Suppression of β-catenin signaling in colon carcinoma cells by a bacterial protein

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Abstract
Colorectal cancer is one of the leading causes of cancer-related death worldwide. The adenomatous polyposis coli (APC) gene is mutated in hereditary colorectal tumors and in more than 80% of sporadic colorectal tumors. APC mutations impair β-catenin degradation, leading to its permanent stabilization and increased transcription of cancer-driving target genes. In colon cancer, impairment of β-catenin degradation leads to its cytoplasmic accumulation, nuclear translocation, and subsequent activation of tumor cell proliferation. Suppressing β-catenin signaling in cancer cells therefore appears to be a promising strategy for new anticancer strategies. Recently, we discovered a novel Vibrio cholerae cytotoxin, motility-associated killing factor A (MakA), that affects both invertebrate and vertebrate hosts. It promotes bacterial survival and proliferation in invertebrate predators but has unknown biological role(s) in mammalian hosts. Here, we report that MakA can cause lethality of tumor cells via induction of apoptosis. Interestingly, MakA exhibited potent cytotoxic activity, in particular against several tested cancer cell lines, while appearing less toxic toward nontransformed cells. MakA bound to the tumor cell surface became internalized into the endolysosomal compartment and induced leakage of endolysosomal membranes, causing cytosolic release of cathepsins and activation of proapoptotic proteins. In addition, MakA altered β-catenin integrity in colon cancer cells, partly through a caspase- and proteasome-dependent mechanism. Importantly, MakA inhibited β-catenin-mediated tumor cell proliferation. Remarkably, intratumor injection of MakA significantly reduced tumor development in a colon cancer murine solid tumor model. These data identify MakA as a novel candidate to be considered in new strategies for development of therapeutic agents against colon cancer.

KEYWORDS
apoptosis, cancer, lysosomes, MakA, proliferation, β-catenin

Abbreviations: APC, adenomatous polyposis coli; Bax, BCL2-associated X protein; BCG, Bacillus Calmette-Guerin; CK1, casein kinase 1; GFP, green fluorescent protein; GSK3β, glycogen synthase kinase; LAMP1 and LAMP2, lysosomal-associated membrane proteins 1 and 2; LEF, lymphoid enhancer-binding factor; MakA, motility-associated killing factor A; NF-κB, nuclear factor kappa B; PEI, polyethylenimine; PI, propidium iodide; SEM, scanning electron microscopy; TCF, T-cell factor; VEGF, vascular endothelial growth factor.

Aftab Nadeem and Kyaw Min Aung contributed equally to this work.

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INTRODUCTION

Cancer is not a single disease but a set of complex events with distinct genetic and histological features. However, all cancers are characterized by several biological traits, among which uncontrolled cell proliferation is the most fundamental. The observation that spontaneous tumor regression sometimes may occur in cases associated with microbial infection has inspired the idea that bacterial cells and/or products may have potential for development of new anticancer therapies, for example, as immunotherapeutic agents in cancer therapy. In 1890, the bone surgeon Dr William Coley, New York City, invented the “Coley's toxin” treatment. Coley noticed that cancer patients who came down with infections after surgical treatment seemed to do better than people who did not get infections. He suggested that infections could stimulate the immune system to also fight the cancer. However, there is still lack of evidence to support the use of Coley’s toxin for treatment or prevention of cancer. Nevertheless, several bacterial species are being explored as possible agents against cancer. Initially developed as a vaccine against tuberculosis, the Bacillus Calmette-Guerin (BCG) is also a bacterial agent approved for treatment of bladder cancer since the late 1970s.

Among different types of cancer, colon cancer is considered the third most common type of cancer and the second leading cause of cancer-related death in industrialized countries. Colon cancer originates from neoplastic transformation of epithelial cells of the colon. Both environmental and genetic factors play important roles in the development of colon cancer. In particular, the Wnt signaling pathway has emerged as a key pathway for colon cancer tumorigenesis. Wnt/β-catenin signaling is an evolutionarily conserved intracellular signaling pathway in several organisms ranging from worms to mammals and is responsible for regulating multiple steps during embryonic development, cell proliferation and differentiation. Wnt signaling regulates cytoplasmic and nuclear levels of β-catenin. The degradation of β-catenin occurs via a “destruction complex” that consists of different protein components, including the scaffold protein Axin, the tumor suppressor protein adenomatous polyposis coli (APC), glycogen synthase kinase (GSK3β) and casein kinase 1 (CK1). Mutations in Wnt pathways trigger the development of multiple types of cancer including melanoma and colon cancer. The familial forms of colon cancer are caused by a loss of APC function, often owing to truncating mutations in the gene. A mutation in the APC gene leads to dysfunction of the destruction complex, which is important for β-catenin degradation. This in turn leads to a reduction in β-catenin proteolysis, thus enhancing its nuclear translocation. In the nucleus, β-catenin binds to T-cell factor/lymphoid enhancer factor and contributes to activation of target genes, including VEGF, cyclin D1 and COX-2, thereby increasing tumor cell migration and proliferation. Oncogenes such as β-catenin represent attractive molecular targets for the development of cancer therapy. Unlike, for example, inactivating mutations of p53, activation of β-catenin is a gain-of-function mutation. As such, it is feasible to use well-established drug development strategies to develop β-catenin signaling inhibitors. Extracellular Wnt inhibitors, including the secreted frizzled-related proteins, which act at the cell surface to inhibit Wnt signaling through its receptors, have been pursued as potential therapeutics.

Clinical treatment of established colon cancer remains a major challenge. Although there are several strategies available to combat colon cancer, including surgery, chemotherapy and radiation therapy, unfortunately, all come with major side effects. Thus, there is an urgent need to find novel candidate anticancer molecules that can be developed as therapeutics to combat colon cancer. Among current approaches, studies of bacterial proteins and peptides have revealed promising bioactive molecules with anticancer treatment potential. We recently identified the protein, motility-associated killing factor A (MakA) from Vibrio cholerae as a virulence factor against Caenorhabditis elegans and zebrafish, that is, acting in both invertebrate and vertebrate hosts. In the present study, we investigated the effect of MakA on mammalian cells. Our data demonstrate that MakA readily targeted cancer cells and suppressed β-catenin signaling in colon carcinoma cells. Moreover, we demonstrated its potential as a therapeutic against cancerous tissue growth in a murine solid colon tumor model.

MATERIALS AND METHODS

2.1 Cell lines and cell culture

Human colon cancer cells (HCT8; RRID:CVCL_2478, DLD1; RRID:CVCL_0248, HCT116; RRID:CVCL_0291 and CaCo-2; RRID:CVCL_0025), breast cancer cells (MCF7; RRID:CVCL_0031), bladder cancer cells (5637; RRID:CVCL_0126), neuroglioma cells (H4; RRID:CVCL_1239), mouse colon cancer cells (CT26; RRID:CVCL_7254) and pancreatic cancer cells (SUIT-2; RRID:CVCL_3172) were obtained from American Type Culture Collection (ATCC) and maintained in RPMI-1640 or DMEM media supplemented with nonessential amino acids (1:100), sodium pyruvate (1 mM), penicillin (20 Units), streptomycin (20 μg/mL) and 10% fetal bovine serum (FBS) at 37°C, 5% CO2.
The DLD1 and HCT8 cell lines are derived from the same patient. Normal, noncancerous colon cell lines (CCD-18Co; RRID:CVCL_2379 and CCD 411 CoN; RRID:CVCL_2871) were obtained from ATCC, maintained in DMEM media supplemented with nonessential amino acids (1:100), sodium pyruvate (1 mM), penicillin (20 Units), streptomycin (20 μg/mL) and 10% FBS at 37°C, 5% CO2. All experiments were performed with mycoplasma-free cells. All human cell lines have been authenticated using short tandem repeat (STR) profiling within the last 3 years.

2.2 Isolation of human and murine bone marrow-derived monocytes

For isolation of human monocytes, venous blood was collected from donors using the cell preparation tubes (CPT) and the peripheral blood mononuclear cells (PBMCs) were obtained according to the manufacturer’s protocol (Becton Dickinson, NJ). For monocyte isolation, 1 x 10^8 to 2 x 10^8 PBMCs were plated in T-75 cell culture flasks and allowed to adhere in CO2 incubator at 37°C for 2 hours. Nonadherent cells were removed by washing twice with RPMI-1640. Adherent cells were harvested using a cell scraper, washed in phosphate-buffered saline (PBS) with 1% fetal calf serum (FCS) and resuspended in a complete media. For isolation of mouse monocytes, bone marrow cells were harvested from both femurs and tibias of C57BL/6 mice. Mono­cytes were isolated using an EasySep Mouse Monocytes Isolation Kit (STEMCELL, Vancouver, BC, Canada). Viability of the cells was determined prior to subsequent functional assays.

2.3 MakA purification and labeling

MakA was purified as previously described. For cellular uptake, MakA was labeled with Alexa Fluor 568 using an Alexa Fluor 568 protein labeling kit (Thermo Fisher) according to the manufacturer’s instructions.

2.4 Antibodies

Rabbit polyclonal anti-Bcl2 (Cell Signaling, Cat#2876), rabbit monoclonal anti-Bax (abcam, Cat#ab32503), rabbit polyclonal anti-cytochrome c (Cell Signaling, Cat#4272), rabbit polyclonal anti-MakA (GeneCust), mouse monoclonal anti-capase-3 (Cell Signaling, Cat#9668), rabbit polyclonal anti-actin (Sigma-Aldrich, Cat#A2066), mouse monoclonal anti-β-catenin (BD Biosciences, Cat#610153, Rabbit anti-phospho-β-catenin (Ser33/37/Thr41), (Cell Signaling, Cat#9561), mouse anti-Axin2 (R&D Systems, Cat#MAB6078-SP), mouse monoclonal anti-tubulin (Sigma-Aldrich, Cat#T6074) antibody, rabbit polyclonal anti-histone H3 (Abcam, Cat#ab1791), rabbit polyclonal anti-cleaved caspase-3 (Cell Signaling, Cat#9661T), rabbit monoclonal anti-Cyclin D1 (Thermo Fisher, Cat#MAS-14512), mouse monoclonal anti-VEGF (Santa-Cruz, Cat#SC7269), mouse monoclonal anti-phospho-GSK-3β (Santa-Cruz, Cat#SC373800), mouse anti-GSK-3β (BD Biosciences, Cat#610202, Clone 7/GSK-3b), anti-Ki-67 (BD Biosciences, Cat#550609), goat anti-rabbit HRP-conjugated IgG (AgriSera AB, Cat#AS09602) or rabbit anti-mouse HRP-conjugated IgG (Dako, Cat#PO260).

2.5 Plasmids and transfection

Cav1-GFP was a gift from Dr Ari Helenius (Addgene plasmid # 14433; http://n2t.net/addgene:14433; RRID:Addgene_14433). Transfection of DNA constructs was performed using polyethylenimine (PEI)-mediated transient transfection according to the manufacturer’s directions (Sigma). GFP-LAMP1 (#C10596) was used for transient transfection of HCT8 cells according to the manufacturer’s instructions (Thermo Fisher).

2.6 Live cell microscopy

Cells were grown in a 96-well plate (1 x 10^3/well, Tecan Group Ltd) overnight and treated with MakA at different concentrations for 48 hours at 37°C, 5% CO2. For visualization of nuclear fragmentation and membrane permeability, cells were stained with propidium iodide (PI) and counterstained with nuclear staining, Hoechst 33342. Active caspase 3/7 was visualized with FAM-FLICA Caspase-3/7 according to the manufacturer’s instructions (Immunochemistry). Fluorescence and bright-field images were captured with a fluorescence microscope (Nikon, Eclipse Ti). Images were processed using the NIS-Elements (Nikon) and ImageJ software.

For live cell confocal microscopy, HCT8 or CCD-18Co cells were grown on a coverslip or coverslip bottomed, 8-well chamber slides (3 x 10^4/well, ibidi) and treated with Alexa-568-labeled MakA for 18 or 24 hours at 37°C, 5% CO2. The co-localization of MakA with cell nuclei or mitochondria was detected by incubating the cells with a nuclear marker, Hoechst 33342 (Thermo Fisher) or mitochondrial marker, Mitotracker (Thermo Fisher), according to the manufacturer’s instructions. In addition, accumulated MakA in lysosomes was detected by co-staining the cells with Lysotracker (Thermo Fisher) or LAMP1-GFP (Thermo Fisher) according to the manufacturer’s instructions. Cells were imaged on a Leica SP8 inverted confocal system (Leica Microsystems) equipped with an HC PL APO 63x/1.40 oil immersion lens.

2.7 Cell death assays and flow cytometry

For cell death experiments, cells were grown on a 96-well plate (1 x 10^4/well, Tecan Group Ltd) overnight and treated with MakA at different concentrations for 48 hours at 37°C, 5% CO2. Loss of cell viability with increasing doses of MakA was quantified by [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega) cell viability assay, according to the manufacturer’s instructions. MTS absorbance
Colon carcinoma cells were grown on a 6-well chamber slide (3 × 10^4/well, Millipore) overnight, followed by treatment with unlabeled or Alexa Fluor-labeled MakA for 24 hours, fixed with 4% paraformaldehyde (20 minutes), permeabilized with Triton X (0.25%, 15 minutes). Cells were incubated with anti-Galectin 3 (1:100), anti-Cathepsin L (1:100), anti-β-catenin (1:300), anti-tubulin, anti-MakA, anti-GSK3β, anti-GM130, anti-CLTC or anti-Ki67 (1:300) primary antibodies overnight at 4°C, followed by incubation with corresponding Alexa-488- or Alexa Fluor-labeled secondary antibodies (1:200) for 1 hour at room temperature (RT). Nuclei were counterstained with DAPI (5 minutes, RT) and slides were mounted with Fluoromount aqueous mounting medium (Sigma), examined using an EZC1 Eclipse laser scanning confocal microscope (Nikon), using a 63×/1.40 oil immersion lens. Images were captured and processed using the NIS-Elements (Nikon) or LasX (Leica Microsystems) software. The nuclear intensity of β-catenin was quantified using Image J.

2.9 Scanning electron microscopy

HCT8, colon carcinoma cells were grown on a coverslip overnight, followed by treatment with MakA (250 nM, 24 hours), fixed in 2.5% gluteraldehyde and 4% PFA containing 0.1 M sodium cacodylate for 1 hour at RT followed by washing in 0.1 M sodium cacodylate buffer. The samples were dehydrated in a series of ethanol gradients, critical point dried and coated with 1 nm iridium. Images were acquired by field-emission scanning electron microscopy (FESEM; Carl Zeiss Merlin GmbH) at an accelerating voltage of 5 kV using an in-chamber secondary detector at variable magnifications.

2.10 Western blot analysis

Colon carcinoma cells were grown on a 6-well chamber slide (3 × 10^5/well, Thermo Scientific) overnight, followed by treatment with MakA for 24 or 48 hours. For inhibition experiments, cells were pretreated with the pan-caspase inhibitor zVAD (25 μM, Enzo Life Sciences) or the calpain inhibitor Leupeptin (1 mM, Sigma Aldrich) in 24 or 48 hours. Cells or animal tumor tissues were rinsed with PBS, lysed in ice-cold lysis buffer (20 mM Tris-HCl pH 8, 300 mM KCl, 10% glycerol, 0.25% Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1× complete protease inhibitor (Roche) and phosSTOP (Roche). Cell lysates were then mixed with 4× sample buffer and boiled for 10 minutes prior to separation by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking (PBST, 0.1% and skim milk, 5%, RT, 1 hour), membranes were incubated with primary antibodies at 4°C (5% skim milk, overnight). After washing with PBST (0.1%) membranes were incubated with the appropriate HRP-conjugated secondary antibodies in blocking buffer (5% skim milk, RT, 1 hour). Protein bands were detected with a chemiluminescence reagent (Bio-Rad) using a ChemiDoc imaging system.

2.11 TCF/LEF, TOPFlash reporter assay

HCT8 cells were transfected using polyethylenimine (PEI) with a β-catenin reporter plasmid, M50 Super 8x TOPFlash that contain TCF/LEF binding sites cloned into pTA-Luc (Clontech). The plasmid, M51 Super 8x FOPFlash, was used as a control that contained mutant TCF/LEF binding sites cloned into pGL3 (Promega). M50 Super 8x TOPFlash and M51 Super 8x FOPFlash were gifts from Randall Moon (Addgene). Cells were treated with increasing concentrations of MakA, 24 hours post-transfection. Luciferase activity was determined with the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fisher) or Dual-Luciferase reporter assay system (Promega) using an Infinite M200 microplate reader (Tecan).

2.12 Hemolytic assay

The hemolytic activity of MakA was determined by measuring the release of hemoglobin from lysed red blood cells (RBCs). A 1% (w/v) erythrocyte suspension was prepared from human blood by washing with PBS repeatedly by centrifugation at 1200g for 5 minutes and resuspending cells in PBS, pH 7.4. Erythrocyte suspension was treated with MakA (2 μM) in a 96-well plate and incubated at 37°C for 24 hours. The Triton X-100 (0.1%) was used as the positive control and PBS was the negative control. Images were captured at the end of the treatment.

2.13 Clonogenic and tumor cell migration assay

To assess the effect of MakA on tumor cell proliferation, a clonogenic assay was performed. HCT8 cells were seeded in a 24-well plate (500 cells/well) overnight and treated with increasing concentrations of MakA (125-500 nM) for 10 days, fixed (4% PFA, 30 minutes) and stained with crystal violet. The number of colonies (>50 cells) was assessed in triplicate wells. A dose-dependent decrease in tumor cell
migration was quantified by transwell migration/invasion assay as previously described. The number of migrated cells was counted after staining with Hoechst 33342. Fluorescence and bright-field images were captured with a fluorescence microscope (Nikon, Eclipse Ti). Images were processed using the NIS-Elements (Nikon) and ImageJ software.

**Figure 1**

Legend on next page.
2.14 | Animal model

A colon cancer murine solid tumor model was established using the CT26 cell line and BALB/c mice. Equal numbers of CT26 cells (1 x 10⁶) were inoculated into the flanks of these mice subcutaneously. Animals were randomly distributed into two groups 1 week postinoculation. The animals received treatment intratumorally on Days 9, 12, 15 and 18: MakA (25 μg); or saline (vehicle). On Day 21, the mice were sacrificed and changes in tumor size were observed and photographed. Additionally, tumor volume (width² x length x π/6) was calculated and plotted as a violin plot. Mice were maintained 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 the Institutional Animal Ethics Committee (License No: PRO/136/December 2016-December 2019), NICED, Kolkata, India. Two independent experiments were performed.

2.15 | MakA uptake by tumor tissue

The presence of MakA after intratumor injection in tumor-bearing mice was monitored as follows at the end of the experiment. The mice were sacrificed and tumor tissues were snap-frozen in liquid nitrogen, embedded in O.C.T. compound (VWR), and successive 14-μm sections were collected from the center of the tumor mass. Subsequently, the tissue sections were placed on a positively charged microscope slide (Superfrost/Plus; Thermo Fisher Scientific). Frozen tissue sections were fixed with 4% paraformaldehyde (20 minutes) and permeabilized with Triton X (0.25%, 30 minutes). Tissue sections were incubated with rabbit polyclonal anti-MakA primary antibodies overnight at 4°C, followed by incubation with corresponding Alexa-555-conjugated secondary antibodies (1:200) for 1 hour at room temperature (RT). Nuclei were counterstained with DAPI (10 minutes, RT) and slides were mounted with Fluoromount aqueous mounting medium (Sigma), examined using an EZC1 Eclipse laser scanning confocal microscope (Nikon), using a 63×/1.4 plan Apo λs lens. Images were captured and processed using the NIS-Elements (Nikon) and ImageJ software.

2.16 | Statistical analysis

Results are presented as mean ± SD. Statistical analysis was performed using Student’s t-test, one-way or two-way analysis of variance (ANOVA) or the Mann-Whitney test at different statistical levels of significance: *P ≤ .05 and **P ≤ .01. 

3 | RESULTS

3.1 | MakA induces cell death in cancer cells

MakA has recently been identified as a putative virulence factor in V. cholerae with a role in defense against predators as manifested in studies with C. elegans as model host organism. The MakA protein also promotes lethal infection in zebrafish, yet the molecular and cellular basis for its cytotoxicity and effect on intracellular organelles remains unknown. To investigate its effect on mammalian cells, we examined the cytotoxic effect of MakA against a panel of carcinoma cells of different tissue origin (Figure 1A and Figure S1A). Importantly, MakA reduced the viability of all carcinoma cell lines tested in a concentration-dependent manner. The loss of cell viability was quantified by a decrease in absorbance using the MTS/formazan assay (Figure 1A and Figure S1A). Among all the carcinoma cell lines tested, colon carcinoma cells (Figure 1A and Figure S1A) were the most sensitive and hence used for further studies aimed at understanding the mechanisms involved in MakA-induced cell death.

3.2 | MakA induces apoptosis in colon carcinoma cells

The process of apoptosis, also referred to as programmed cell death, is characterized by highly complex and sophisticated molecular mechanisms, involving an energy-dependent cascade of molecular events. Flow cytometry analysis following Annexin V/PI staining was employed to monitor the occurrence of apoptotic cells. The results
FIGURE 2  Legend on next page.
show that MakA induced dose-dependent apoptosis in HCT8 cells. The number of cells undergoing both early-stage (Annexin V-positive/PI-negative) and late-stage apoptosis (Annexin V-positive/PI-positive) were increased significantly \( (P \leq 0.01) \) in a dose-dependent manner compared to vehicle-treated cells (Figure 1B,C). The presence of apoptotic cell death in response to MakA was confirmed by nuclear fragmentation and chromatin condensation in HCT8 and DLD1 cells as visualized by Hoechst 33342 staining using fluorescence and confocal microscopy (Figure S1B-D). The regulation of apoptosis involves activation of caspases, an increase in the Bax/Bcl2 ratio and release of cytochrome c from the mitochondria to the cytoplasm.\(^{21}\) Therefore, we investigated if MakA affects the expression of these apoptosis modulator proteins. MakA treatment caused induction of the proapoptotic protein Bax and a decrease in expression of the antiapoptotic protein Bcl2 in HCT8 cells. (Figure 1D,E and Figure S1E,F). Additionally, we observed activation of caspase 3 in response to MakA in HCT8 cells (Figure 1D). Activation of caspase 3 and Bax in response to MakA was further confirmed by fluorescence confocal microscopy (Figure 1F,G). To address if caspases play an important role in MakA-induced apoptotic tumor cell death, we pretreated HCT8 colon carcinoma cells with zVAD-fmk, a pan-caspase inhibitor, followed by treatment with MakA. A dose-dependent decrease in tumor cell viability was partially rescued by zVAD-fmk (Figure 1H). Flow cytometry analysis confirmed a significant decrease in the number of early-stage apoptotic cells in the HCT8 cells pretreated with zVAD and then challenged with MakA when compared to cells only treated with MakA (Figure 1I,J). Together, these results suggest that MakA induces potent apoptotic cell death, partly dependent on activation of caspase 3.

### 3.3 MakA binds to the tumor cell surface and induces morphological changes in the tumor cell membrane

To investigate the cellular uptake of MakA in colon carcinoma cells, HCT8 or DLD1 cells were treated with Alexa-568-labeled MakA (250 nM, 24 hours) and visualized by confocal microscopy. MakA bound to the cell surface, with the majority of the protein being internalized and accumulated in the perinuclear region (Figure 2A).

Importantly, the perinuclear accumulation of MakA caused disruption of Golgi structure in CaCo-2 cells although it failed to colocalize with the Golgi marker, GM130 (Figure S2A-B). To address if cell membrane association of MakA leads to membrane perturbation, HCT8 cells were treated with MakA (250 nM, 24 hours) and analyzed by scanning electron microscopy (SEM). SEM images revealed drastic changes in tumor cell membrane morphology in response to MakA (Figure 2B). Furthermore, MakA induced formation of flower- and cone-shaped structures in HCT8 cells. (Figure 2B). Overall, the results suggest that MakA association with the cell membrane plays an important role in tumor cell membrane perturbation.

### 3.4 MakA accumulates in the endolysosomal compartment and induces lysosomal leakage

To investigate the molecular mechanism of MakA-induced tumor cell death, we next investigated its subcellular localization. For this purpose, HCT8 cells were treated with Alexa-568-MakA (250 nM, 24 hours). We then monitored Alexa-568-MakA localization in intracellular compartments by co-staining the cells with Mitotracker (mitochondria marker), Hoechst 33342 (nuclei marker) or lysotracker (lysosomes marker). MakA failed to colocalize with Mitotracker or Hoechst 33342 (Figure 2C,D). However, the majority of the intracellular Alexa-568-MakA showed strong colocalization with lysotracker (Figure S3). LAMP1 and LAMP2 (lysosomal-associated membrane proteins 1 and 2) are distributed among endolysosomal organelles and LAMP1 is routinely used as an endolysosomal marker. To investigate if MakA colocalizes with LAMP1, the HCT8 cells were transfected with Lysosomes-GFP (GFP-LAMP1) and treated with Alexa-568-MakA (250 nM, 24 hours). Live-cell confocal microscopy revealed that the majority of the intracellular Alexa-568-MakA colocalized with GFP-LAMP1 (Figure 2D). We then investigated if MakA caused changes in the distribution of LAMP1 in tumor cells. Indeed, there was a major redistribution of GFP-LAMP1 in cells treated with Alexa-568-MakA compared to vehicle-treated control cells. Unlike vehicle-treated cells, where the majority of GFPLAMP1 was distributed in vesicular structures throughout the cytoplasm, Alexa-568-MakA cells showed accumulation of GFP-LAMP1 in the perinuclear region. In addition, we also observed the formation of tube-like...
structures, positive for GFP-LAMP1 in MakA-treated HCT8 cells (Figure 2D-F). By live cell confocal microscopy, we found that the GFP-LAMP1-positive tubular structures were mobile and had the tendency to move toward the cell membrane (Video S1 and S2). A majority of the GFP-LAMP1-positive tubular structures colocalized with alpha tubulin (Figure 2E). We then investigated if perinuclear accumulation of GFP-LAMP1 may indicate lysosomal dysfunction. First, to examine the effect of MakA on lysosome acidification, HCT8 cells were treated with MakA (250 or 500 nM, 24 hours) or vehicle and stained with acridine orange. MakA caused a concentration-dependent decrease in lysosome acidification as shown by a decrease in the number of acridine orange-positive acidic vesicles (Figure 3A,B). To investigate how lysosomal membrane permeabilization was affected by MakA, cells treated with MakA (500 nM, 24 hours) or vehicle were stained for Galactin3 or cathepsin L. The lysosomal permeability is accompanied by the release of cathepsins including cathepsin L from lysosomes into the cytosol where they may trigger cellular responses, including apoptosis.22 Another sensitive method to quantify the lysosomal permeability is visualization of Galactin3 puncta.23 MakA treatment caused the appearance of punctate Galactin3 and leakage of majority of the cathepsin L protein from the lysosomes into the cytosol (Figure 3C-D). Overall, these results suggest that MakA accumulates in lysosomes and induces lysosomal dysfunction of the tumor cells.

3.5 Role of caspases in MakA-mediated suppression of β-catenin signaling in colon cancer cells

The APC gene product induces β-catenin degradation, which depends on phosphorylation by the serine-threonine kinase glycogen synthase

**FIGURE 3** MakA causes lysosomal permeability of tumor cells. A, Dose-dependent decrease in lysosomal acidification due to MakA as observed by the decrease in acridine orange (AO)-positive vesicles (n = 3). Scale bars = 20 μm. B, Histogram indicating MakA-mediated decrease in AO-positive vesicles for data presented in (A). Data points represent individual cells pooled from the three independent experiments. Significance was determined from biological replicates using a two-tailed, t-test. (***P ≤ .01, n = 50 cells). C,D, Representative immunofluorescence images of vehicle (Tris 20 mM) or MakA (500 nM, 24 hours) treated HCT8 cells. Arrowhead (white) indicates punctate appearance of Galectin 3 and Cathepsin L release to the cytosol in MakA-treated cells. Scale bar = 10 μm
FIGURE 4 Legend on next page.
kinase Ž (GSK3β).24 The β-catenin protein is a key molecule of the canonical Wnt/β-catenin signaling pathway, which has oncogenic capacity leading to development of human colon cancers.6 The most MakA-sensitive cell lines (HCT8 and DLD1) have constitutive β-catenin activity due to loss of function of APC mutations. To determine if MakA can affect β-catenin signaling in human colon cancer cells, we used HCT8 and DLD1 cells carrying an APC gene mutation. MakA caused fragmentation and a decrease in expression of β-catenin in both cell lines (Figure 4A,B). We then investigated whether MakA may modify the subcellular distribution of β-catenin. HCT8 cells were treated with MakA and stained for β-catenin using specific antibodies and visualized by confocal microscopy. Unlike vehicle-treated cells, where β-catenin was bound to the cell surface or accumulated in the tumor cell nuclei, MakA treatment caused perinuclear accumulation of β-catenin and a significant decrease (P ≤ .01) in its nuclear staining (Figure 4C,D). The endocytic pathways, caveolin- and clathrin-mediated endocytosis, play an important role in the activation of Wnt/β-catenin signaling.25,26 To determine if the perinuclear accumulation of β-catenin is due to disruption of caveolin and clathrin, HCT8 cells were treated with MakA and changes in the redistribution of the caveolin and clathrin proteins were monitored by confocal microscopy (Figure S4A,B). In contrast to the vehicle-treated cells, MakA caused redistribution of these proteins to the intracellular aggregate or ring-like structure as monitored using the markers GFP-Cav1 and CLTC, respectively. Together these results suggest that MakA-mediated collapse of the endocytic machinery may play an important role in the inhibition of β-catenin translocation to the tumor cell nuclei. Additionally, HCT8 cells treated with MakA or vehicle were fractionated into cytoplasmic and nuclear fractions, and immunoblot analysis was performed using anti-β-catenin antiserum. Consistent with the confocal microscopy data, a decrease in nuclear accumulation of full-length β-catenin was observed concomitant with an increase in the fragmented form, as determined by immunoblot analysis (Figure 4E,F). Next, we investigated if MakA induced β-catenin fragmentation and/or if the decrease in expression could be regulated by activation of caspases or calpains because these proteases were previously demonstrated to mediate β-catenin fragmentation under certain conditions.27,28 Unlike the calpain inhibitor leupeptin, the pan-caspase inhibitor zVAD blocked the caspase-3-mediated fragmentation of β-catenin (Figure 4G,H and Figure S6A). Taken together, these results suggest that caspases but not calpains play an important role in MakA-induced fragmentation of β-catenin.

### 3.6 | Role of proteasome in MakA-mediated suppression of β-catenin signaling in colon cancer cells

To determine the role of the proteasome in MakA-mediated suppression of β-catenin signaling, HCT8 cells were treated with increasing concentrations of MakA and expression of β-catenin or β-catenin-dependent proteins was monitored by Western blot analysis. Importantly, MakA caused a dose-dependent decrease in β-catenin expression (Figure 5A,B). This decrease in overall β-catenin expression coincided with an increase in phosphorylation of β-catenin at the ser33/37/thr41 residues (Figure S5A). The phosphorylation of GSK3β was decreased in response to MakA, suggesting its activation (Figure S5B). To further understand the inhibitory effects of MakA on β-catenin signaling, we first assessed the effects of MakA treatment on the expression of β-catenin downstream targets. MakA caused a concentration-dependent decrease in the expression of axis inhibition protein, Axin-2 (Figure 5A,B). Axin, a main component of the canonical Wnt/β-catenin signaling pathway, plays a double role in regulation of Wnt/β-catenin signaling, that is, it inhibits the Wnt/β-catenin signal transduction by promoting β-catenin degradation and facilitates recruitment of GSK3 to the plasma membrane to activate Wnt signaling.29 Studies by Lustig et al also reveal that Axin-2 is highly expressed in several human tumors and tumor cell lines due to Wnt/β-catenin signaling.30 In addition, we observed a decrease in the expression of Cyclin D1 and VEGF in both HCT8 and DLD1 cells (Figure 5C). Quantification of Western blots for cyclin D1 and VEGF is shown in Figure S6B. The MakA-mediated decrease in β-catenin and expression of its downstream targets prompted the idea that MakA might
FIGURE 5  Legend on next page.
facilitate proteasomal degradation of β-catenin. To test this possibility, HCT8 cells were treated with MakA and then challenged with the proteasome inhibitor MG132. Based on Western blot analysis of the whole-cell lysate (Figure 5D,E), we found that the MakA-mediated decrease in the level of β-catenin was markedly less efficient in the presence of proteasome inhibitor. To determine if MakA colocalizes with β-catenin, HCT8 cells were treated with MakA and stained for both β-catenin and MakA using specific antibodies. We observed strong colocalization of β-catenin and MakA at the cell membrane and moderate colocalization at the perinuclear region of the tumor cell (Figure 5F,G). Importantly, MakA failed to colocalize with GSK3β, which served as a negative control (Figure S5C). Nuclear DNA-binding T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) proteins and their transcriptional cofactor β-catenin represent the key components of the canonical branch of the Wnt/β-catenin signaling pathway.31 To examine whether the reduced level of nuclear β-catenin and its colocalization with MakA influences β-catenin activity, TCF/LEF reporter activity was quantified using the TOP-flash luciferase assay as previously described.32 MakA caused a dose-dependent decrease in the activity of β-catenin (Figure 5H). Taken together, these results suggest that MakA colocalizes with β-catenin and inhibits its transcriptional activity in colon cancer cells.

3.7 MakA is therapeutically active against colon cancer

β-catenin plays an important role in tumor cell proliferation, a predominant feature of tumor cells.6 The findings that MakA caused β-catenin fragmentation and a decrease in its total expression prompted us to investigate further the effect of MakA on tumor cell proliferation. MakA caused a dose-dependent decrease in tumor cell proliferation, as observed by a decrease in the number of tumor cell colonies (Figure 5I). To assess if this decrease is due to cell death or a change in the cell proliferation-related proteome, we treated colon carcinoma cells with MakA and stained for the cell proliferation marker Ki67. A significant (P < .01) increase in the number of Ki67-negative cells was observed in colon carcinoma (HCT8 and DLD1) cells treated with MakA (Figure 5J and Figure S6C,D). Importantly, MakA also caused reduction in the expression of VEGF (Figure 5C) involved in colon cell migration and invasion.33 Therefore, we aimed to investigate if MakA reduces tumor cell migration. Indeed, we observed a MakA-mediated, dose-dependent decrease in tumor cell migration of HCT8 cells (Figure 5K). We also tested the effect of MakA on primary cells, such as human red blood cells and monocytes and murine monocytes (Figure 5L and Figure S6A). MakA caused no detectable cytolytic effect on these primary cells (Figure S7A). Furthermore, the nontransformed colon cells CDD18-Co and CDD841CoN were less sensitive to MakA challenge at the concentration that effectively killed human colon carcinoma cells, HCT8 and murine colon carcinoma cells, CT26 (Figure 5L). Importantly, the cellular uptake of Alexa-568-MakA was limited to the cytoplasmic vesicular staining in CDD18-Co. In addition, weak colocalization was observed between Alexa-568-MakA and GFP-LAMP1. Unlike HCT8 cells, lysosomal integrity remained unaffected in CDD18-Co as evidenced by no detectable change in the cellular distribution of GFP-LAMP1 (Figure S7B,C) in response to MakA.

**FIGURE 5** MakA colocalizes with β-catenin and inhibits tumor cell proliferation. A. Western blot analysis of HCT8 cells treated with increasing concentrations of MakA (24 hours). B. Histograms indicating quantification of Western blots (A) for β-catenin and Axin2 presented as relative to control (Ct) vehicle. Data are representative of two independent experiments; bar graphs show mean ± SD. Significance was determined from replicates using a one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons test (post-test) against vehicle control (Ct). **P < .05 or ns = not significant. C. Western blot analysis of HCT8 and DLD1 cells treated with MakA (250 nM, 24 hours). D. Western blot analysis of HCT8 cells treated with MakA (250 nM, 24 hours) in the presence or absence of proteasome inhibitor, MG132 (10 μM). E. Histograms indicating quantification of Western blots (D) for β-catenin presented as relative to control (Ct) vehicle. Data are representative of two or three independent experiments; bar graphs show mean ± SD. Significance was determined from replicates using a one-way ANOVA with Tukey’s multiple comparisons test post-test against vehicle control (Ct). **P < .05 or ns = not significant. F,G. Immunofluorescence analysis of HCT8 cells treated with MakA (250 nM, 24 hours). Cellular localization of MakA was detected using anti-MakA antiserum (red), while anti-β-catenin was detected with anti-β-catenin antibodies (green). The white-dotted line was used for calculation of the Pearson coefficient for colocalization (R). Nuclei were stained with DAPI. Scale bar, 10 μM. H. HCT8 cells were transfected with a TCF/LEF TOPFlash reporter plasmid, M50 Super 8x TOPFlash, or FOPFlash control, M51 Super 8x FOPFlash (Addgene) using polyethyleneimine (PEI). Twenty-four hours post-transfection, the cells were treated with increasing concentrations of MakA (24 hours). Luciferase activity was determined with the dual-luciferase reporter assay system. A dose-dependent reduction in luciferase activity was detected 24 hours of MakA treatment. Mean ± SD; one-way ANOVA with Dunnett’s multiple comparison test. (*) P < .05, **P < .01, NS = nonsignificant difference, n = 5. I. MakA inhibited HCT8 cell proliferation in a concentration-dependent manner as quantified by colony forming assay. One-way analysis of variance test was performed to calculate statistical significance (P < .01), n = 3. J. Immunofluorescence analysis of vehicle (Tris 20 mM) or MakA (250 nM, 24 hours) treated HCT8 cells. Inhibition of HCT8 cell proliferation by MakA (250 nM, 24 hours) was quantified by an increase in the number of Ki67-negative-stained cells. Scale bar = 20 μM. K. A dose-dependent decrease in HCT8 cell migration was quantified by transwell migration assay as previously described.30 Significance was determined from biological triplicates using a two-tailed, t-test (**P < .01). L. Histogram indicates loss of HCT8 and CT26 cell viability with increasing concentrations of MakA (48 hours, n = 3). The nontransformed CDD18-Co and CDD 841 (n = 3) CoN cells or primary human (n = 2 individual donors) or mouse monocytes (n = 2 individual mice) were relatively resistant to MakA challenge. Loss of cell viability was measured by decrease in MTS absorbance. Mean ± SD of three independent experiments; two-way ANOVA with Tukey’s multiple comparison test. (*) P < .05, **P < .01, ns = no significant difference.
FIGURE 6  Legend on next page.
Next, we examined if MakA is cytotoxic to tumor cells in an in vivo model. The colon cancer murine solid tumor model was established by injecting murine colon carcinoma (CT26) cells subcutaneously into the flanks of mice (Figure 6A). Mice were randomly distributed into two treatment groups: (a) vehicle or (b) MakA (25 μg/dose), (5 mice per group). Vehicle or MakA was given by intratumor injection on Days 9, 12, 15 and 18. Mice were sacrificed on Day 21 and changes in tumor volume were measured. MakA treatment resulted in a significant decrease (P ≤ 0.01) in tumor volume compared to the vehicle-treated control group (Figure 6B,C). To investigate the appearance of MakA in the tumor mass, samples of tumor tissues from vehicle- or MakA-treated mice were lysed and the presence of MakA was assessed by Western blot analysis using MakA-specific antibodies. Interestingly, MakA was detected in all analyzed samples of tumor tissue from MakA-treated mice (Figure 6D). Further analyses showed that MakA treatment caused reduction in the expression of β-catenin and a β-catenin-dependent tumor cell proliferation marker, cyclin D1 (Figure 6D-F). Importantly, MakA was retained in the tumor mass and could be detected both intracellularly and bound to the cell membrane in the tumor tissue sections analyzed 72 hours after injection (Figure 6G). Overall, these results suggest that MakA inhibits tumor cell proliferation by causing stable changes in the tumor cell proteome. In addition, MakA is therapeutically active against colon cancer.

4 | DISCUSSION

In the present study, MakA was identified as a potential therapeutic agent that reduced the tumor load in a colon cancer murine solid tumor model. Molecular analysis of MakA-treated colon carcinoma cells suggested loss of β-catenin and overall reduction in its nuclear translocation. MakA-mediated reduction in β-catenin expression was partially explained by proteolytic cleavage, through a caspase and proteasome-dependent mechanism. Importantly, caspases were shown to play an important role in MakA-mediated apoptosis of colon carcinoma cells. Taken together, these effects suggest that using MakA to target β-catenin can be a promising new approach to colon cancer treatment, by reducing the Wnt signaling that is important for colon cancer development.

There are several known examples of virulence factors from pathogenic bacterial species, including *Helicobacter pylori*, *Bacteroides fragilis*, *Salmonella enterica* serovar Typhimurium and *Fusobacterium nucleatum*, which modulate the function of the Wnt/β-catenin signaling pathway via different mechanisms. However, in contrast to MakA, those bacterial virulence factors actually promote tumorigenesis. In the case of *F. nucleatum*, a Gram-negative oral pathogen, the FadA adhesion protein from *F. nucleatum* stimulates the growth of colorectal cancer cells by activating Wnt/β-catenin signaling. Recently, it was reported that FadA selectively stimulates the growth of colorectal cancerous cells through activating Annexin A1 (ANXA1), a member of the Annexin family of Ca2+-dependent phospholipid-binding proteins. The AvrA protein, a type III secretion system effector protein from *S. Typhimurium*, also activates β-catenin signals and enhances colonic tumorigenesis. Earlier studies also showed that *H. pylori* infection can promote cancer stem cell characteristics in gastric cancer cells by activating Wnt/β-catenin signaling in a process dependent on the cytotoxic CagA protein. Wnt/β-catenin activation in addition contributes to an anti-inflammatory state in the bacterial replicative niche due to reciprocal regulation of the transcription factors NF-κB and β-catenin. Unlike these bacterial molecules, in our study we found that MakA mediated a clear suppression of β-catenin signaling and tumor cell proliferation. MakA could mediate β-catenin-mediated Wnt signaling both directly and indirectly. The indirect mechanism involves β-catenin and is mediated by MakA-dependent activation of Caspase 3. MakA co-localizes with β-catenin at the cell membrane and at the intracellular perinuclear region of the tumor cell. We propose that this colocalization may directly regulate the exposure of β-catenin to the proteasome, which leads to proteasome-mediated β-catenin degradation.

Increased knowledge of the genetic and epigenetic alterations of Wnt/β-catenin signaling involved in progression of human cancer has led to several approaches targeting Wnt/β-catenin signaling as a means to develop cancer therapies. Use of bacteria as anticancer agents through enhancing human immunity has been described in several studies. Prodigiosin, a natural red pigment produced by *Serratia marcescens*, has exhibited promising anticancer activity through an unknown mechanism. Recently, it was demonstrated that Obatoclax, a synthetic prodigiosin analog, inhibits Wnt/β-catenin...
signaling and reduces cyclin D1 levels, suggesting potential as a therapeu
tic in advanced breast cancers.\textsuperscript{46} Furthermore, prodigiosin pro-
duced by \textit{Serratia marcescens} subsp. \textit{lawsoniana}, also known as
\textit{Chamaecyparis lawsoniana}, was reported to have anticancer activities
when used in human choriocarcinoma (JEG3) and prostate cancer cell lines (PC3) in vitro, as well as in JEG3 and PC3 tumor-bearing nude mice in vivo.\textsuperscript{47} Several pharmacologically active small molecular antag-
onists of the protein–protein interaction between the transcription factor \(\beta\)-catenin and TCF have shown promising results in preclinical settings.\textsuperscript{59} In addition to these approaches, several studies have linked
the protective effect of nonsteroidal anti-inflammatory drugs on colo-
rectal cancer with molecular pathways linked to nuclear inhibition of
\(\beta\)-catenin.\textsuperscript{49,50} Recently, it has been shown that a small molecule,
MSAB, binds to \(\beta\)-catenin and stimulates its proteasomal degradation,
therefore suppressing oncogenic Wnt/\(\beta\)-catenin signaling.\textsuperscript{51} In addi-
tion, nonsteroidal anti-inflammatory drugs such as indomethacin dis-
rupt lysosomal function, which may contribute to reduced \(\beta\)-catenin/ TCF signaling activity in colon carcinoma cells.\textsuperscript{52,53}

Morphological changes, formation of different flower-like and cone
shaped structures, induced by cell membrane bound MakA were
observed by scanning electron microscopy analysis. It appears that
MakA binding to the tumor cell membrane may lead to its oligomeri-
zation followed by increase in cell membrane tension and formation
of different flower-like and cone-shaped structures. This may ulti-
ately lead to permeability of the cell membrane as observed by
increase in number of PI-positive tumor cells when exposed to MakA.
In addition, we observed disruption of intracellular organelles includ-
ing caveolin, clathrin, lysosomes and Golgi. Our findings also highlight
the potential of MakA to be developed as a therapeutic agent against
colon cancer. MakA shows selectivity in targeting cancer cells with
minimal toxicity toward nontransformed cells. Use of full-length MakA
protein as an anticancer therapeutic for targeting malignant cancers
may be challenging and should prompt efforts to identify therapeuti-
cally active peptides of MakA in future studies. Therapeutic peptides
provide promising and novel approaches for treatment of cancer.\textsuperscript{54} A
peptide less than 50 amino acids can be easily synthesized and may
have low toxicity compared to the full-length protein. The peptides
can presumably more easily penetrate tissues and cells, and therefore
may be more efficient as long as they retain high target specificity and
selectivity.\textsuperscript{55}

Lysosomes are membrane-enclosed cytoplasmic organelles with
an important role in catabolic processes of the cell. However, recent
findings suggest that lysosomes are not “waste bags” of the cell but play
an important role in a number of cellular processes, including
metabolism, endocytosis, apoptosis, cell proliferation, migration,
autophagy regulation and other modes of cell death.\textsuperscript{56,57} Cancer cells
are strongly dependent on efficient lysosomal function. Accordingly,
malignant transformation causes changes in lysosomal content, cellu-
lar distribution and function. Therefore, changes in the lysosomal
compartment cause cancer cells to become more sensitive to
lysosome-targeting agents, which offers the possibility for specific
eradication of cancerous cells and tumors.\textsuperscript{57} In our study, we
observed the lysosomal accumulation and rapid redistribution of the
GFP-LAMP1 in MakA-treated cells. In cells treated with MakA, GFP-
LAMP1 accumulated in the perinuclear region and thereafter partially
translocated to tubulin-positive structures. In addition, we observed
the inhibition of lysosome acidification and release of cathepsin L in
colon carcinoma cells after treatment with MakA. Lysosomal dysfunc-
tion may lead to apoptosis, an evolutionarily conserved and well-
controlled form of cellular suicide. The molecular features of apoptotic
cell death include nuclear fragmentation and condensation, plasma
membrane blebbing and the activation of several effector molecules
including caspase proteases and BCL2-associated X protein (Bax).\textsuperscript{56,58}

In the present study, MakA was shown to (a) induce apoptotic cell
death, (b) accumulate in tumor cell lysosomes causing lysosomal dys-
function, (c) reduce \(\beta\)-catenin levels by activating proteasome- and
caspase-mediated proteolytic fragmentation and disruption of
\(\beta\)-catenin/TCF reporter activity, (d) inhibit tumor cell proliferation and
(e) reduce tumor burden in a colon cancer murine solid tumor model
(Figure 6H). To the best of our knowledge, MakA is the first bacterial
protein reported to lower \(\beta\)-catenin levels. Taken together, our results
suggest that the MakA protein may be considered a novel candidate
for development of new therapeutic strategies against colon cancer.

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT
Data supporting the findings of this study are available from the
corresponding author upon request.

ETHICS STATEMENT
Mouse experiments were performed per the principles and guidelines
of the ethical committee for animal care of National Institute of Chol-
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sent study was approved by Institutional Animal Ethics Committee
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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