Bacterial Ribonuclease MazF-Mediated Apoptosis as Potential Cancer Therapy

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Abstract:

Programmed cell death is a dynamic and critical mechanism of cell suicide in eukaryotes and prokaryotes. MazF is a ribonuclease protein involved in bacterial intracellular programmed death. This protein cleaves mRNAs at ACA sequences, leading to inhibition of protein synthesis and triggering cell death. Given that cancer is heterogenic and has varied susceptibility to treatment, we examined the impact of MazF proteins on the growth and viability of three cancer cell lines: MCF7, HT29, and AGS. These cell lines were transfected with ACA-less mazF mRNAs and evaluated for MazF-mediated cell death. The data illustrated that efficient MazF translation leads to a significant reduction in cell viability and is modulated by structural elements of ACA-less mazF mRNAs. In the presence of MazF, the levels of activated caspase-3 and -7 were significantly elevated in transfected cells, confirming the occurrence of apoptosis. We also quantified mRNA translation on a single-cell basis in MCF7 and AGS cell lines to examine MazF-mediated inhibition of protein synthesis. MazF expression significant decreases the levels of protein translation in the examined cell lines. This is the first report of MazF as a potential anti-cancer agent via induction of apoptosis in MCF7, AGS, and HT-29 cell lines.

Introduction:

Despite recent advances in treatment and diagnostic measures, cancer continues to plague the medical community and remains a major cause of mortality and morbidity worldwide. Traditional cancer therapies also suffer from various limitations (e.g., the lack of specificity) and side effects including both temporary (e.g., diarrhea, nausea, loss of hair, and reduced resistance to infection) and long-term effects (e.g., decreased heart, lung, and kidney health). In an attempt to avoid some of these drawbacks, recent treatment efforts have focused on introducing certain drugs or small toxins with mild toxicities and tolerable immunogenicity.
As small proteins, ribonucleases have been a promising option in cancer therapy at the level of gene expression and function as anti-angiogenic, neurotoxic, antitumor, or immunosuppressive agents against cancer. These proteins occur naturally in both prokaryotic and eukaryotic cells. Bacteria generate diverse groups of RNase proteins with various cytotoxic activities from anti-tumor effects to involvement in bacterial programmed cell death (PCD) and population stability.

Bacteria are routinely exposed to harsh conditions, such as amino-acid starvation, antibiotics, temperature change, and DNA damage, that lead to the regulation of cell growth and cell death. Toxin-antitoxin (TA) is an intracellular death mechanism present in almost all bacteria, comprising of bicistronic operons containing a toxin and an antitoxin. In the TA system, toxins target processes such as DNA replication, mRNA stability, protein synthesis, and ATP synthesis while their corresponding antitoxins prevent toxin activities under normal conditions. MazF is a well-studied toxin in bacteria that halts protein translation but does not affect DNA or RNA synthesis. MazF protein targets specific sites in single-stranded mRNAs, 16S rRNA, and transfer-messenger RNAs (tmRNAs), tRNA-mRNA hybrids that bind to the aminoacyl site of ribosomes. MazF degrades RNAs at either the 5’or the 3’ ends of the first A residue of ACA sequences. Similar to RNase A, the 2-OH group at the cleavage site is crucial for the function of MazF.

Shimazu et al. reported that MazF has the ability to impede protein synthesis and become involved in Bak-dependent apoptosis in mammalian cells. However, the injection of MazF DNA into solid tumors, in spite of tumor shrinkages, did not prevent tumor regression. This failure stems from the occurrence of mutations in the mazF gene, degradation of MazF or the lack of continued expression of MazF in cells. Therefore, DNA delivery may not be an optimal approach to deliver this RNase into cancer cells or tumors.

In vitro transcribed mRNA (IVT mRNA)-based therapy, unlike other nucleic acid-based therapies, offers exceptional advantages in the medical field. Translation in both dividing and non-dividing cells,
functionality in the cytoplasm, lack of immunogenic CpG motifs, expression independent of a strong promoter or terminator, and no risk of genomic integration unlike viral and plasmid DNA have made mRNA therapies valuable treatments against cancer\textsuperscript{14,15}. The success of this therapy depends on many factors, such as the quantity of \textit{in vitro} transcription products, intracellular stability of IVT mRNA and its translational efficiency, which can be influenced by caps, internal ribosome entry sites (IRES), poly-As, Kozak sequences, codon composition and adjacent nucleotides\textsuperscript{16}. In general, mRNA-based therapies, in spite of their advantages, still face skepticism stemming from the instability and short half-life of mRNAs\textsuperscript{16}.

In the present study, we evaluated the RNase activity of MazF in human MCF7 breast-, AGS gastric-, and HT29 colorectal-adenocarcinoma cell lines for the first time. The influences of mRNA structural elements, i.e., cap, IRES, poly-A tail, and Kozak sequence, on effective translation of transfected mazF mRNA in these cell lines were also investigated.

\textbf{Materials and Methods}

\textit{Plasmid Construction:}

\textit{pT7-mazF} vector was generated by replacing GFP with ACA-less \textit{mazF} in pcDNA3.3\textunderscore eGFP (Addgene, Watertown, MA, USA, cat. 26822) containing the T7 promoter. ACA-less \textit{mazF} was amplified from pBApo\textit{-mazF} (kindly provided by Takara Bio Inc. Kusatsu, Japan) by using forward (5'-ATGATAATATGGCCACAACCATGGTAAGCCGATACGT-3') and reverse (5'-GATAATTCTTAATTCATCACCCAATCAGTACGTTAATTTTGGC-3') primers. \textit{pIRES-mazF-1} vector was developed by substituting GFP with ACA-less \textit{mazF} in pcDNA3.1(+) IRES (Addgene, cat. 51406) through overlapping PCR. The ACA-less \textit{mazF} fragment and the corresponding linear plasmids were ligated using the NEB builder HIFI DNA (New England Biolabs, Ipswich, MA, USA, cat. E2621S) assembly kit according to the manufacturer's instructions. Finally, the ligated products were electroporated into \textit{Escherichia coli}
DH10B. Antisense \textit{mazF} mRNA was synthesized against the 5’ end of the \textit{mazF} sequence. All designed primers and antisense fragments were synthesized by Integrated DNA Technologies.

\textbf{mRNA Synthesis:}

\textit{T7-\textit{mazF}} and T7-GFP templates were amplified from pT7-\textit{mazF} and pcDNA3.3_eGFP, respectively, with forward (5′-TTGGACCCTCGTACAGAAGCTTAATACG-3′) and reverse (T120-CTTCCTACTCAGGCTTTATTCAAAGACCA) primers. pcDNA3.1(+) IRES and pIRES-\textit{mazF}-1 were used as the DNA templates for T7-ires-GFP and T7-ires-\textit{mazF} amplicons with forward (5′-AACCCACTGCTTACGACAAATACGAT-3′) and reverse (5′-CCCTCTAGACTCGACTCTAGAAAGTGTCTCATGCCGG-3′) primers. The PCR products were used to generate mRNAs via a MEGAscript T7 kit (Thermo Fisher Scientific, Waltham, MA, USA, cat. AM 1334). \textit{In vitro} transcription (IVT) reactions were assembled with ATP, CTP and GTP from a MEGAscript T7 kit, Pseudo-UTP (Ψ) (Trilink BioTechnologies, San Diego, CA, USA, cat. N1019), Anti Reverse Cap Analog, 3′-O-Me-m7G (5′) ppp(5′)G (Trilink BioTechnologies, cat. N-7003), DNA templates, and T7 Polymerase (from MEGAscript T7 kit) and incubated at 37ºC overnight. To destroy the DNA template and exclude the 5′ triphosphates, the IVT reactions were first treated with Turbo DNase (from MEGAscript T7 kit) and then Antarctic phosphatase (New England Biolabs, cat. M0289S). This was followed by purification of IVT utilizing the RNeasy Mini Kit (Qiagen, Hilden, Germany, cat. 74104). The quality of the RNA sample was analyzed by formaldehyde agarose gel electrophoreses. The mRNAs were stored at -80 ºC.

\textbf{Cell Culture:}

HEK293, Human embryonic kidney 293 cells (ATCC®, Manassas, VA, USA, CR-1573) and MCF7 (ATCC® HTB-22) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose and 2mM L-glutamine (ATCC® 30-2003). AGS (ATCC® CRL-1739) cells were grown in F12K medium (ATCC®...
while HT-29 (ATCC® HTB-38) cells were cultured in McCoys 5A medium (ATCC® 30-2007). All media were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc., Flowery Branch, GA, USA. cat. S11095) and 1% penicillin/streptomycin (Thermo Fisher, cat. 15140122). Cells were maintained in fresh medium every 2 days at 37 °C with 5% CO₂.

Transfection

All cell lines were seeded in concentrations of 5x10⁵ cells per well or 2.5x10⁴ cells per well in 6-well or 96-well plates, respectively. Transfection was carried out using Lipofectamine® MessengerMAX mRNA Transfection Reagent (Thermo Fisher, LMRNA003) following manufacturer’s instructions.

Western Blot Analysis

Western blot assays were conducted using the anti-cleaved PARP monoclonal antibodies (Cell Signaling Technology, Danvers, MA, USA, cat. 9541S) and anti-actin monoclonal antibodies (Cell Signaling Technology, cat. 4970S). The transfected cells, in 6-well plates, were suspended in the lysing buffer (Cell Signaling Technology, cat. 9803S). The proteins present in the lysate were separated on a 10% SDS-PAGE gel and then transferred onto a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA, cat. 1620174) via semi-dry electroblotting machine. The membranes were blocked (TBE, 5% dry milk powder, 0.1% Tween-20) for 1 hour at room temperature and incubated with antibodies overnight at 4 °C. Horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, cat. 7074S) and chemiluminescence substrate (Thermo Fisher, cat. 34580) were used to visualize actin and cleaved PARP.

Cell Viability Assay

The viability of transfected cells was analyzed via trypan blue exclusion. The cells were seeded in 6-well plates and transfected with mazF mRNA the following day. The transfected cells were trypsinized and counted using a hemocytometer.
The toxicity of mazF mRNA on different cell lines was determined using the MTT assay following the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA cat. G4000) instructions.

**Immunofluorescent Microscopy**

The activation of caspase -3 and -7 was visualized under a fluorescence microscope (Motic AE31, Kowloon, Hong Kong) using an Image-iT LIVE Red caspase -3 and -7 Detection Kit (Thermo Fisher, cat. I35102).

**Caspase Activity**

Caspase -3 and -7 activity was measured using Caspase-Glo® 3/7 assay (Promega, cat. G8091) following the manufacturer’s protocol.

**Detection of Nascent Protein Synthesis**

The transfected cells were prepared for analysis using the Click-iT Plus OPP Protein Synthesis Assay Kit (Thermo Fisher, C10458) following the manufacturer’s instructions, with one modification: cells were fixed using 4% paraformaldehyde for 15 minutes instead of the recommended 3.7% formaldehyde. Following staining, samples were imaged using a GE IN Cell Analyzer 2500HS then analyzed using GE InCarta software (version 1.5). For analysis, we created a mask using HCS Nuclear Blue Stain.

**Statistical Analysis**

All experiments were conducted in triplicates. Data are shown as the mean ± standard deviation. The data were analyzed through t-tests. The difference was considered statistically significant when P< 0.05.
Results:

*mazF* mRNA Structure

mRNA therapy is an attractive approach for delivering genetic constructs into cells to treat a disease. mRNA therapy owes its success to its superior transfection and expression efficiency, simplicity and safety profile\(^\text{18}\). These advantages led us to examine whether ACA-less *mazF* mRNA can be effectively transfected and translated in cancer cells. To this end, a T7-*mazF* construct (pT7-*mazF*) was designed. To compare the expression efficacy of cap-dependent and IRES-dependent mRNAs for *in vitro* transcription purposes, we developed a pIRES-*mazF*-1 plasmid. Additionally, several ACA-less *mazF* mRNAs lacking one or more critical structural elements such as a cap, an IRES, poly-As, or Kozak sequences were constructed and their effective translations in mammalian cells were measured.

Our results indicated that capped-*mazF*-polyadenylated or IRES-*mazF*-polyadenylated mRNA could be translated in cells while the omission of any one of these mRNA structural elements, i.e., cap/IRES or poly-As, was enough to block MazF protein expression. However, the lack of Kozak sequences did not have any significant impacts on the expression of IRES-*mazF* mRNAs.

In mammalian cells, synthesized RNAs undergo nucleoside modifications that distinguish them from exogenous RNA\(^\text{14}\). Kormann et al. suggested that the replacement of one of four basic nucleoside triphosphates with a modified nucleotide blocks the interaction of mRNA with Toll-like receptors TLR3, TLR7, TLR8 and retinoic acid-inducible gene I (RIG-I), which leads to the elimination of the immunogenicity of exogenous mRNA\(^\text{19}\). In comparison to other modified nucleotides, the incorporation of pseudouridine into synthesized mRNA has been reported to yield a higher level of mRNA translation and increase the safety profile of such RNA therapies\(^\text{18}\). Therefore, *mazF* transcripts containing pseudouridine were also evaluated for their translational ability in mammalian cells. In order to attribute MazF-mediated cell death to the translation of transfected *mazF* mRNA, antisense *mazF* RNAs complementary to the 5' end of *mazF*
mRNA were co-transfected into AGS cells. Our results indicate that the partially double-stranded *mazF*
mRNA-antisense hybrid was sufficient to block effective translation of *mazF* mRNA resulting in reduced
cell death compared to cells transfected with *mazF* mRNA alone (Figure 1 i-j).

To examine the expression of *mazF* and GFP mRNAs in cancer cells, four versions of *mazF* and GFP
mRNAs were synthesized, i.e., capped and polyadenylated mRNA (C-mRNA-A), capped and
polyadenylated mRNA carrying pseudouridine (C-mRNA-psU-A), IRES and polyadenylated *mazF* mRNA
(IRES-mRNA-A), and IRES and polyadenylated mRNA with pseudouridine (IRES-mRNA-psU-A). The mRNAs
were then dephosphorylated with Antarctic phosphatase to prevent mRNAs from being recognized by
RIG-I, which is a sensor for viral RNAs 20. HEK293, MCF7, AGS and HT29 cells were then transfected with
these *mazF* or GFP mRNAs. Monitoring transfected cells by fluorescence microscopy showed that cells
began to express GFP 6 hours after transfection. Cells exclusively translated C-GFP-A or mRNAs possessing
an IRES sequence and a poly-A tail (Figure 1 a-e). However, the fluorescence intensity of the IRES-GFP-A
was lower than that of capped GFP. IRES-GFP-A was expressed only in the AGS cell line (Figure 1d), while
the expression of IRES-GFP-A was not detectable in HT29, MCF7, and HEK293 cell lines.

Following the transfection, our results demonstrated that the expression of *mazF* mRNA in cells
arrested cell proliferation over the course of transfection. Significantly, HEK293 and AGS cells (Figure 1 f-
j) underwent extreme morphological changes 19 hours post transfection, while MCF7 and HT29 cells had
a severely reduced number of attached cells at 24 and 36 hours post transfection, respectively, suggesting
that MazF proteins were able to induce death in cancer cells.

The viability of MCF7, AGS, and HT29 cells expressing *mazF* mRNAs was determined using trypan
blue exclusion. As illustrated in Figure 2, when the cells were transfected with one of the four types of
mRNA mentioned above, the viability of cells was drastically reduced. An MTT-based cell viability assay
also indicated that the expression of MazF protein hampered cell metabolic activity and increased the
number of dead cells (data not shown). These data confirm that the expression of the MazF protein induces death in the examined cancer cell lines.

**MazF-Mediated Apoptosis in Cancer Cells**

During stationary phase, MazF protein mediates cell death in *E. coli* by cleaving cellular mRNAs at ACA sequences. According to the findings of Shimazu et al., MazF protein has a similar ribonuclease function in human HEK293 cell lines, i.e., the induction of apoptosis. To validate the induction of apoptosis in different cancer cells, we examined whether activated Caspase-3 and -7 are present in transfected cells. The Caspase-3 and -7 detection assay contains aspartic acid-glutamic acid-valine-aspartic acid (DEVD), a caspase substrate that has a high affinity for activated caspases. Our results showed that the expression of MazF caused apoptosis reactions in MCF7, AGS, and HT29 cells (Figure 3). MCF7, AGS, and HT29 cells exhibited MazF-mediated apoptosis 12, 18, and 36 hours post transfection, respectively. Additionally, the activity of caspase was quantified in these cell lines (Figure 3). Further, we investigated whether cleaved PARP, a marker of cells undergoing apoptosis, was present in the cell lines translating mazF mRNA. As shown in Figure 4, the cleaved PARP is detectable in AGS cells transfected with mazF mRNA. Altogether, these results demonstrate that MazF could induce apoptosis in cancer cells.

**MazF-Mediated Inhibition of Protein Synthesis in Cancer Cells**

In *E. coli*, the activation of MazF protein leads to the inhibition of protein synthesis through digestion of cellular mRNA. Hence, we evaluated the inhibition of protein synthesis by MazF in MCF7, AGS, and HT29 cell lines by quantifying mRNA translation on a single-cell basis. In this assay, an O-propargyl-puromycin (OPP) is incorporated into *de novo* peptide chains and the generated chain is labeled by photostable Alexa Fluor® dye. Fluorescence signals are then used to measure nascent protein
synthesis. As illustrated in Figure 5, MazF proteins significantly blocked synthesis of new proteins in the cells. Thus, our results confirm the translational interference role of MazF in the examined cell lines.

In *E. coli*, MazF cleaves mRNA at ACA sequences. GFP mRNA has 21 ACA sequences in the coding region. The co-transfection of *C-mazF-A* mRNAs with pcDNA3.3-egfp resulted in the decrease of GFP signal in transfected cells (Figure 6). This observation indirectly confirms that MazF is able to degrade mRNA and eventually block protein synthesis in transfected cells.

Discussion

Even with recent advancements in the medical field, cancer remains a significant concern. Introduction of novel therapies can bestow hope to many patients worldwide. Ribonuclease provide promising new treatment options that could be effective at transcription and translation levels. However, it quickly became evident that certain ribonucleases, due to their instability, the lack of cytotoxic or cytostatic activity, and the presence of ribonuclease inhibitors (RI) within cells making them poor candidates for cancer therapy.

MazF is a small and stable ribonuclease in bacterial cells that cleaves mRNAs, tRNA, and rRNA at ACA sequences. Under normal conditions, MazF binds a small, labile protein, i.e., MazE, and remains nontoxic in the cells. Under stressful conditions, MazE is degraded allowing MazF to arrest cell proliferation and induce death within bacterial cells. The absence of a MazF inhibitor in mammalian cells encouraged us to investigate its potential application in cancer therapy. Shimazu et al. demonstrated that MazF is able to halt the growth of and induce apoptosis in T-REx-293 cells, a human kidney cell line.

Given that cancer is a heterogeneous group of diseases with various sensitivities to different treatments, here we examined the impact of MazF on the growth and viability of three additional cancer cell lines, namely, MCF7, HT29 and AGS.
In this study, we demonstrated that the delivery of mazF mRNA into cancer cells enhances translation of the encoded protein. mRNA is an attractive candidate in non-viral gene therapy due to its ability to be active in the cytosol without entering the nucleus. However, mRNA lability has always overshadowed the advantages of mRNA-based therapy. In order to address this concern, we studied the necessity of the main structural elements of mRNA to boost the translation efficiency of MazF in in vitro cell line conditions. Our results demonstrated that the presence of Cap/IRES and poly-A elements enables the translation of the encoded protein within cells. We also synthesized and delivered GFP and mazF mRNA into HEK293, MCF, AGS and HT29 cell lines. mazF mRNA deficient for caps or poly-As resulted in no induction of death in transfected cells. These results may suggest synergy between cap structure and poly-A tail in translation efficiency, a finding reported by various researchers. This cooperation stems from mRNA circulation, where the cap-eIF4E-eIF4G-PABP-poly-A complex hampers the function of exonucleolytic nucleases in order to degrade mRNA.

The 5’ ends of eukaryotic mRNAs contain a methylated m7GpppN cap structure that is crucial for mRNA splicing, stabilization, transport, and translation. In IVT reactions, the cap structure is linked to the synthesized mRNA through an enzymatic reaction. However, this capping process caps either the 5’ side or both ends of the mRNA, rendering half of the synthesized mRNAs nonfunctional. Recently, several cap analogues have been designed to optimize the capping process in in vitro conditions. One example of these cap analogs is the anti-reverse-cap analogue (ARCA), which contains a methylated 3’-OH group that enforces ARCA to localize in the proper orientation.

Although the cap is a fundamental element in mRNA stability and translation, some viral and cellular mRNAs possess internal ribosome entry sites (IRES) that carry out their translation in a cap-independent manner. IRES sequences directly recruit ribosomes and start translation at either a non-AUG codon or an overlapping +1 frame gene. The discovery of IRES has led to an exciting path in cancer therapy. This element enables researchers to design bicistronic, tricistronic or tetracistronic operons.
encoding two or more genes under the control of a single promoter. Here, we recruited the IRES sequence from encephalomyocarditis virus (EMCV) RNA to synthesize uncapped mRNAs. We then compared the expression of capped and cap-independent mRNAs in cell-culture systems. As mentioned previously, both IRES-mazF-A and C-mazF-A were expressed in the examined cell lines in a time-dependent manner. However, each cell line differs in the expression of capped and IRES-dependent mRNAs and AGS cells are more likely to express IRES-GFP-A than other cell lines. This indicates the probability that the type of mRNA and cell line have significant influences on the expression of capped and cap-independent mRNAs. Our results of transfected IVT mRNA in cells concur with that of other research groups. Avci-Adali et al. reported that, in spite of the high transfection efficacy, human endothelial cells and BJ human foreskin fibroblasts express fewer copies of capped GFP proteins in comparison to HEK293.

Another problem that overshadows the advantages of the IVT mRNA technique is mRNA immunogenicity. Several investigations have indicated that certain innate immune receptors such as TLR3, TLR7, TLR8 and RIG-I may be stimulated by exogenous RNA. The incorporation of modified nucleosides, e.g., pseudouridine or 2-thiouridine ($s^2$U) in IVT mRNA, and the elimination of 5′ triphosphates from synthesized mRNA can suppress the activation of TLRs and RIG-I. Previous research showed that inclusion of pseudouridine in mRNA structures significantly enhances the translation efficiency of the encoded proteins when compared to mRNAs possessing unmodified uridines. This difference stems from the diminished activity of protein kinase ribonuclease (PKR) in the presence of mRNAs that contain modified nucleotides. It seems that pseudouridine has the ability to enhance stability and boost the expression of IVT mRNAs in comparison to other modified nucleotides. Our results, however, varied slightly from those of previous studies. Our data showed that the presence of modified nucleotides in the structure of IRES-GFP did not lead to an increased expression of encoded proteins in cells. Our data also highlighted the role of each cell line on the expression of IVT mRNAs containing either modified or non-modified nucleotides.
As a part of their defense responses, eukaryotic and prokaryotic cells have the capability to degrade mRNA and subsequently suppress protein synthesis. For example, when bacteria are exposed to harsh conditions (e.g., extreme amino acid starvation or antibiotic-blocked transcription or translation), they use MazF proteins to cleave mRNAs and therefore block protein synthesis. In mammalian cells, some of the host antiviral responses that lead to inhibition of protein synthesis and eventually to induction of apoptosis in virus-infected cells including the interferon response, RNaseL-mediated degradation of 28S and 18S ribosomal RNAs, and the activation of protein kinase-R. However, the dependency of cancer on protein synthesis can be targeted by inhibition of nascent protein synthesis ribonuclease-based therapy. We analyzed the impact of MazF proteins on the inhibition of protein synthesis, which resulted in the induction of apoptosis in MCF7, AGS and HT29 cell lines. In this study, nascent protein synthesis was determined through measuring the incorporation of O-propargyl-puromycin (OPP) into newly synthesized proteins. Our observations confirmed that the overexpression of MazF leads to a drastic drop in the levels of protein translation in the examined cell lines. These results are consistent with the study conducted by Shimazu et al. which demonstrated that the level of protein synthesis was reduced in T-REx-293 cells 24 hours post transfection with MazF mRNA. In addition, our results suggested that the reduction of protein synthesis mediated by MazF was cell line dependent.

In mammals, the inhibition of protein synthesis leads to apoptosis and cell death, although the exact mechanisms remain unknown. Here, our data illustrated that MazF not only stopped the proliferation of cells but also significantly reduced the number of living cells 18 to 24 hours post transfection in tested cell lines. In order to confirm the occurrence of apoptosis, the activation of caspase-3 and -7 and the presence of caspase 3’s substrate was evaluated in transfected cells. Our observations suggested that in the presence of MazF, the level of caspase-3 and -7 significantly increased and apoptosis occurred in the cells. These data confirm the antiproliferative action and apoptotic ability of MazF in cancer cells. Interestingly, MazF was able to induce apoptosis in MCF7 cells that are supposedly apoptosis-
resistant. These cells are deficient for caspase-3 and also are resistant to ionizing radiation (IR)-induced apoptosis \(^{39}\). Essman et al. demonstrated that the exposure of MC7 to methylxanthine caffeine and the staurosporine analogue UCN-01 did not lead to the activation of the initiator caspase-9 and the cells remained resistant to apoptosis \(^{39}\). Therefore, according to our results, MazF may serve as a suitable therapeutic candidate against MCF7. As we have demonstrated, MazF successfully induced death in AGS and HT29 cell lines. These cell lines have been reported to possess long non-coding RNA GACAT1 (IncGACAT1) and long non-coding RNA (IncRNA) Gas5, respectively, that stimulate cell proliferation and invasion \(^{40,41}\). These non-coding RNAs contain several ACA sites in their sequences that potentially make the non-coding RNAs more vulnerable to the action of MazF.

In conclusion, we described the use of MazF, a bacterial ribonuclease, for induction of apoptosis in three different cell lines. We also investigated the influence of IRES, a cap structure, and the incorporation of modified nucleotides on the expression of the encoded protein. Our results showed that although cell lines have a varying preference for translation of the delivered mRNAs, the delivered \(mazF\) mRNA was expressed and the MazF protein was able to inhibit \(de\ novo\) protein translation. Thus, it seems MazF has great potential to serve as a therapeutic agent for cancer treatment.

**Additional Information**

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**Competing Interests Statement**

The authors declare that they have no competing financial and non-financial interests.
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Author Contributions Statement

Saffarian Abbas Zadeh and Tzeng defined the scope of work, designed the experiments, analyzed the results and prepared the manuscript; Saffarian Abbas Zadeh constructed all the vectors used in this study; Saffarian Abbas Zadeh, MacPherson, Huang, and Ding carried out the designed bioassays; Powell and Bruce measured and analyzed the impact of MazF on the levels of nascent protein synthesis reported in Figure 5. All authors read and approved the final manuscript.

Figure Legends

Figure 1: The translation of GFP and mazF mRNAs in cancer cells 24 hours post transfection. a) and c), cells were transfected with C-GFP-A mRNAs; d) AGS was transfected with IRES-GFP-A; b), e), f) are non-transfected AGS cells; g) AGS cells were transfected by GFP mRNA; h), AGS cells were transfected by mazF mRNA; i) AGS cells were co-transfected by mazF mRNA and antisense mRNAs; j) AGS cells were transfected by antisense mRNA and served as the control. a-e are fluorescence images and f-j are bright-field images.

Figure 2: The expression of MazF results in a significant reduction in cell viability in transfected a) AGS and b) MCF7 cell lines. (n=3)

Figure 3: MazF-mediated apoptosis in transfected MCF7 (a1-3) and HT29 (b1-3) cell lines; a1 and b1: The expression of MazF leads to caspase activity in transfected cells; a2-3, b2-3: Fluorescent cells under the fluorescence microscope indicating the activation of caspase in transfected cells. (n=3)

Figure 4: The presence of cleaved-PARP in lysates of transformed AGS cells 18 hours post induction of MazF visualized via Western blot. β-actin was used as the loading control and blotted separately. Images for β-actin and cleaved-PARP gels were cropped and aligned. The original exposures and contrast levels were maintained. The original Western blot raw data and construction of the figure are in the Supplementary information file. Ladder: Precision Plus protein™ (BioRAD, cat. 1610373).
Figure 5: Impaired protein translation in a1) MCF7 and b1) AGS mediated by MazF. a2, b2: The reduction of fluorescence (red signals) indicated the inhibition of nascent protein synthesis in transfected cells. The nuclease was stained with NuclearMask Blue stain, (n=3).

Figure 6: Impact on pcDNA3.3-egfp expression in C-mazF-A mRNA transfected HEK 293 cells. a) Abundant GFP expression in HEK293; b) Reduced GFP expression in C-mazF-A mRNA transfected HEK 293 cells.