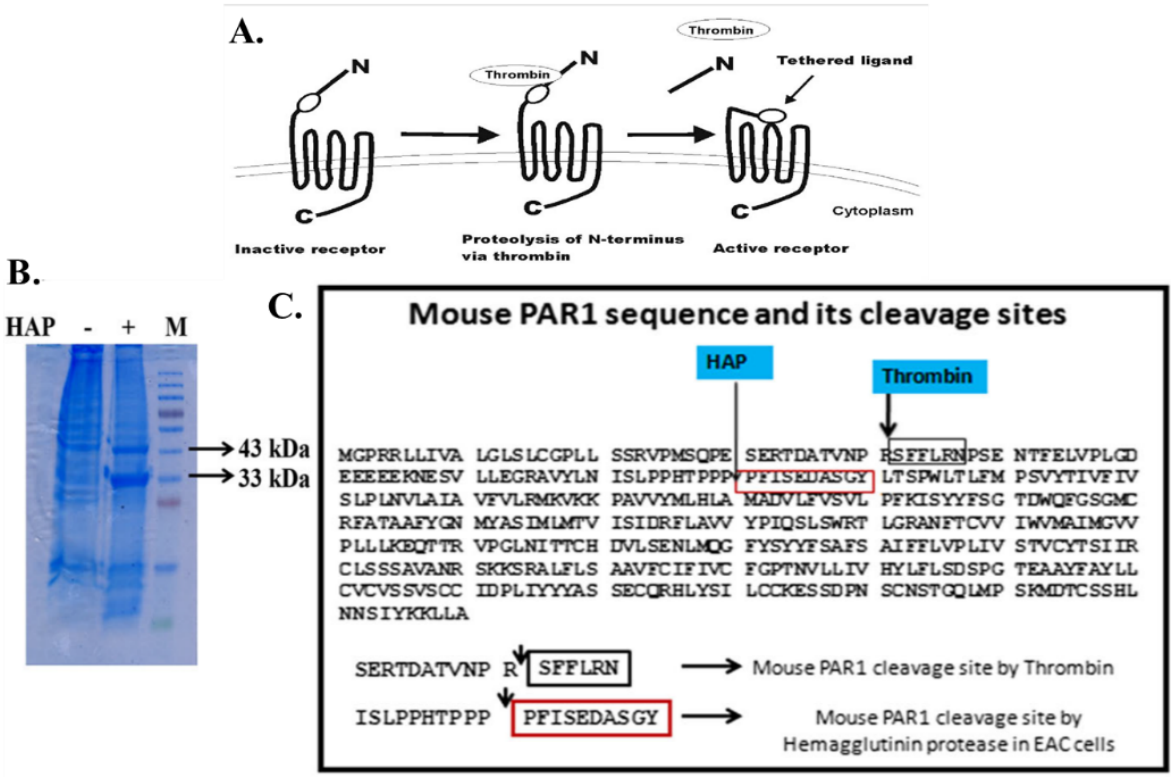


Fig.15. A) Schematic representation of thrombin mediated N-terminal cleavage of PAR1 and liberation of the active tethered ligand, B) SDS-PAGE demonstrates the

HAP mediated PAR1 cleavage, and C) shows results of N-terminal sequencing, where we observed the unique HAP mediated PAR1 cleavage site compared to the thrombin mediated one.

5.6.2 Flowcytometry analysis of novel pro-apoptotic peptide 'PFISED' mediated apoptosis in human and mice colon cancer cells

A gradient of peptide concentration (25μM, 50μM, 100μM and 200μM) was used for 16 h in human (HT29) and mice (CT26) colon cancer cells to standardize the optimal dose of peptide. The pro-apoptotic peptide induced apoptosis in more than 90% cells at 100 μM concentration in both HT29 (Fig.16 A) and CT26 (Fig.16 B), but no apoptosis was observed in normal mouse peritoneal macrophage cells (Fig.16 C) or in normal human fibroblast cells (MRC-5)(Fig.16 D).

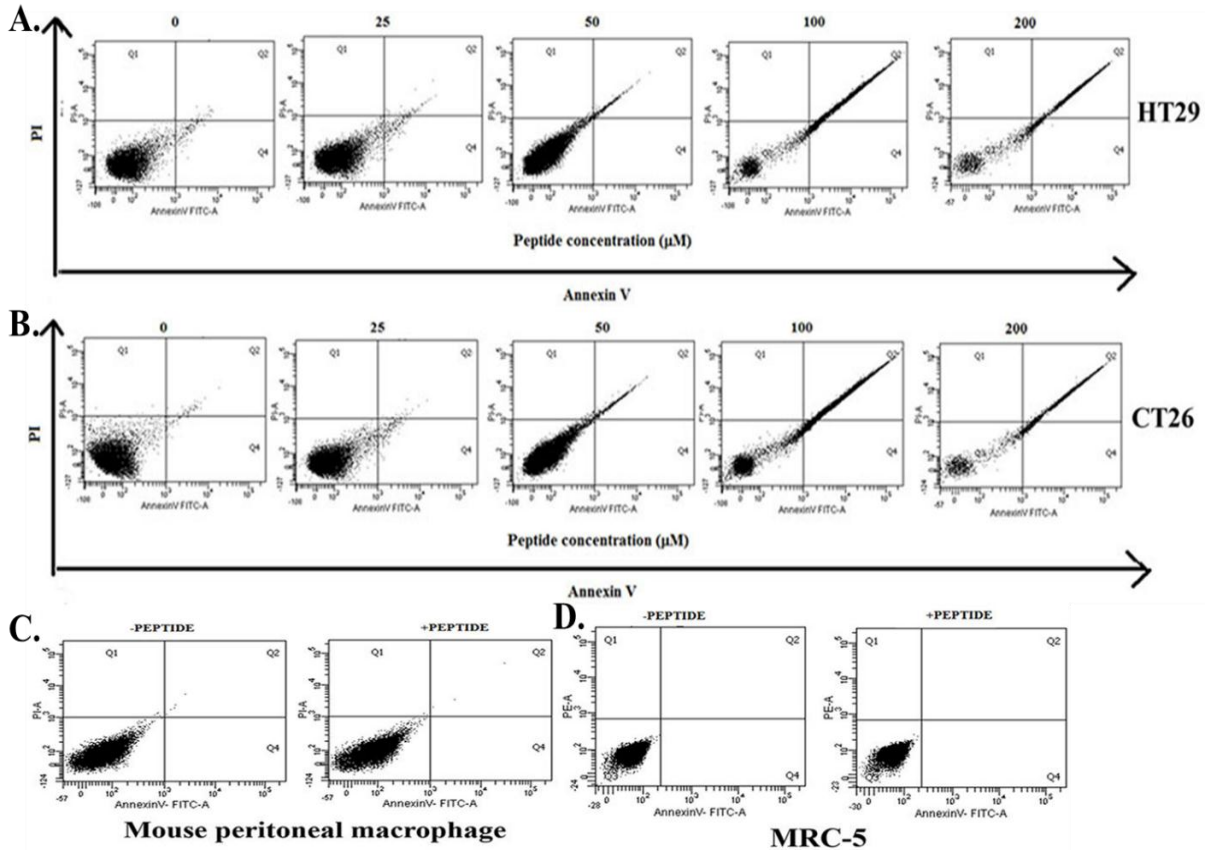


Fig.16. Dose dependent response of peptide (PFISED) in human and mice colon cancer cells: A, B) Both human (HT29) & mice (CT26) colon cells

showed apoptosis in response to peptide in a dose dependent manner, at an optimal concentration of 100μM. C, D) whereas, no such apoptotic response was observed in either normal mouse peritoneal macrophage cells or normal fibroblast cells (MRC-5) as a result of peptide treatment.

5.6.3 Mice survival kinetics assay

Intraperitoneal tumors were induced with 10^6 number of CT26 cells in Swiss albino mice and the survival kinetics observed. After 7 days of inoculation, the mice were separated into four groups of 10 animals each. In the tumor control group, mice survival rate was 20% after 30 days and 0% after 60 days. In peptide treated group, mice were treated with 500 μl of 100 μM peptide at a weekly interval. The peptide treated group after 30 days showed 100% survival and after 60 days survival rate was 0%. The rate of survival in jumble peptide "FEPIDS" group was similar as the tumor control group (Fig.17 A). These results suggest the anti-tumor effects of the peptide.

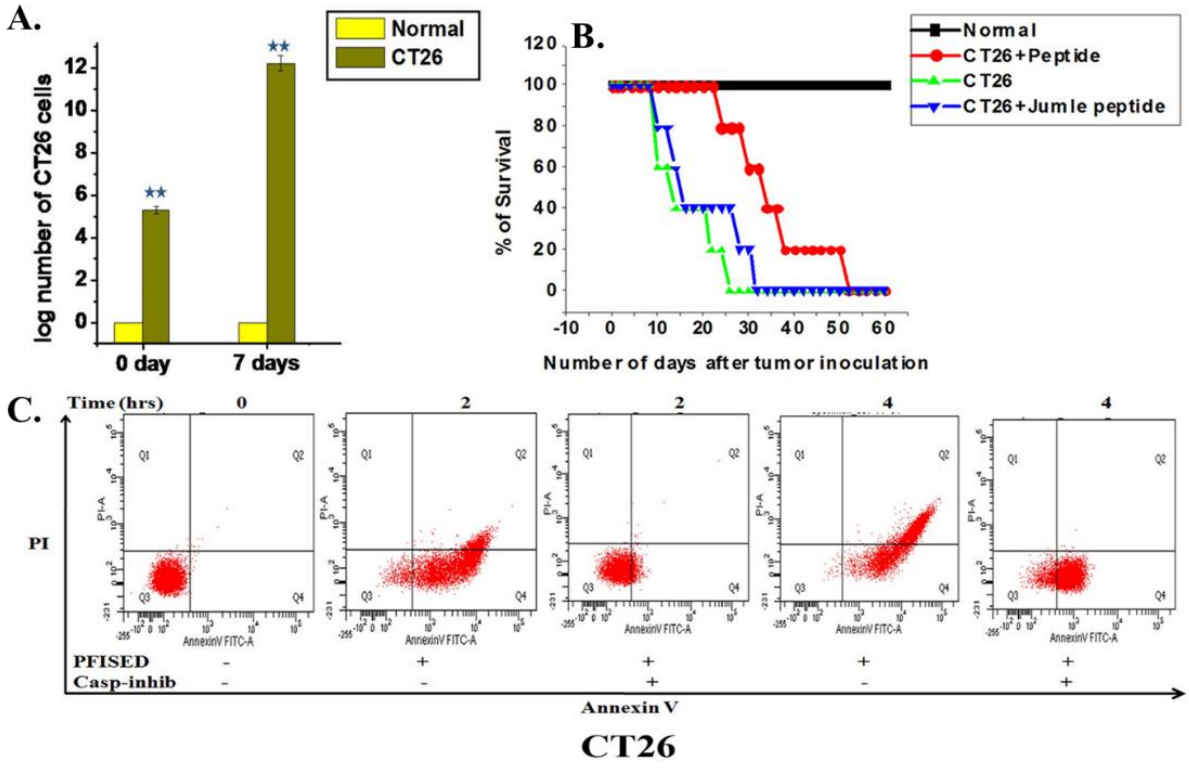


Fig.17. Survival kinetics assay on CT26 induced intraperitoneum mice model: A, B) Effect of peptide in CT26 induced intraperitoneum mice model showed a significant increase in survival rates. C) Flowcytometric analysis with a caspase inhibitor revealed that improved survival rates were actually an immediate consequence of apoptosis of cancerous cells upon peptide treatment.

5.6.4 PFISED mediated over expression and activation of PAR1

Human and mice colon (HT29 & CT26) cancer cells and normal mice colon tissue (explants culture) were treated with 100 μM of peptide for 16 h and cDNA was prepared from the total RNA pool of peptide treated and untreated cells. Real time PCR revealed 3 to 4 fold over expression of PAR1 in both mice and human colon cancer cells (Fig.18 A, B). Interestingly there was no significant change in PARs expression levels in normal colon tissues (Fig.18 C). The Ct values showed that the level of expression of PARs (PAR1-4) were always lower in normal colon tissues when compared with malignant cells. Furthermore, PAR1 expressions at the protein levels were determined in HT29 cells treated with 100 μM of peptide for 16 h. The cells were then fixed for immunofluorescence assays. Results showed the level of PAR1 increased as a result of peptide treatment in HT29 cells (Fig.18 D).

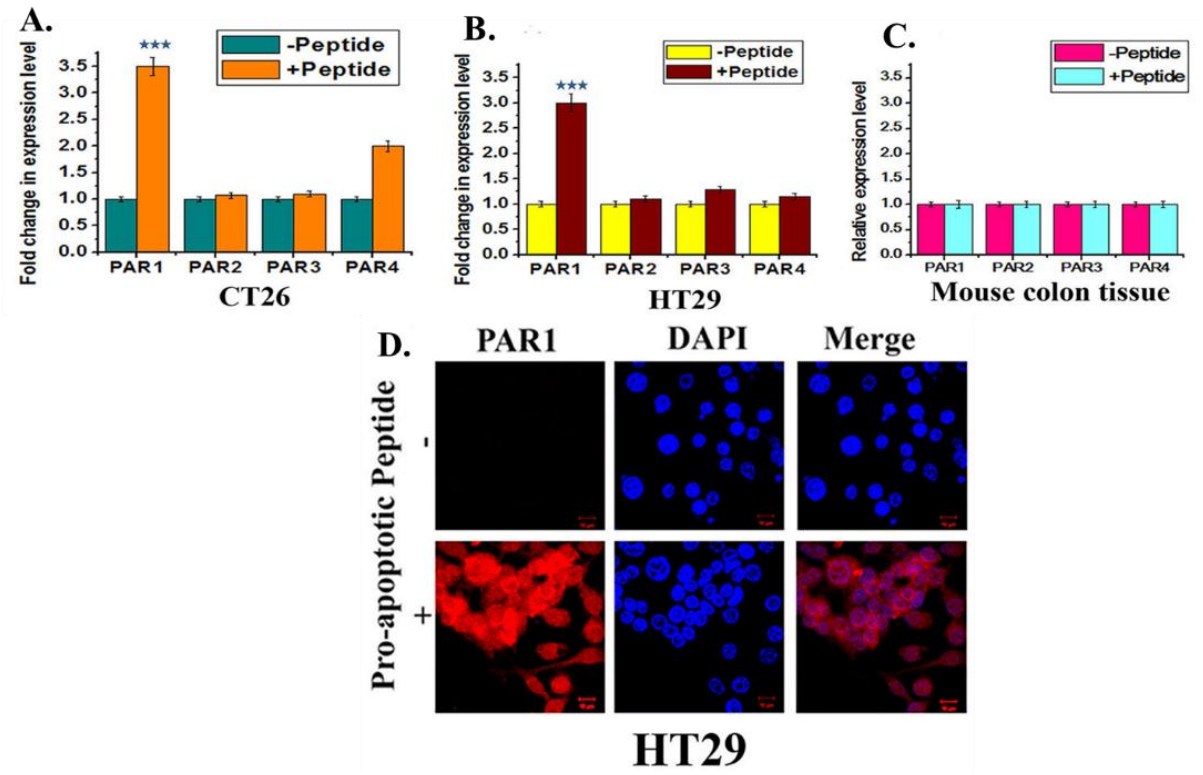


Fig.18. Peptide induced PAR1 activation: A,B) Real Time PCR showed almost 3 to 4 fold over expression of PAR1 RNA compared to the other PARs in both HT29 and CT26 cells upon peptide treatment. C) There was no significant change in PARs expression level in normal mouse colon tissue in response to peptide treatment. D) Immunofluorescence revealed over expression of PAR1 in HT29 cells after peptide treatment.

5.6.5 PFISED induced PAR1 mediated activation of NFκB, MAP kinase pathways and increased cellular ROS level in colon cancer cells

To investigate the pro-apoptotic peptide induced major signaling pathways and the possible involvement of PAR1, HT29 cells were pre-incubated with PAR1 inhibitor followed by 100 μM of peptide for 16 h. Cells were then fixed for immunofluorescence assay. Results showed that peptide treatment prompted the nuclear translocation of both p50 and p65 (Fig.19 A,B) along with the

phosphorylation of p38 (Fig.19 C) and increased ROS levels (Fig.19 D). Simultaneously, presence of PAR1 inhibitor was able completely block the peptide mediated activation of NFκB (Fig.19 A,B) or p38 phosphorylation (Fig.19 C) and ROS generation (Fig.19 D).

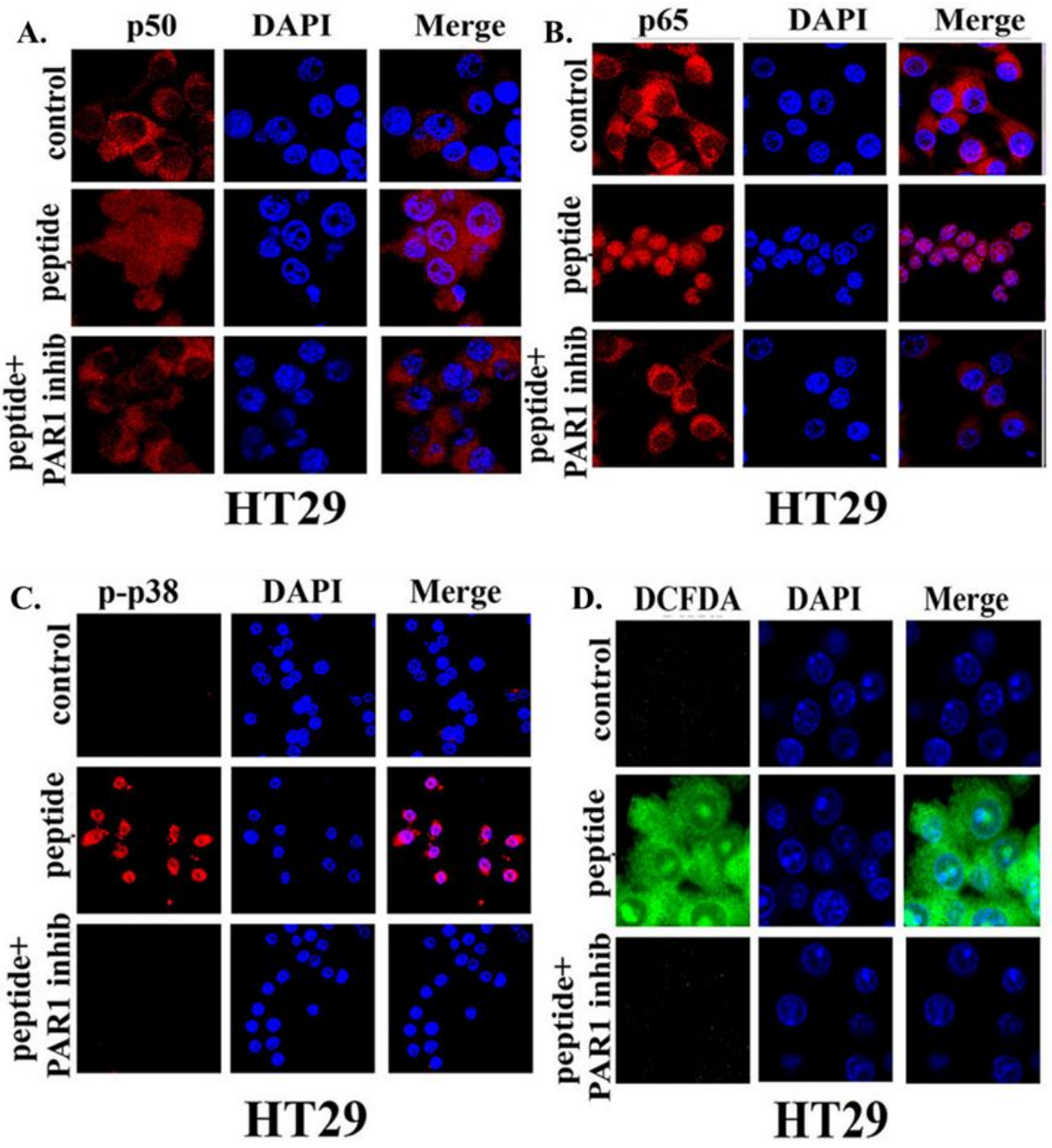


Fig.19. Peptide induced NFκB and MAP kinase pathways along with cellular ROS generation in human colon cancer cells: A,B) Immunofluorescence showed peptide mediated nuclear translocation of p50 and p65,

respectively, (C) and phosphorylation of p38. D) DCFDA staining observed under fluorescence showed that peptide increased the cellular ROS levels in both HT29 cells.

5.6.6 PFISED induced PAR1 mediated cellular apoptosis

We found HAP induced over expression of PAR1 and further prompted the activation of MAP kinase and NFκB pathways in a PAR1 dependent manner, in human colon cancer cells. To determine whether these signaling pathways were responsible for the peptide mediated cellular apoptosis, HT29 cells were pre-incubated for 2 h with either NFκB inhibitor or p38 inhibitor or both followed by 100 μM of the peptide "PFISED" treatment for 16 h. When cells were pre-incubated with 3 μM NFκB inhibitor, 32% cells showed apoptosis. When pre-incubated with 10 μM p38 inhibitor, 35% cells showed apoptosis. Interestingly, both inhibitors together showed apoptosis in 24% cells only, while peptide treatment showed 100% cellular apoptosis (Fig.20 A). Simultaneously, pre-incubation with a PAR1 inhibitor followed by peptide treatment showed 25% cellular apoptosis.

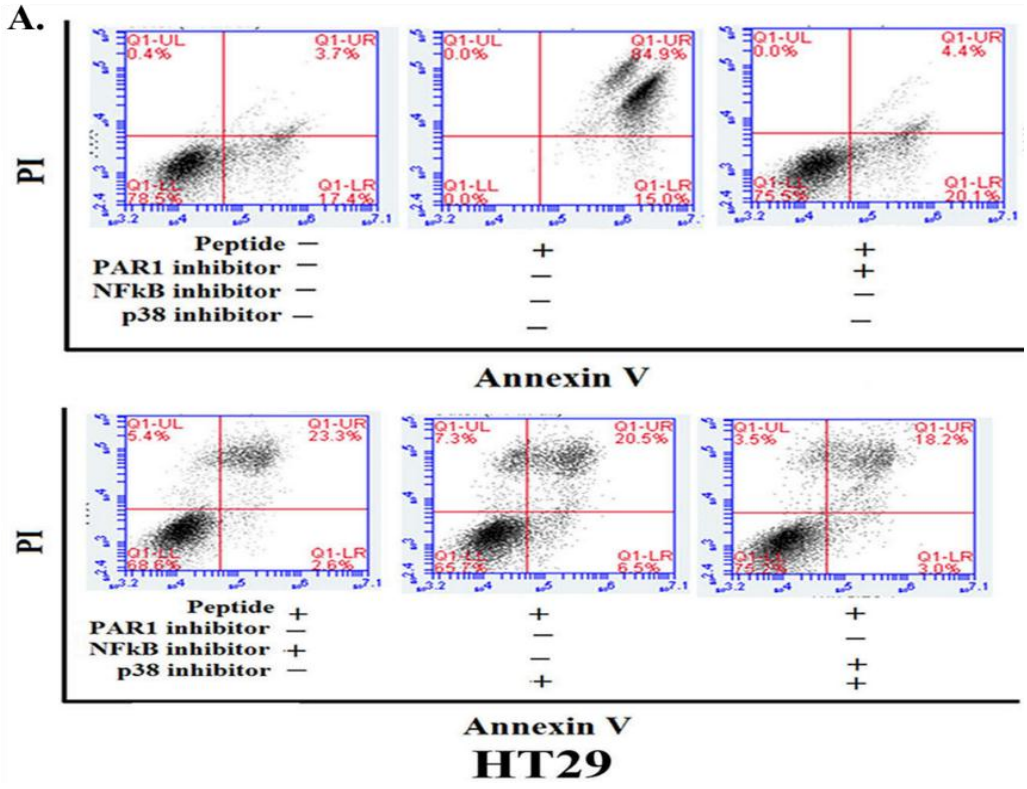


Fig.20. PFISED mediated cellular apoptosis was analyzed by FACS in presence and absence of different inhibitors (NFκB, p38 and PAR1): A) Pro-apoptotic peptide mediated cellular apoptosis was analyzed by FACS in presence and absence of different inhibitors (NFκB, p38 and PAR1) on HT29 cells.

CHAPTER 6

DISCUSSIONS

DISCUSSIONS

Colorectal cancer is an alarming health problem worldwide, where it is the third most common in men and the second most common in women, accounting for 8% of all cancer-related deaths making it the fourth most recurrent cause of death due to cancer [Consensus Document for Management of Colorectal cancer, ICMR, 2014]. Mostly due to poor prognosis and failure of chemotherapeutic regimens such metastatic diseases rarely ensue to complete remission. All the modern chemotherapy drugs target and eliminate the rapidly-growing cancer cells in respect to the slow-growing healthy cells. However, while doing so, often they end up harming fast-growing healthy cells resulting in serious side effects. Therefore, the identification of novel oncogenic targets in cancer treatment is critical now more than ever. In the present study, we have demonstrated that hemagglutinin protease (HAP) secreted by *V. cholerae* has apoptotic and hence anticancer activity on both human and mouse Colon cancer cells. HAP was able to induce cytotoxicity in the cancer cells by activation of the intrinsic pathway of apoptosis, furthermore, HAP treatment could notably enhance the lifespan of tumor-bearing mice. Apart from HAP, very few reports have indicated implementation of bacterial proteases in cancer therapy, instances include the potent antitumor activities of a protease isolated from a Gram-negative *Serratia marcescens* kums 3958 in Meth-A tumors of BALB/c mice [8], where the degenerating effects on the tumor tissues were likely attributed to its proteolytic activities [208]. The 35 kDa HAP is one of the vital secretory proteins in *V. cholerae,* and its functional activities very much like its counterpart from *Serratia marcescens* kums 3958 is associated with its protease activity. Our studies have illustrated that HAP affected colon cancer cell viability in a dose-dependent manner, where dosage as low as 1μg/ml was optimal for cell death and apoptosis in both human and mice cells. Results have demonstrated that purified HAP at 1μg/ml induced apoptotic effects in both human (HT29) and mice (CT26) colon cancer cells, while the same at 1.5μg/ml concentrations exhibited necrosis in mice colon cancer cells. Therefore, the dosage of choice throughout the rest of our study was allotted to 1μg/ml. Apoptosis demands an intricate network of protein-protein interactions that are predominantly regulated by the ratio of anti-apoptotic and pro-apoptotic proteins of the BCL2 family. We observed an increased ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 with elevated levels of pro-apoptotic p53 as well as a rise in the concentrations of cytochrome C into the cytosol

from mitochondria, along with the activation of caspase 9 and caspase 3. Our results signify that HAP-induced apoptogenic signals lead to tumor cell death via mitochondria-dependent intrinsic pathway of apoptosis. We predominantly utilized colon adenocarcinoma-derived CT26 cells for evaluation of the anticancer activities of HAP in vivo. Results showed a weekly administration of 1μg/ml of HAP was able to improve the survival rates of intraperitoneally induced cancerous mice to 40% in 30 days.

As mentioned earlier, the diverse nature of cancer and the high rate of recurrence [209], indicates a steady demand for the identification of novel oncogenic targets for cancer treatment. A G-coupled receptor, PAR1 emerges as such a promising oncogenic target owning to its participation in the invasive and metastatic processes of cancer of breast, ovaries, lung, colon, prostate, and melanoma [210,211]. The PARs are unique in their requirement of proteases as ligands, e.g. PAR1 is activated by serine proteases like thrombin, factor Xa and activated protein C [212, 85]. Barring its active involvement in the regulation of tumorigenicity, invasion, and metastasis in several cancers, the PARs, predominantly PAR1 have also been reported to either induce or inhibit apoptosis depending on the dosage of its physiological agonist thrombin or synthetic receptor activators [213,214,140]. Our studies up until now have indicated the antitumor activities of HAP secreted by *V. cholerae*, and further investigations on the signaling pathways associated with the HAP mediated apoptosis of Colon cancer cells have revealed that it is PAR1 dependent. Our results have shown that HAP causes PAR1 activation and overexpression in both HT29 and CT26 cells, whereas in normal colon tissues there is no PAR1 expression as a result of HAP treatment. Reports suggest that the thrombin mediated PAR1 activation leads to activation of a group of signaling pathways, namely the phosphatidylinositol 3-kinase (PI3K) [215], protein kinase C [216], c-Jun N-terminal kinase (JNK) [217], p38 MAPK [218], RhoGTPase [219], and NFκB [132]. Our results demonstrated that HAP was able to induce NFκB and MAP kinase activation. Nuclear translocation of p50 and p65, along with phosphorylation of p38 followed a 1h treatment of 1μg/ml of HAP. Further cementing our observations and signifying the role of PAR1 in NFκB and MAP kinase activation, pre-incubation with a PAR1 inhibitor ML161 [220], was able to completely abolish the HAP mediated signal transduction. Cellular apoptosis

along with both NFκB and MAP kinase activations have long been associated with cellular ROS levels and our results exhibited similar effects on ROS generation as a result of HAP treatment, where 1μg/ml of HAP was able to significantly induce cellular ROS generation in both HT29 and CT26 cells. Interestingly enough, while pre-incubation with inhibitors of either NFκB or MAP kinase was only able to partially hamper ROS generation, implementation of both inhibitors completely arrested ROS production. PAR1 inhibitor ML161 also blocked ROS generation, allowing us to infer that HAP mediated PAR1 activation induces cellular ROS levels in an NFκB or MAP kinase-dependent pathway. Further analysis showed similar consequences of inhibitor pre-incubation followed by HAP treatment on cellular apoptosis as on ROS generation, where either NFκB or MAP kinase inhibitors were able to provoke only 40% and 50% apoptosis respectively, while both inhibitors could completely abolish any apoptosis in spite of HAP treatment. The PAR1 inhibitor Ml161 completely blocked HAP-mediated apoptosis, therefore indicating that HAP-mediated PAR1 activation leads to induction of both NFκB and MAP kinase pathways followed by ROS generation and culminating in cellular apoptosis in both HT29 and CT26 cells. The expression of PAR1 is higher in malignant cells as compared to healthy normal cells [8], consequently, HAP-induced PAR1 mediated apoptosis shows a promising approach toward a receptor-mediated targeted therapy of cancer.

V.cholerae hemagglutinin protease (HAP) treatment brings about overexpression and activation of PAR1 compared to other PARs. Being a predominant oncogenic marker, the evaluation of PAR1 molecular alterations is as relevant as targeting the same for cancer therapy. Previous reports have indicated that PAR1 overexpression is governed at both transcription and translational levels, depending primarily on two transcription factors namely, Sp1 and AP2, which compete with one another for their binding to similar DNA sequences within the PAR1 promoter region [221]. While AP2 facilitates down-regulation of PAR1, Sp1 promotes overexpression of the same, such that a decrease in the AP2/Sp1 ratio leads to PAR1 overexpression [222]. ChIP assays clearly demonstrated that HAP treatment facilitated the binding of Sp1 in respect to AP2 on the PAR1 promoter region, also HAP treatment caused activation and nuclear translocation of Sp1, whereas levels of AP2 decreased as a result of HAP treatment.

Thus the ratio of AP2/Sp1 decreases resulting in PAR1 up-regulation which in turn facilitates binding of additional HAP molecules with the PAR1 receptors and thereby triggering its downstream signaling pathways. Further investigations revealed that HAP mediated PKCζ phosphorylation is key in the nuclear translocation of Sp1 leading to PAR1 overexpression. Previous reports confirmed that PKCζ phosphorylation is primarily regulated by PI-3Kinase [223, 224]. We employed a PI-3K inhibitor wortmannin and observed that it completely blocked the HAP-mediated PI3K activation along with the phosphorylation of PKCζ. Thus the HAP mediated overexpression and nuclear translocation of SP1 was inhibited resulting in the normalization of the PAR1 expression levels. Our results further showed that wortmannin was able to completely abolish the HAP-induced apoptosis, which indicates that HAP mediated PI-3K activation is the major regulatory pathway of HAP induced cellular apoptosis, that in turn is regulated by HAP mediated PAR1 activation and its downstream signaling pathways.

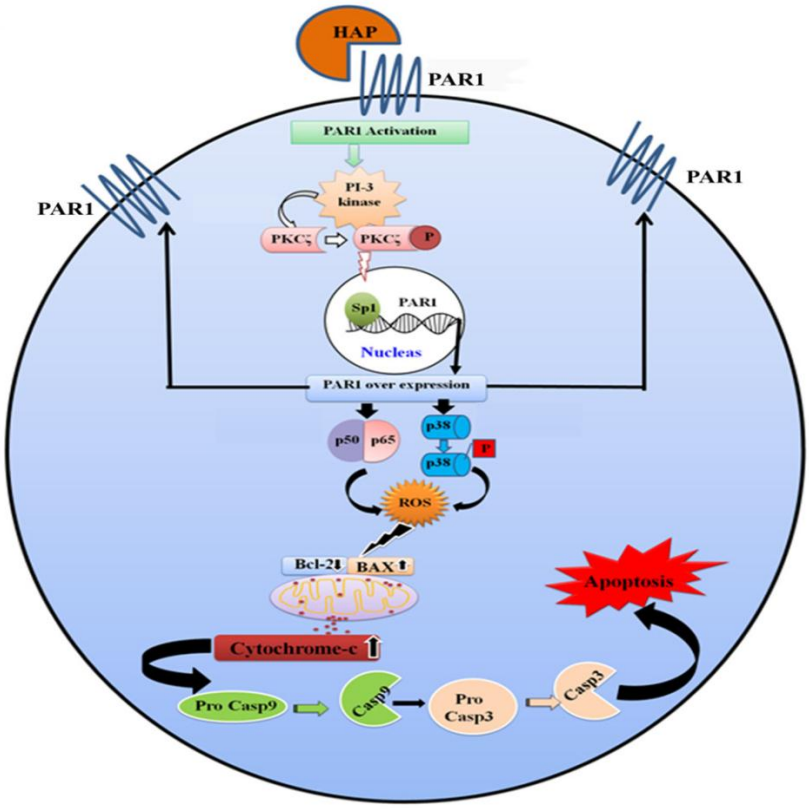


Fig 21. Schematic representation of the entire HAP mediated signaling cascade.

DISCUSSIONS

So far our studies have demonstrated that *V. cholerae* HAP is a potent antitumor agent for both human and mouse Colon cancer cells. Furthermore, HAP induces apoptosis by ROS-mediated intrinsic pathway via PAR1 activation. PAR1 itself is more prevalent in malignant cells compared to their healthy counterparts. Therefore, we can regard HAP as a potential candidate for targeted therapy of cancer. The success of a novel cancer therapy depends on their selectivity toward cancer cells and limited toxicity for normal cells. Apart from its antitumor properties, HAP is a dominant pathogenic factor aiding in the pathogenesis of *V. cholerae,* hence its outright applications in therapeutics may prove unsafe giving rise to undesired side effects. As mentioned earlier, PARs are unique in their requirement of proteases as ligands for their downstream signaling, the activating proteases bind to and cleave the N-terminus of PARs, generating a novel N-terminal domain termed "tethered ligand" that binds intramolecularly to trigger transmembrane signaling. A specific N-terminal peptide sequence of PAR1 itself gives the activation signal [8,225]. Previous studies have reported that HAP cleaves PAR1 at a site distinct from its conventional ligand Thrombin, consequently, we designed a novel peptide "PFISED", after determining the HAP mediated PAR1 cleavage site, thereby excluding the obligation of HAP usage altogether. Our results demonstrated that the novel peptide similar to HAP induced apoptosis in both human and mouse colon cancer cells, but no such response was observed either in normal human macrophage cells (MRC-5), nor in normal mouse peritoneal macrophage cells or in normal mouse colon tissues. The pro-apoptotic peptide also enhances the survival rates of intraperitoneally colon cancer-induced mice. Activation and overexpression of PAR1, along with activation of both NFκB and p38 pathways and induced ROS generation as a result of peptide treatment were observed. Preincubation with inhibitors of PAR1 was able to completely abolish Peptide mediated apoptosis, whereas, inhibitors of NFκB and p38 could block apoptosis both individually and in combination similar to results with HAP. Therefore, we can conclude that PAR1 acts as a receptor for the pro-apoptotic peptide "PFISED" and its overexpression in malignant cells increase the probability of receptor-peptide interactions within malignant cells in respect to normal cells leading to a targeted therapy where the malignant cells undergo apoptosis more frequently compared to the normal/healthy cells in the same environment.

DISCUSSIONS

Therapeutic peptides are a novel and promising approach to the development of anti-cancer agents. Peptides outcompete conventional cancer medicines with their small size, ease of synthesis and modifications, tumor penetrating ability, and good biocompatibility [179,182], thus peptides have evolved as promising therapeutic agents in the treatment of cancer. In the present study, we have demonstrated that hemagglutinin protease (HAP) secreted by *V. cholerae* induces apoptosis in colon cancer cells (HT29 & CT26) by ROS mediated intrinsic pathways of apoptosis and significantly enhances the survival rates of cancer-induced mice. Moreover, HAP promotes activation and overexpression of PAR1 in colon cancer cells. Consequently, we designed a novel peptide "PFISED" after determining the unique HAP mediated N-terminal cleavage site of PAR1. While the thrombin-mediated PAR1 activation leads to cell proliferation and growth, our novel peptide-induced PAR1 activation results in apoptosis of both human and mouse colon cancer cells, without any adverse effects in normal mouse peritoneal macrophages or fibroblast cells (MRC-5). Therefore, we can conclude that the pro-apoptotic peptide induced the activation and overexpression of PAR1 triggering the downstream signaling of NFκB and p38 pathways leading to ROS generation and cellular apoptosis. The peptide-mediated overexpression of PAR1 enhances receptor density for peptide in malignant cells as compared to normal cells, thereby significantly promoting apoptosis in malignant cells. Thus the novel pro-apoptotic peptide exhibited sharp selectivity in terminating malignant cells without altering the survival of healthy normal cells in the same environment.

CHAPTER 7

SUMMARY

SUMMARY

- Hemagglutinin protease (HAP) was purified from culture supernatants of *V. cholerae* O1 El Tor strain C6709 by anion exchange chromatography (DE-52), followed by gel filtration chromatography (G-100).
- The survival kinetics shows that HAP treatment increases the survival rates of intraperitoneally colon cancer induced mice.
- Further *in vitro* studies with both human (HT29) and mice (CT26) colon cancer cell lines have shown HAP induced apoptosis via FACS, also western blot analysis on expression levels of different proteins of apoptotic pathway shows activation of the intrinsic pathway of apoptosis.
- Study of the oxidative stress via DCFDA staining shows that HAP increased the cellular ROS level in both HT29 and CT26 cells indicating an association with HAP mediated apoptosis.
- Immunofluorescence and western blot analysis have shown the involvement of MAP kinase pathway, and NFκB to be the downstream effector molecule in HAP mediated signaling.
- Real-time PCR results show that HAP treatment significantly upregulates the PAR1 mRNA in both HT29 and CT26 cells, but not in normal colon tissues.
- Further investigations have shown the role of two transcription factors, namely SP1 and Ap-2 in the HAP mediated up-regulation of PAR1 via a PI3 kinase pathway.
- Moreover, HAP mediated PAR1 cleavage site lead us to design and synthesize a novel activating peptide "PFISED."
- The peptide showed apoptosis in human and mouse colon (HT29 and CT26) cancer cells, but not in normal human fibroblast cells (MRC-5), normal mouse peritoneal macrophage cells, or normal mouse colon tissues.
- Treatment with this peptide enhanced the survival kinetics of CT26 induced mice.
- The peptide also induced overexpression and activation of PAR1 and its downstream MAP kinase & NFκB signaling pathways leading to enhanced cellular ROS levels, resulting in cell death.
- Therefore, our studies successfully demonstrated that HAP and the novel proapoptotic peptide "PFISED" designed out of the HAP mediated cleavage of

the PAR1 tethered ligand are potential therapeutic candidates for treatment of colorectal cancer.

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Apoptosis
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Molecular targeting of breast and colon cancer cells by PAR1 mediated apoptosis through a novel pro-apoptotic peptide

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Abstract
A novel activating peptide was designed and synthesized from *V. cholerae* hemagglutinine protease (HAP) mediated cleavage site of mouse PAR1. The peptide "PFISED" interacts with PAR1 in a new site which is different from its thrombin mediated conventional activation site and induced a series of new downstream signaling pathways. The peptide showed apoptosis in human and mouse breast (MCF-7 and EAC) and colon (HT29 and CT26) cancer cells where as in the same peptide concentration in normal human breast epithelial cells (MCF-10A), normal human fibroblast cells (MRC-5), normal mouse peritoneal macrophage cells and normal mouse breast and colon tissues did not show any effect. Treatment with this peptide enhanced the survival kinetics of EAC induced mice. The peptide mediated apoptosis was inhibited in presence of PAR1 inhibitor and was significantly reduced in si-PAR1 treated cells that indicate the activating peptide "PFISED" induced PAR1 mediated apoptosis of colon and breast cancer cells. This peptide induced over expression and activation of PAR1 and its downstream MAP kinase and NFκB signaling pathways. These signaling pathways enhanced the cellular ROS level to kill malignant cells. We report a novel pro-apoptotic peptide which can selectively kill malignant cells via its specific target receptor PAR1 which is over expressed in the malignant cells and can be used as a molecular target therapy for cancer treatment.

Keywords Pro-apoptotic peptide · Apoptosis · PAR1 · Targeted therapy

Introduction

Conventional therapies are accompanied by severe, dose-limiting side-effects. Ineffectiveness of conventional anti-cancer therapy prompted oncologist to develop new techniques and drugs to combat this disease. Chemotherapy, radiation, and surgery are the conventional treatment modalities for cancer. The success achieved with these approaches has been limited due to factors like chemoresistance to drugs and non-specificity leading to peripheral toxicity. Cancer cells divide more quickly than healthy cells and chemotheraputic drugs target these cells. However in the process, they also harm fast growing healthy cells causing side effects. To combat these problems, the concepts of targeted therapy using apoptotic inducers were developed. Several proteases and peptides have been reported to have anti-tumor activity [1–4].

Discovery of several protease and peptide receptors and tumor-related peptides is expected to create more effective and selective anti-cancer drugs in the future [5–7]. Conventional medicines have two major limitations compared to peptides: poor delivery to tumors due to their large size and dose-limiting toxicity to the liver and bone marrow due to nonspecific uptake into the reticuloendothelial system. The use of such macromolecules has therefore been restricted to different kinds of malignancies [8–13]. Peptides possess many advantages such as small size, ease of synthesis and modification, tumor penetrating ability, and good biocompatibility [14, 15]. Peptides have evolved as promising therapeutic agents in the treatment of cancer [14].

In our previous study we showed that hemagglutinin protease secreted by *V. cholerae* has anti-cancer property [16]. It induced cytotoxicity in cancer cells by activating

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the intrinsic pathway of apoptosis. It inhibited tumor cell proliferation in a breast cancer adenocarcinoma model. Treatment of HAP could significantly improve lifespan in treated tumor bearing mice [16]. Protease signaling is mostly regulated via the activation of protease activated receptors (PARs). PAR plays important roles in both cancer progression and apoptosis of malignant cells depending on different stimuli [17–19]. PARs are of four types, PAR1 to 4. PAR1 has long been thought to be involved in tumour invasion, metastases associated with melanomas, as well as with cancer of the breast, colon, lung, pancreas and prostate [20, 21]. *V.cholerae* hemagglutinin protease (HAP) showed PAR1 induced apoptosis in mouse breast cancer cells [16]. In our previous study we showed that HAP induced a new PAR1 cleavage site that also caused PAR1 activation. Here we have identified the "tethered ligand" that binds intramolecularly to trigger transmembrane signaling. Since HAP mediated PAR1 activation caused apoptosis in breast cancer cells, hence we hypothesized that the new "tethered ligand" sequence will provide a new pro-apoptotic peptide that may able to induce PAR1 mediated apoptosis.

In this study we designed a novel pro-apoptotic peptide "PFISED" from HAP mediated new PAR1 cleavage site and showed its anti-cancer activity for breast and colon cancer cells. Here we showed PAR1 mediated apoptosis of breast and colon cancer cells via MAP kinase and NFκB pathways. These signaling pathways enhanced the cellular ROS level. PAR1 is over expressed in malignant cells and the level of ROS in malignant cells is reported to be higher than the normal healthy cells. Malignant cells cross the threshold level of ROS faster than the normal healthy cells and induced apoptosis. The novel pro-apoptotic peptide "PFISED" induced PAR1 mediated apoptosis of breast and colon cancer cells without altering the survival of healthy cells and may be used as a novel targeted therapy for cancer treatment.

Materials and methods

Chemicals and reagents

PAR1 inhibitor (ML161) and other general chemicals used in the present study were of analytical grade and purchased from Sigma-Aldrich (USA). Inhibitors of p38 (SB203580) and NFκB (MG132) were purchased from Calbiochem (USA). FBS, antibiotics (streptomycin, penicillin), RPMI-1640 and Trizol were purchased from Invitrogen (USA). All the antibodies (anti p38, anti p-p38, anti p65, anti p50, anti PAR 1, anti Actin and anti tubulin) were purchased from Santa Cruz Biotechnology (USA), the apoptosis detection kit was purchased from BD Bioscience (USA), MMLV reverse transcription was purchased from Fermentas and SYBR green supermix was purchased from Bio-Rad (USA). si-RNA of human PAR1 (CGGUCUGUUAUGUGUCUA UdTdT) was purchased from Euorigentec (Belgium). Jet PRIME, transfection reagent was purchased from Polyplus Transfection (France) and used for the transfection of si-RNA of PAR1 and the experiment was performed as per manufacture's protocol. Cell-permeable Z-VAD-FMK (Sigma Aldrich, Cat No. V116) which acts as caspase inhibitor was dissolved in DMSO, aliquoted and stored at − 80 ℃, and then diluted as needed in PBS for experiments. The final concentration of DMSO in the solution injected into the animal was less than 0.5%.

Synthesis of pro-apoptotic peptide "PFISED"

The pro-apoptotic peptide "PFISED", the jumbled peptide "FEPIDS" and the peptide sequence of 2nd extra cellular loop of mouse PAR1 "KEQTTRVPGLNITTCHDVLSENLMQGFYS" were commercially synthesized and purchased from Euorigentec (Belgium). 95% HPLC grade purified peptide was used for this study. Mouse PAR1 is a 430 amino acid long protein in which the second extra cellular loop is 29 amino acid long peptide sequence ranging from 246 to 275 amino acids.

Cell culture

MCF-7 and EAC cells were cultured in RPMI-1640 medium and HT29, CT26, mouse peritoneum macrophage cells and MRC-5 were cultured in high glucose DMEM medium supplemented with 10% Fetal Bovine Serum (FBS) with 100 U/ml penicillin and 100 µg/ml streptomycin in humidified air/CO_2 (5% CO_2) at 37 °C. MCF-10A was maintained in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 20 ng/ml Human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone and 5% horse serum. EAC and CT26 cells were supplied by the Chittaranjan National Cancer Institute (CNCI), Kolkata, MCF-10A cells was supplied by Saha Institute of Nuclear Physics (SINP), Kolkata and the other cells were purchased from National Centre for Cell Science (NCCS), Pune, India.

Flowcytometric analysis for apoptosis

Quantitative evaluation of apoptosis was performed by using the flowcytometry methods of double staining using an FITC-conjugated annexin-V/propidium iodide (PI) staining. Cells (10^6 cells) were incubated for 16 h with different doses of Peptide (25 µM, 50 µM, 100 µM and 200 µM). After Peptide treatment cells were washed with PBS and incubated with PI and Annexin-V for 15 min at 37 °C. Excess PI and Annexin-V were then washed with PBS and cells were analyzed by FACS Aria II using Cell Quest software [22, 23].

Western blotting for the analysis of different signaling pathways

Cell lysate was prepared by RIPA buffer after appropriate treatment and the expression of p38, p-p38, PAR1, actin and tubulin was determined. Nuclear localization of NFκB partner p50 and p65 was determined by cytosol and nuclear extraction buffer. 30 µg of cell lysate was loaded onto 10% SDS-PAGE and proteins were transferred to PVDF membrane, blocked with 5% non-fat milk and incubated overnight at 4 °C with specific primary antibodies (1:5000). The membranes were then washed and incubated with either AP or HRP conjugated secondary antibody at room temperature (RT) for 1 h. Finally proteins were detected by AP substrate or by chemiluminescence detection kit (ECL, Amersham Bioscience, UK) [24].

Nuclear cytosolic fractionation

For NFκB analysis, cells were treated with 100 µM of peptide for 16 h. The untreated and peptide treated cells were then lysed with cytosolic extraction buffer (10 mM HEPES (pH 7.5), 0.1% NP-40, 1.5 mM $Mgcl_2$, 10 mM Kcl, 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF and 0.5 mM DTT) to obtain cytosolic fractions. After centrifugation, pellets were then lysed with the nuclear extraction buffer (20 mM HEPES (pH 7.5), 2.5% Triton X-100, 1.5 mM $Mgcl_2$, 0.4 M Nacl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF and 0.5 mM DTT) to obtain nuclear fractions [25]. These fractions were used for Western blotting to determine nuclear translocation of p50 and p65. For experiments where PAR1 was inhibited, cells were initially pre-incubated with 0.5 µM PAR1 inhibitor (ML161) for 2 h and then incubated with 100 µM of peptide for 16 h. Cells were then harvested and nuclear and cytoplasmic fractions were separated as described above.

Detection of mRNA expression level of PAR upon peptide treatment by Real-Time quantitative RT-PCR

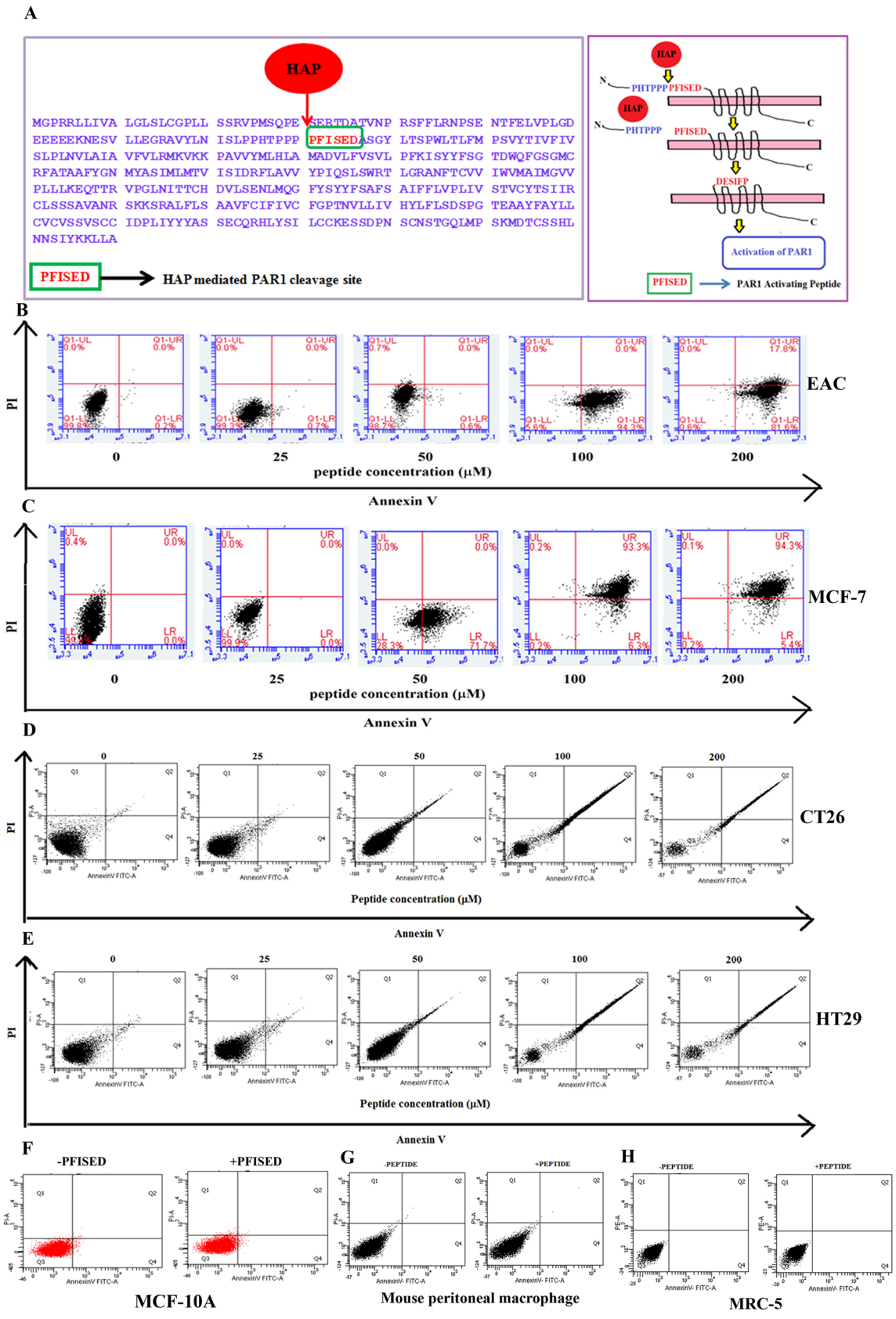
Total cellular RNA was isolated from 100 µM peptide treated and untreated EAC, MCF-7, CT26, HT29, MCF-10A cells and mouse breast and colon tissue using Trizole reagent (Invitrogen). 2 µg of the isolated total RNA was reverse transcribed using MMLV reverse transcriptase (Fermentas) and random hexamer primer (Fermentas) to synthesize the cDNA first strand. Thereafter, the amplified cDNA first strand was used in the subsequent analysis by Real-Time quantitative RT-PCR. The sequences of the polynucleotide primers for mouse PARs that were used in the PCR reactions are described in our previous publication [26]. GAPDH was used as an internal control to normalize the results. Human primers sequences are as follows; Human GAPDH (F'): 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, Human GAPDH (R'): 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′, Human PAR1 (F'): 5′-GTAGTCAGCCTCCCACTAAAC-3′, Human PAR1 (R'): 5′-CACAGACACAAACAGCACATC-3′, Human PAR2 (F'): 5′-CCTCAGTGTGCAGAGGTA TTG-3′, Human PAR2 (R'): 5′-AGCAGAATCAGCAGC CATATT-3′, Human PAR3 (F'):5′-CCCATCATCCTTCCG ATTCTAC-3′, Human PAR3 (R'): 5′-GTGGATGAGAGT CGTGTAACAG-3′, Human PAR4 (F'): 5′-GTCACTAGC AGAGGTCACTTTG-3′, Human PAR4 (R'): 5′-GCCTCT TAAAGTGCTGGGATTA-3′. Real-Time quantitative RT-PCR was performed using SYBR green reagent (iQ SYBR green supermix, Bio-Rad). The 25 µl of the PCR mixture contained 10 pM of each primer (the same combination of forward and reverse primers as used for semi quantitative RT-PCR), 12.5 µl of SYBR green reagent (Bio- Rad) and 2.0 µl of cDNA. The samples were placed in 96-well plate and sealed with optical sealing tape. PCR reactions were carried out by using an iCycler iQ multicolour real time PCR detection system (Bio-Rad). The thermal cycling conditions used for Real Time PCR were: denaturation at 95 °C for 30 s, annealing either at 50 °C (for mouse specific primers of PARs) or at 55 °C (for human specific primers of PARs) for 30 s and extension at 60 °C for 30 s. Relative quantification was performed based on comparative cycle threshold (Ct) method as described by Kageyama et al. [27]. Briefly, the Ct of the target amplicon and the Ct of the internal control GAPDH were determined for each sample. The ΔCt value for each experimental sample was subtracted from the calibrator to obtain the ΔΔCt. The arithmetic calibrator $2^{-\Delta\Delta Ct}$ was used to calculate the amount of target relative to calibrator. The data were represented as percentage compared to control and derived by averaging the results obtained from three independent experiments. All real time PCR primer sequences were standardized in our lab.

Immunofluorescence

Pro-apoptotic peptide treated (with 100 µM of peptide for 16 h) and untreated cells were fixed with 2% paraformaldehyde for 10 min at RT. Cells were then permeablised with 0.1% Triton X-100 in 0.1% sodium citrate solution and blocked with 5% serum. Cells were incubated over night with primary antibody (1:500) against p65, p50, p-p38 and PAR1 at 4 °C. Cells were washed with PBS and then incubated with TRITC conjugated secondary antibody. Nuclei were stained with DAPI [28, 29] and viewed under confocal microscope (Zeiss-LSM510) at 63X resolution.

Detection of ROS by DCFDA staining

The in situ ROS level was measured by oxidation of 2′,7′-dichlorofluorescin diacetate (DCFDA) to highly


A
HAP
MGPRRLLIVA LGLSLCGPLL SSRVPMSQPE SERTDATVNP RSFFLRNPSE NTFELVPLGD
EEEEEKNESV LLEGRAVYLN ISLPPHTPPP PFISEDASGY LTSPWLTLFM PSVYTIVFIV
SLPLNVLAIA VFVLRMKVKK PAVVYMLHLA MADVLFVSVL PFKISYYFSG TDWQFGSGMC
RFATAAFYGN MYASIMLMTV ISIDRFLAVV YPIQSLSWRT LGRANFTCVV IWVMAIMGVV
PLLLKEQTTR VPGLNITTCH DVLSENLMQG FYSYYFSAFS AIFFLVPLIV STVCYTSIIR
CLSSSAVANR SKKSRALFLS AAVFCIFIVC FGPTNVLLIV HYLFLSDSPG TEAAYFAYLL
CVCVSSVSCC IDPLIYYYAS SECQRHLYSI LCCKESSDPN SCNSTGQLMP SKMDTCSSHL
NNSIYKKLLA
PFISED
HAP mediated PAR1 cleavage site
HAP
PHTPPP PFISED
HAP
PHTPPP
PFISED
DESIFP
Activation of PAR1
PFISED
PAR1 Activating Peptide
B
PI
Annexin V
0
25
50
100
200
peptide concentration (μM)
EAC
C
PI
Annexin V
0
25
50
100
200
peptide concentration (μM)
MCF-7
D
PI
Annexin V
Peptide concentration (μM)
CT26
E
PI
Annexin V
Peptide concentration (μM)
HT29
F
-PFISED
+PFISED
MCF-10A
G
-PEPTIDE
+PEPTIDE
Mouse peritoneal macrophage
H
-PEPTIDE
+PEPTIDE
MRC-5

◂**Fig. 1** The novel pro-apoptotic peptide "PFISED" induced apoptosis in human and mice breast and colon cancer cells but not in normal human and mice cells. **A** The design of the pro-apoptotic peptide "PFISED" is shown schematically. **B**–**E** Dose dependent response of the pro-apoptotic peptide on EAC, MCF-7, CT26 and HT29 cells was analyzed by FACS to study cellular apoptosis. **F**–**H** 100 µM Peptide was used to study its effect on human normal breast epithelial cells (MCF-10A), normal mouse peritoneal macrophage cells and normal human fibroblast cells (MRC-5)

fluorescent 2′,7′-dichlorofluorescin (DCF). Pro-apoptotic peptide "PFISED" treated (100 µM peptide for 16 h) and untreated MCF-7 and HT29 cells were incubated with DCFDA for 20 min at 37 °C, washed with PBS and viewed under confocal microscope (Zeiss-LSM510) at 63X resolution [30].

Explant culture of normal breast and colon tissue

Normal breast tissue was harvested from lactating female mice and the colon tissue was harvested from normal female mice. Tissues were sectioned into approximately 1 mm × 1 mm slices. These tissue sections were randomized and cultured in 48-well flat bottom plates in presence of RPMI-1640 and DMEM medium respectively supplemented with 10% FBS, 1X Insulin-Transferrin-Selenium (ITS) and 1X penicillin and streptomycin. Tissue slices were treated with 100 µM peptides for 16 h. Treated and untreated tissue samples were used for cDNA preparation for real time PCR of PARs and whole cell lysate preparation for western blotting for PAR1 expression [31].

Maintenance and preparation of EAC and CT26 cells from mice peritoneal cavity

EAC and CT26 cells were maintained in Swiss albino mice through serial intraperitoneal (I.P) inoculation at 10 day intervals. 10^6 number of viable EAC cells were implanted into the peritoneal cavity of each mouse (22–25 g body weight) and allowed to multiply. EAC cells were isolated from the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells were incubated at 37 °C for 2 h. The cells of macrophage lineage adhered to the Petri dishes. The non-adherent cell population were collected gently and washed with PBS. More than 98% of this separated cell population was morphologically characterized as EAC [32–34]. Similar technique was also used for maintenance and preparation of CT26 cells.

Study the peptide induced apoptosis in presence and absence of caspase inhibitor (Z-VAD FMK) in EAC and CT26 induced intra peritoneum mice model

10^6 numbers of EAC or CT26 cells were inoculated into the peritoneal cavity of Swiss albino mice (22–25 g body weight) and allowed to multiply. Normal animals without tumor inoculation were kept in a separate group. After 7 days of tumor inoculation, animals were divided into four different groups in (i) normal set (non-tumor-bearing); (ii) tumor-bearing set; (iii) peptide-treated tumor bearing set where 500 µl of 100 µM Peptide was injected intraperitonially; and (iv) caspase inhibited peptide treated tumor bearing set (pre-incubated with caspase inhibitor before peptide treatment). In the 4th group mice were intraperitoneally injected with caspase inhibitor (Z-VAD FMK at a dose of 30 mg/KG body weight) 30 min prior to peptide treatment. After 2 and 4 h of peptide treatment tumor cells were isolated from each group except the normal non tumor bearing set and progression of apoptosis was measured with Annexin V and PI staining.

EAC and CT26 induced tumor in intra peritoneum mice model and effect of the pro-apoptotic peptides on its survival

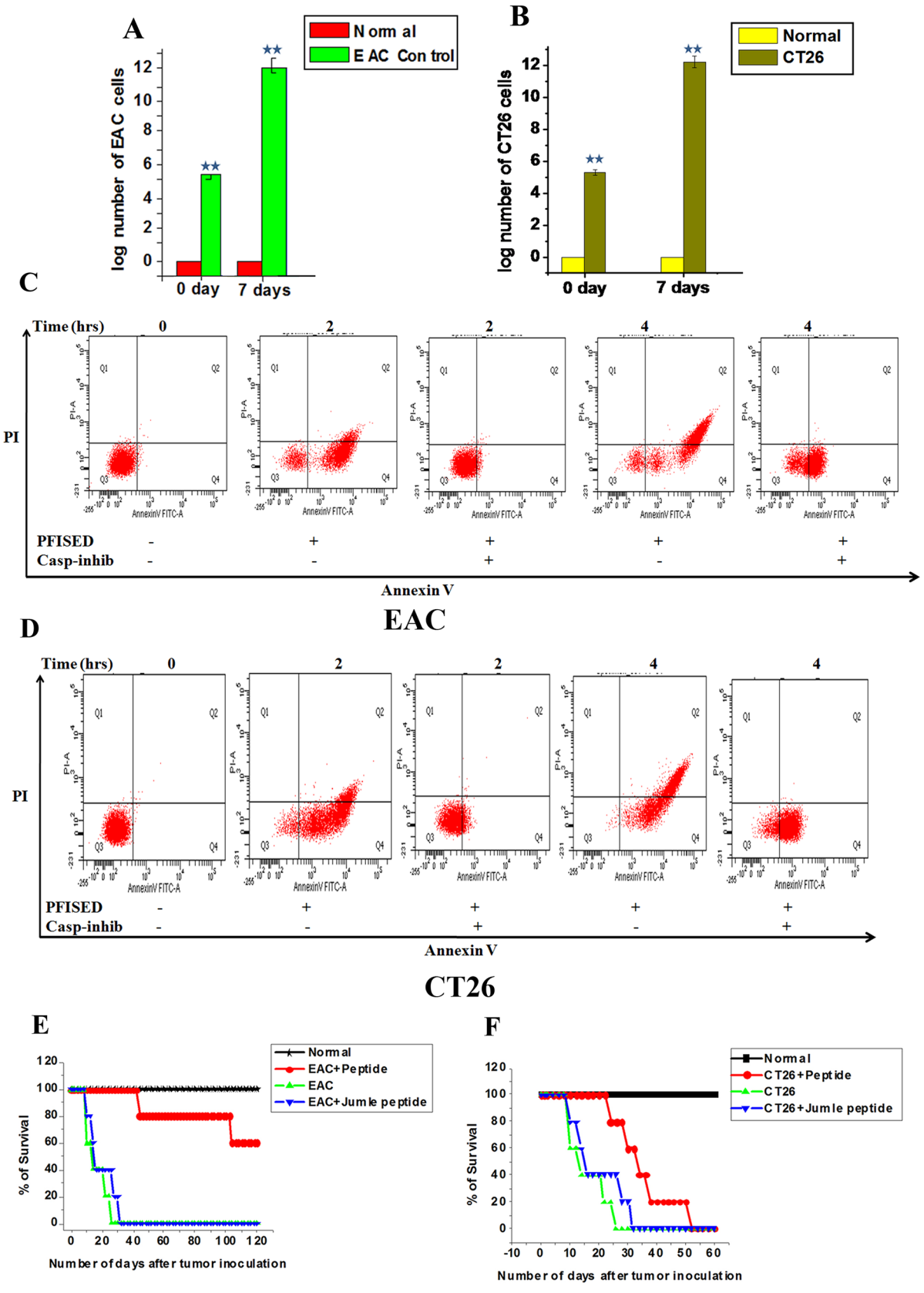
Survival kinetics was studied by implanting 10^6 numbers of EAC or CT26 cells into the peritoneal cavity of Swiss albino mice (22–25 g body weight) and allowed to multiply. Animals were divided into four different groups in (i) normal set (non-tumor-bearing); (ii) tumor-bearing set; (iii) peptide-treated tumor bearing set where 500 µl of 100 µM Peptide was injected intraperitonially after one week of tumor cells (EAC or CT26 cells) inoculation and injected once in a week; and (iv) jumble peptide treated tumor bearing set. The life span of each group of mice was evaluated by measuring the percentage of survival rate in each group at 10 days interval by using a formula:

(Number of live animals in a group/number of initial animals in that group) × 100

In each group ten animals were present and all animal experiments were performed at least three times. The data were represented by averaging the results obtained from three independent experiments.

Isothermal titration calorimetry (ITC)

ITC experiments were conducted using an iTC200 Microcalorimeter at 25 °C. The pro-apoptotic peptide "PFISED" and the peptide sequence of the second extracellular loop of mouse PAR1 "KEQTTRVPGLNITTCHDVLSENLMQGFYS" were dissolved in 1 mM ammonium bicarbonate buffer, pH 7.0 and degassed prior to titration to ensure no


A
Normal
EAC Control
log number of EAC cells
0 day
7 days
B
Normal
CT26
log number of CT26 cells
0 day
7 days
C
Time (hrs)
PI
PFISED
Casp-inhib
Annexin V
D
EAC
Time (hrs)
PI
PFISED
Casp-inhib
Annexin V
CT26
E
Normal
EAC+Peptide
EAC
EAC+Jumle peptide
% of Survival
Number of days after tumor inoculation
F
Normal
CT26+Peptide
CT26
CT26+Jumle peptide
% of Survival
Number of days after tumor inoculation

◂**Fig. 2** The novel pro-apoptotic peptide "PFISED" induced apoptosis in EAC and CT26 cells in intraperitoneal mice model and enhances the survival kinetics of EAC and CT26 induced mice. **A**, **B** Numbers of viable EAC and CT26 cells were measured after 7 days of tumor inoculation by a hemocytometer and trypan blue exclusion method that indicate the formation of tumor in EAC and CT26 inoculated groups. **C**, **D** 2 and 4 h of peptide treatment induced apoptosis in EAC and CT26 cells in intraperitoneally tumor induced mice but in caspase inhibitor treated group the pro-apoptotic peptide failed to induced apoptosis in EAC and CT26 cells in mice model. **E**, **F** Effect of pro-apoptotic peptide on the survival kinetics was studied in EAC and CT26 induced tumor in breast and colorectal intraperitoneal mice model

bubble formation takes place. The pro-apoptotic peptide "PFISED" (400 μM) was injected into a fixed volume of second extracellular loop of PAR1 (20 μM) after an interval of 180 s. A corresponding blank experiment was employed by injecting an equal volume of the pro-apoptotic peptide "PFISED" with the same concentration into the buffer solution (without the second extra cellular loop peptide) to subtract the heat of dilution when "PFISED" interacts with the buffer. The isotherms were analyzed using in-built Origin 7.0 software. The data points obtained best fitted the 'one set of sites' model. Equilibrium constant Ka, change in enthalpy (ΔH), and change in entropy (ΔS) connected with "Peptide–Peptide" complex formation were evaluated after fitting the isotherms. The Gibbs energy was calculated using the equation, $\Delta G = \Delta H - T\Delta S$.

Statistical analysis

The experimental results were expressed as mean ± standard deviation. Data were assessed by the Student's unpaired *t* test (two population) by using the software origin 6.1; p value < 0.05 was considered as statistically significant.

Results

Design and synthesis of a novel pro-apoptotic peptide "PFISED"

V. cholerae hemagglutinin protease (HAP) cleaved PAR1 in a site that is different from its conventional thrombin mediated cleavage site. The N-terminal site of cleaved PAR1 interacts with one of its transmembrane domain and activates its downstream signaling pathways. HAP exposed a new N-terminal sequence "PFISED" which by itself could be used as an activating peptide for PAR1. Since HAP induced PAR1 mediated anti-cancer activity, so we used the N-terminal sequence of the cleaved PAR1 "PFISED" as a free peptide that by itself is able to activate downstream signaling pathways of PAR1 and can be used as a novel pro-apoptotic peptide for cancer therapy (Fig. 1A).

The novel pro-apoptotic peptide "PFISED" induced apoptosis in human and mice breast and colon cancer cells

The effect of the pro-apoptotic peptides "PFISED" was studied by annexin V and PI staining. A gradient of peptide concentration (25 μM, 50 μM, 100 μM and 200 μM) was used for 16 h in human and mouse breast and colon cancer cells to standardize the appropriate dose of peptide. For this study we used mouse (EAC) (Fig. 1B) and human (MCF-7) (Fig. 1C) breast cancer cells and mouse (CT26) (Fig. 1D) and human (HT29) (Fig. 1E) colon cancer cells. The pro-apoptotic peptide induced apoptosis in more than 90% cells at 100 μM concentration in all malignant cell types but this peptide "PFISED" did not induce apoptosis in normal human breast epithelial cells MCF-10A (Fig. 1F), in normal mouse peritoneal macrophage cells (Fig. 1G) and in normal human fibroblast cells (MCR-5) (Fig. 1H).

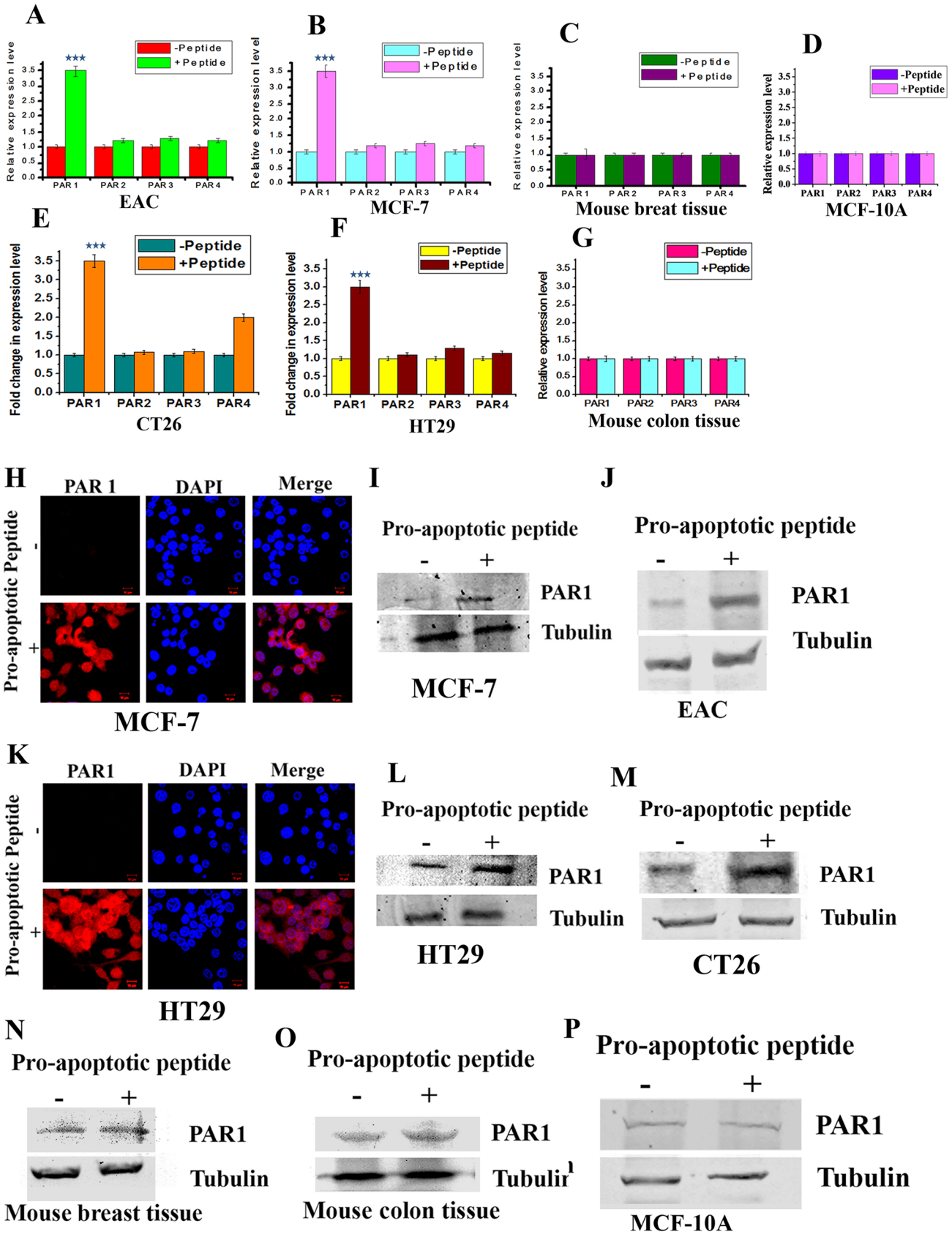
Study the formation of tumor in EAC and CT26 induced intra-peritoneal mice model and study the peptide induced apoptosis in presence and absence of caspase inhibitor in this mice model

10^6 numbers of EAC or CT26 cells were inoculated in Swiss albino mice. Numbers of viable EAC and CT26 cells were measured after 7 days of tumor inoculation by a hemocytometer and trypan blue exclusion method. After tumor inoculation numbers of viable EAC and CT26 cells in intraperetoneal mice model were increased with time, that suggested the formation of tumor in all EAC and CT26 inoculated groups (Fig. 2A, B).

After tumor formation in the peritoneum cavity of Swiss albino mice (after 7 days of tumor inoculation) the mice were separated into three different groups of 10 animals. In tumor control group no peptide was treated and both EAC and CT26 cells were healthy and live, in peptide treated group after 2 and 4 h of peptide treatment tumor cells (both EAC and CT26 cells) under goes apoptosis but in caspase pre-treated group, after 2 and 4 h of peptide treatment, peptide induced apoptosis was inhibited in tumor cells (EAC and CT26) compare to the peptide treated group (Fig. 2C, D). These results suggested that the peptide is able to induce apoptosis of EAC and CT26 cells in mice model (Fig. 2C, D).

The pro-apoptotic peptide treatment increased the survival of EAC and CT26 induced breast and colorectal intraperitoneal mice model

Tumor was induced intraperitoneally with 10^6 numbers of EAC or CT26 cells in Swiss albino mice and the survival kinetics was observed. After 7 days of EAC or CT26 cells


A
Relative expression level
***
-Peptide
+Peptide
PAR 1
PAR 2
PAR 3
PAR 4
EAC
B
MCF-7
C
Mouse breat tissue
D
MCF-10A
E
Fold change in expression level
CT26
F
HT29
G
Mouse colon tissue
H
PAR 1
DAPI
Merge
Pro-apoptotic Peptide
MCF-7
I
Pro-apoptotic peptide
PAR1
Tubulin
MCF-7
J
EAC
K
HT29
L
HT29
M
CT26
N
Mouse breast tissue
O
Mouse colon tissue
P
MCF-10A

◂**Fig. 3** The pro-apoptotic peptide "PFISED" induced PAR-1 activation. **A**, **B**, **E**, **F** Real time PCR showed almost three to four fold over expression of PAR1 RNA compared to the other PARs in EAC, MCF-7, CT26 and HT29 cells upon peptide treatment. **C**, **D**, **G** There was no significant change in PARs RNA expression level in human normal breast epithelial cells (MCF-10A), mouse normal breast and colon tissues on peptide treatment. The above results are graphically represented in the bar diagram. In all panels, *$p < 0.05$, **$p < 0.005$ and ***$p < 0.0005$. **H**, **K** Immunofluorescence revealed the over expression of PAR1 in MCF-7 and HT29 cells upon peptide treatment. **I**, **J**, **L**, **M** Western blot analysis confirmed that peptide treatment induced over expression of PAR1 in MCF-7, EAC, HT29 and CT26 cells, **N**, **O**, **P** but not in normal human breast epithelial cells (MCF-10A) and mouse breast and colon tissues

inoculation, the mice were separated into four different groups of 10 animals. In the tumor control group the rate of survival of mice was 20% after 30 days and 0% after 60 days. In peptide treated group, mice were treated with 500 µl of 100 µM peptide at a weekly interval. In EAC induced mice the rate of survival was 100% after 30 days, 80% after 60 days and 60% after 120 days (Fig. 2E). In CT26 induced mice the initial survival rate was same with EAC induced mice (after 30 days the survival rate was 100%) but after 60 days the survival of CT26 induced mice was 0% (Fig. 2F) that indicate that our dose of peptide is more effective in breast cancer cells (EAC) compared to the colon cancer cells (CT26). The rate of survival in jumble peptide "FEPIDS" group was similar as the tumor control group. These results suggest that the anti-tumor effect of the peptide was due to its pro-apoptotic property.

The pro-apoptotic peptide causes PAR1 over expression in breast and colon cancer cells but not in normal mice breast and colon tissue and normal breast epithelial cells

Human and mouse breast (MCF-7 and EAC) and colon (HT29 and CT26) cancer cells, normal mouse breast and colon tissue (explants culture) and normal human breast epithelial cells (MCF-10A) were treated with 100 µM of peptide for 16 h and cDNA was prepared from the total RNA pool of peptide treated and untreated cells. Real time PCR revealed over expression of PAR1 in mouse and human breast cancer (EAC and MCF7) (Fig. 3A, B) and colon cancer (CT26 and HT29) cells (Fig. 3E, F). Real time PCR showed over expression of PAR1 RNA compared to the other PARs in both malignant cells. Interestingly there was no significant change in PARs expression level in normal mouse breast and colon tissue (explants culture) (Fig. 3C, G) and in normal human breast epithelial (MCF-10A) cells (Fig. 3D). The Ct values showed that the level of expression of PARs (PAR1-4) were always lower in normal mouse breast, colon tissues and the normal breast epithelial cells when compared with the malignant cells. To check the expression of PAR1 at the protein level, breast and colon cancer cells were treated with 100 µM of peptide for 16 h and cells were then either fixed for immonofluorescence or cell extracts were prepared and used for immunoblotting analysis. Normal breast and colon tissue explants were also treated with 100 µM of peptide for 16 h and cell extracts were prepared for immunoblotting analysis. Immunoflorescence showed the level of PAR1 was increased due to peptide treatment in MCF7 (Fig. 3H) and in HT29 cells (Fig. 3K). Western blotting confirmed that PAR1 expression increased in malignant breast (MCF-7 and EAC) (Fig. 3I, J) and in colon (HT29 and CT26) cells (Fig. 3L, M), where as the PAR1 expression level in normal breast epithelial cells, breast and colon tissue was very low compared to the malignant cells and there was no change in PAR1 expression level in normal mouse breast (Fig. 2N), colon tissue (Fig. 3O) and in normal breast epithelial cells (MCF-10A) (Fig. 3P).

Pro-apoptotic peptide treatment caused NFκB, MAP kinase activation and increased cellular ROS level in human breast and colon cancer cells

The pro-apoptotic peptide mediated signaling pathways for cellular apoptosis was studied in human breast (MCF-7) and colon (HT29) cancer cells. DCFDA staining revealed that pro-apoptotic peptide treatment increase cellular ROS level in both human breast (Fig. 4A) and colon (Fig. 4D) cancer cells. We found the pro-apoptotic peptide induced NFκB and MAP kinase activation. Immunofluorescence and western blot analysis revealed that 16 h peptide treatment caused phosphorylation of p38 in MCF-7 (Fig. 4B, C) and in HT29 (Fig. 4E, F) cells. Pro-apoptotic peptide treatment also caused nuclear localization of NFκB partner p50 and p65 in MCF-7 (Fig. 4G–I) and HT29 (Fig. 4J–L) cells.

Pro-apoptotic peptide mediated PAR1 activation induced cellular apoptosis in breast and colon cancer cells by regulating the downstream signaling pathways

We found the pro-apoptotic peptide induced NFκB and MAP kinase activation and also induced over expression of PAR 1 in breast and colon cancer cells. To check whether these signaling pathways were responsible for the peptide mediated cellular apoptosis, MCF-7 and HT29 cells were first pre incubated for 2 h with either NFκB inhibitor or p38 inhibitor or both followed by 100 µM of the peptide "PFISED" treatment for 16 h. When MCF-7and HT29 cells were pre-incubated with 3 µM NFκB inhibitor, 27% and 32% cells showed apoptosis respectively. When MCF-7 and HT29 cells were pre-incubated with 10 µM p38 inhibitor, 22% and 35% cells showed apoptosis respectively but when

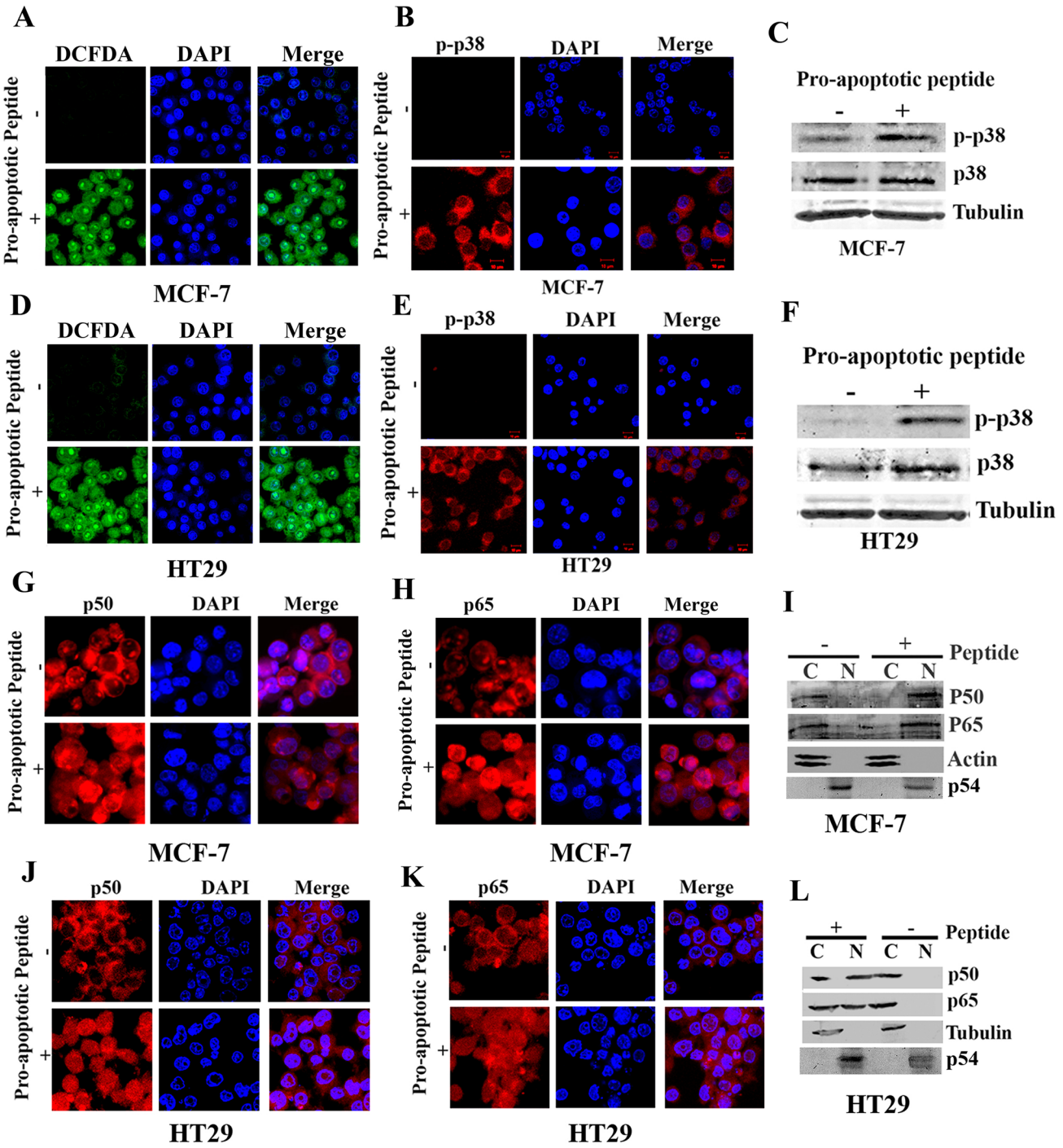


Fig. 4 The pro-apoptotic peptide "PFISED" enhance cellular ROS level and induced NFκB and MAP kinase pathways and in human breast and colon cancer cells. **A**, **D** DCFDA staining revealed that peptide treatment enhances cellular ROS level in MCF-7 and HT29 cells and showed green fluorescence. **B**, **E** Immunofluorescence assay revealed that pro-apoptotic peptide mediated p38 phosphorylation and **G**, **H**, **J**, **K** the nuclear translocation of p50 and p65 in MCF-7 and HT29 cells. **C**, **F**, **I**, **L** Western blot analysis confirmed these results

both inhibitors were used together 19% MCF-7 and 24% HT29 cells showed apoptosis. Peptide treatment on MCF-7 and HT29 showed 100% cellular apoptosis in both the cells (Fig. 5A, B). When MCF-7 and HT29 cells were pre-incubated with PAR1 inhibitor for 2 h and then treated with 100 µM peptide for 16 h there was 17% and 25% cellular

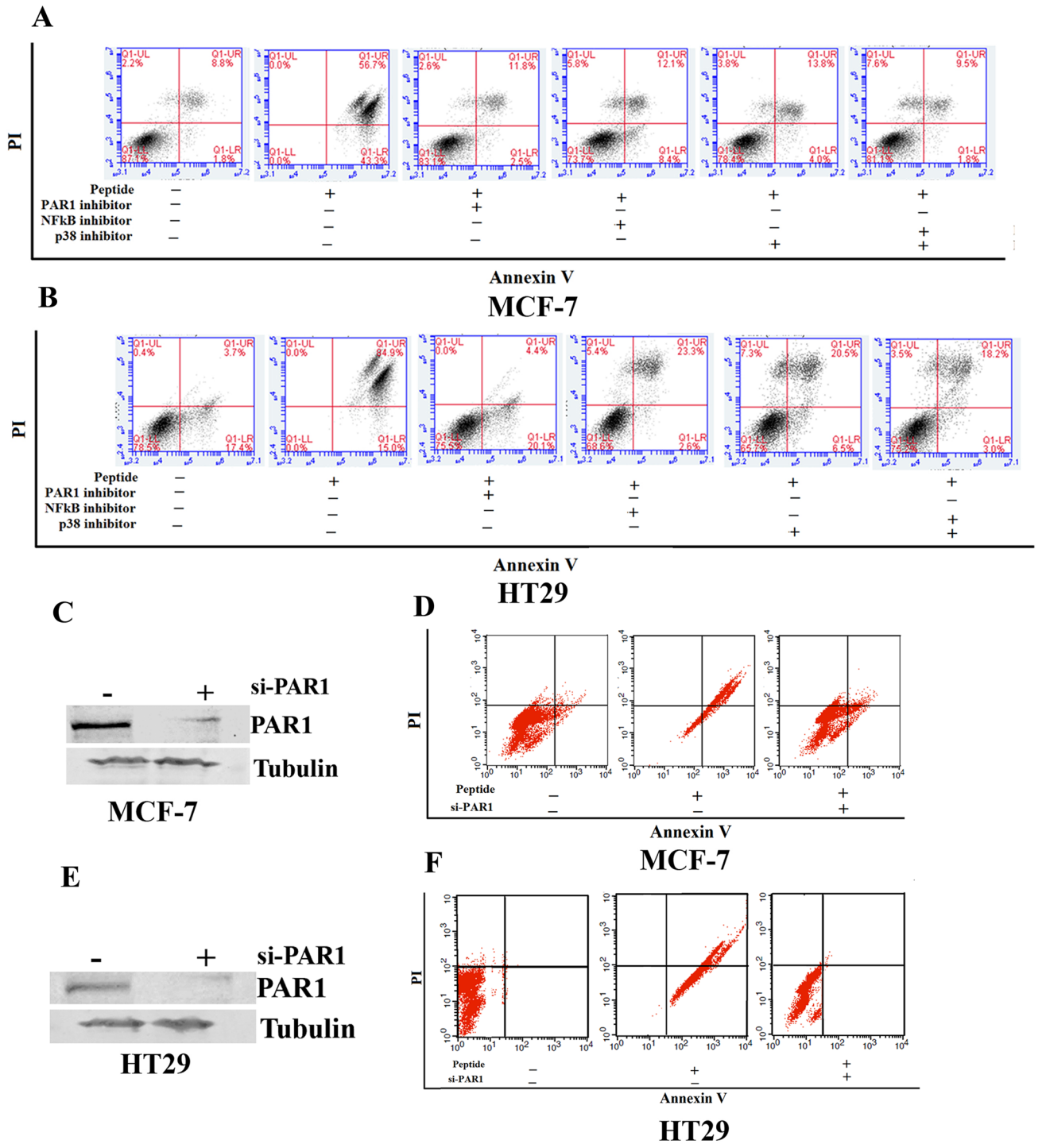


Fig. 5 Peptide mediated PAR1 over expression and activation of its downstream signaling pathways caused apoptosis in breast and colon cancer cells. **A**, **B** Pro-apoptotic peptide mediated cellular apoptosis was analyzed by FACS in presence and absence of different inhibitors (NFκB, p38 and PAR1) on MCF-7 and HT29 cells. **C**, **E** PAR1 down regulation by si-PAR1 transfection confirmed by the western blot analysis and **D**, **F** it results in the inactivation of peptide mediated apoptosis in MCF-7 and HT29 cells

apoptosis (Fig. 5A, B). To study the effect of PAR1 in pro-apoptotic peptide mediated apoptosis, PAR1 was down regulated in MCF-7 and HT29 cells by si-PAR1 transfection. After transfection the level of PAR1 down regulation was confirmed by western blotting (Fig. 5C, E). When si-PAR1 transfected MCF-7 and HT29 cells were treated with the

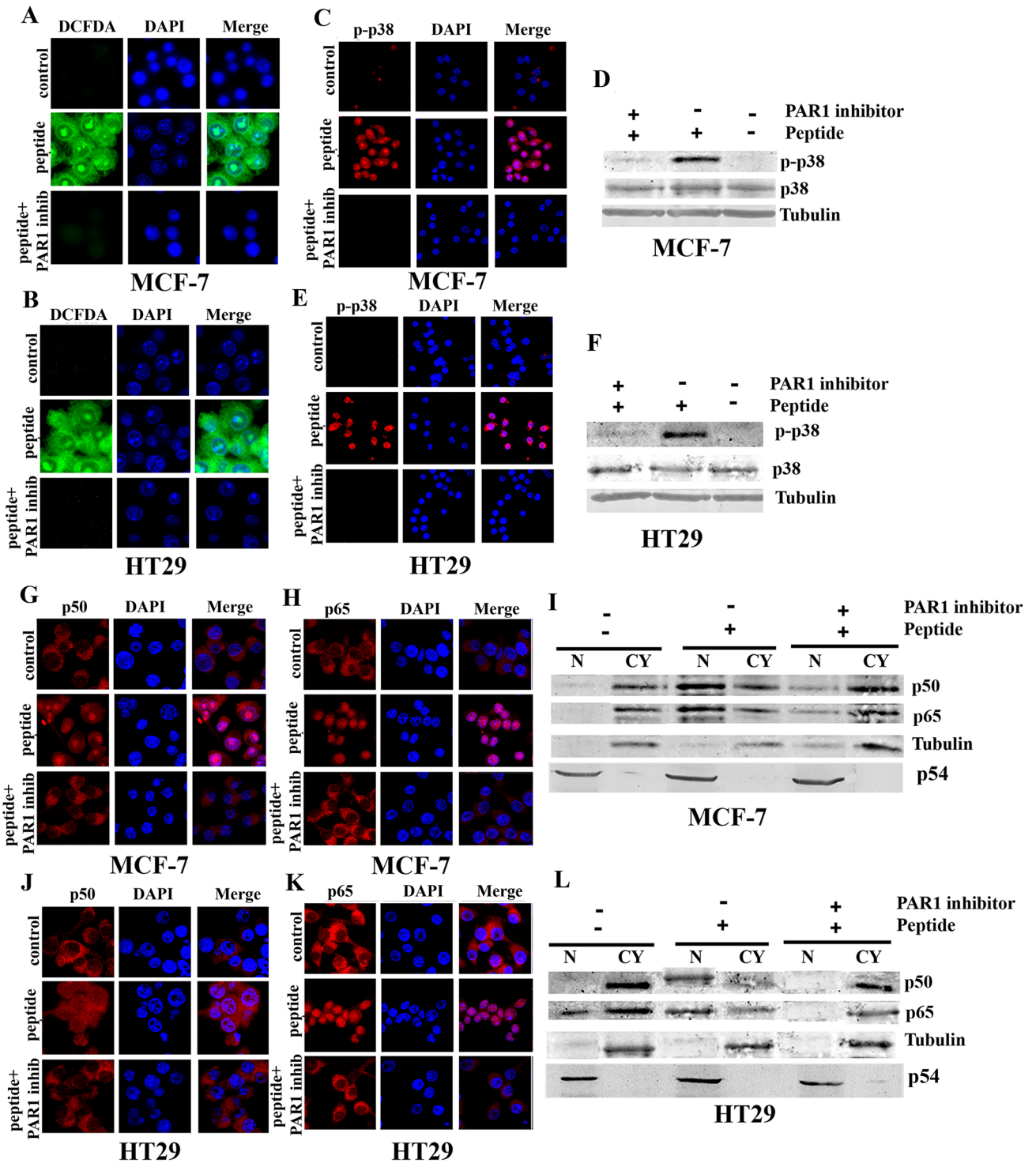


Fig. 6 Peptide mediated PAR1 over expression caused activation of its downstream signaling NFκB, MAP Kinase pathways and enhanced cellular ROS level that results in cellular apoptosis. **A**, **B** Use of PAR1 inhibitor decreased the peptide induced cellular ROS level to its normal state in MCF-7 and HT29 cells that was identified by DCFDA staining. Immunofluorescence assay revealed that use of PAR1 inhibitor block pro-apoptotic peptide mediated **C**, **E** p38 phosphorylation and **G**, **H**, **J**, **K** the nuclear translocation of p50 and p65 in MCF-7 and HT29 cells. **D**, **F**, **I**, **L** Western blot analysis confirmed these results

Table 1 Thermodynamic parameters derived from ITC

Parameters	Pro-apoptotic peptide–extra cellular loop complex
K_a (M^{-1})	1.08×10^5
ΔH (kcal mol^{-1})	−0.318
TΔS (kcal mol^{-1})	0.022
ΔG (kcal mol^{-1})	−0.34

pro-apoptotic peptide they failed to induce apoptosis. These results showed PAR1 activation played the major role in the peptide mediated apoptosis (Fig. 5D, F).

PAR1 inhibition down regulates the pro-apoptotic peptide induced cellular ROS level and NFκB, MAP Kinase pathways

When MCF-7 and HT29 cells were pre-incubated with PAR1 inhibitor, the peptide mediated cellular ROS level decreased to its normal level (Fig. 6A, B). Use of PAR1 inhibitor blocks peptide mediated MAP Kinase (phosphorylation of p38) activation in MCF-7 (Fig. 6C, D) and in HT29 cells (Fig. 6E, F). Use of PAR1 inhibitor also inhibits peptide mediated nuclear translocation of NFκB (p50 and p65) in MCF-7 (Fig. 6G–I) and in HT29 cells (Fig. 6J–L). These results showed the pro-apoptotic peptide mediated PAR1 activation induces its downstream signaling pathways of NFκB and MAP kinase and both pathways could induce the cellular ROS level independently, so PAR1 inhibition or down regulation results deactivation of downstream signaling pathways and inhibition of peptide induced apoptosis.

Study the interaction between the pro-apoptotic peptide and the second extra cellular loop of PAR1 by isothermal titration calorimetry (ITC)

The energetics of binding of pro-apoptotic peptide "PFISED" with the peptide sequence of the second extra cellular loop of mouse PAR1 "KEQTTRVPGLNITTCHDVLSENLMQGFYS" were studied by ITC. The thermodynamic parameters for the association of the pro-apoptotic peptide "PFISED" with the second extra cellular loop of mouse PAR1 are listed in Table 1. The thermogram is shown in Fig. 7A. Gibbs free energy change is negative for this peptide–peptide interaction (ΔG = −0.34 kcal mol^{-1}). The negative free energy change is attributed to a negative enthalpy change and a positive change in entropy. The binding affinity (K_a) of the peptide–peptide interaction is 1.08×10^5 M^{-1}. The amount of Gibbs free energy change (Table 1) and the heat change

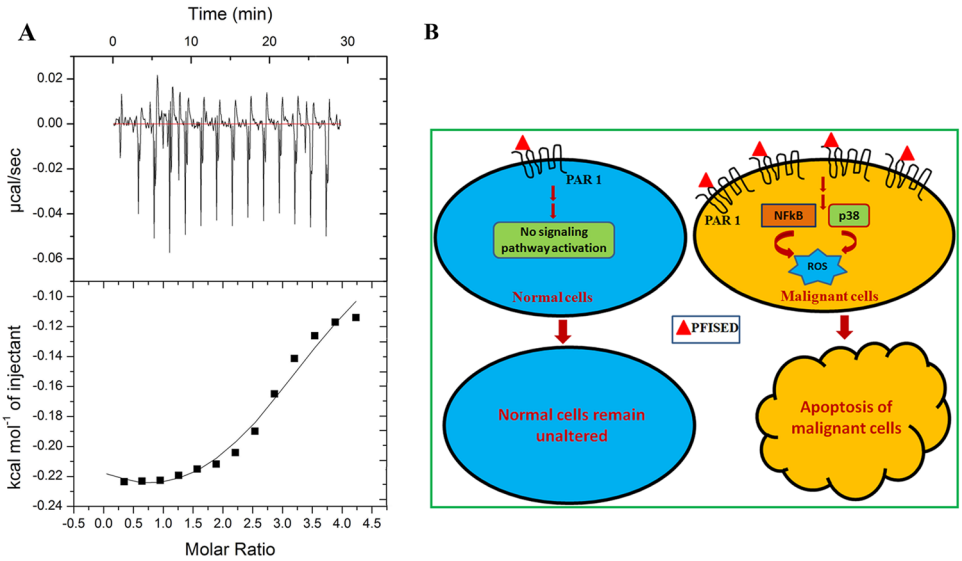


Fig. 7 Mechanism of pro-apoptotic peptide "PFISED" mediated cellular apoptosis in breast and colon cancer cells. **A** The interaction between the pro-apoptotic peptide and the second extra cellular loop of PAR1 was studied by ITC. ITC profile generated from the titration of "peptide–peptide" interaction (between the pro-apoptotic peptide and the second extra cellular loop of PAR1). The upper panel represents the isothermal plot of the peptide–peptide complex formation, whereas the bottom panel displays the integrated binding isotherm generated from the integration of peak area as a function of molar ratio. The solid line represents the best fit data using the "one-site binding model". **B** The pro-apoptotic peptide "PFISED" specifically interacts with PAR1 which is mostly over expressed in malignant cells. So in the same environment the threshold level of PAR1 activation occurs in the cancer cells and this receptor activation induced phosphorylation of p38 and nuclear translocation of p50 and p65 in malignant cells. The activation of MAP kinase and NFκB signaling enhance the cellular ROS level and triggered the apoptotic pathway in malignant cells where as normal healthy cells remain unaltered

for this "peptide–peptide" interaction (0.06 µcal/s) (Fig. 7A) is very low. Moreover this thermogram showed high noise. The signal/noise ratio is very high and the heat change is actually due to injection effect. Usual ITC noise is around 10–100 nanowatts, so a signal below 1 micro calorie is not thermodynamically significant interaction.

Discussion

Therapeutic peptides are novel and promising approach for the development of anti-cancer agents. These peptides are classified for the treatment of cancer into the following groups: Anti microbial/pore forming peptides, Cell permeable peptides and Tumor targeting peptides. The tumor targeting peptides (TTPS) target markers such as receptors expressed on the tumor cell membrane [35] and induced cell death. There are two major types of cell death; apoptosis or necrosis. Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. Cancer happens when too little apoptosis occurs, resulting in malignant cells that will not die. Apoptosis also plays an important role in the treatment of cancer and it is a popular target of many therapeutic strategies.

In an earlier study we had shown that hemagglutinin protesase (HAP) secreted by *V. cholerae* induced apoptosis in breast cancer cells (EAC) by ROS mediated intrinsic pathways of apoptosis and regresses tumor growth in mice model. The success of a novel cancer therapy depends on their selectivity for cancer cells with limited toxicity for normal tissues. Our earlier results showed that HAP induced activation and over expression of PAR1 in breast cancer cells. Increased expression of PAR1 has been reported in different malignant cells [36]. In this study we have designed a novel peptide after determining the HAP mediated PAR1 cleavage site in EAC cells. The protease activated receptor 1 (PAR1), a G-coupled receptor emerges as a promising oncogenic target because of its involvement in the invasive and metastatic process of cancer of the breast, ovaries, lung, colon, prostrate and melanoma [37, 38]. HAP cleaved PAR1 in a new site other than its conventional thrombin mediated cleavage site. The thrombin mediated cleaved N-terminal site of PAR1 itself act as an activating peptide of PAR1 [39–41] so we used the HAP mediated cleaved N-terminal PAR1 site "PFISED" as a new peptide that activates PAR1 and induced PAR1 mediate apoptosis. Thrombin mediated PAR1 activation caused cell proliferation and growth where as the novel pro-apoptotic peptide "PFISED" induced apoptosis in human and mouse breast and colon cancer cells and did not show any adverse effect in normal mouse peritoneal macrophages or in normal human breast epithelial (MCF-10A) and fibroblast cells (MRC-5). This pro-apoptotic peptide treatment also enhances the survival rate of mice in intraperitoneal injected breast and colon cancer cells. The pro-apoptotic peptide "PFISED" treatment started to induce apoptosis after 2 and 4 h of peptide treatment in EAC and CT26 cells in mice model but when we used caspase inhibitor (V-ZAD FMK) 30 min prior to peptide treatment, the pro-apoptotic peptide failed to induce apoptosis in EAC and CT26 cells in mice model. This result confirms that the caspase mediated apoptosis pathway is the reason for increased survival rate in peptide treated EAC and CT26 induced mice model. This pro-apoptotic peptide induced the activation and over expression of PAR1, along with the activation of NFκB and p38 pathways. The use of PAR1 inhibitor blocks the nuclear localization of p50 and p65. PAR1 inhibitor treatment also inhibits the phophorylation of p38. PAR1 activating ligands are reported to interact with the second extra cellular loop of PAR1 [40]. To study the interaction between the pro-apoptotic peptide "PFISED" with the second extra cellular loop of PAR1 we performed Isothermal Titration Calorimetry (ITC). For this purpose we commercially synthesized the peptide sequence of the second extra cellular loop of mouse PAR1 and studied the "peptide–peptide" interaction by ITC. We did not get any significant interaction between the pro-apoptotic peptide and the second extra cellular loop of PAR1. This result suggests that may be the pro-apoptotic peptide "PFISED" interacts with some other extra cellular loop of PAR1.

Considering all these studies it is clear that the pro-apoptotic peptide induced the activation and over expression of PAR1 which triggered its downstream signaling NFκB and p38 pathways and increased the cellular ROS level and induced cellular apoptosis. The pro-apoptotic peptide "PFISED" induced PAR1activation which is mostly over expressed in malignant cells compared to the normal breast and colon cells [42]. The pro-apoptotic peptide mediated over expression of PAR1 increases the receptor density of the peptide in malignant cells. In healthy cells the PAR1 expression is less. When the peptide acts on PAR1 of cancer cells, it significantly enhances apoptotic pathways as compared to normal cells. Thus the novel pro-apoptotic peptide can selectively kill malignant cells without altering the survival of healthy normal cells in the same environment (Fig. 7B).

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Compliance with ethical standards

Animals and ethics statement Mice were maintained as per the principles and guidelines of the ethical committee for animal care of National

Institute of Cholera and Enteric Diseases (NICED). The experimental design of the present study was approved by Institutional Animal Ethics Committee (License No: PRO/136/December 2016-December 2019), NICED, Kolkata, India.

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