An optimized MNK1b aptamer, apMNKQ2, and its potential use as a therapeutic agent in breast cancer

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INTRODUCTION

Breast cancer is the most commonly diagnosed and leading cause of cancer death among women worldwide. Mitogen-activated protein kinase-interacting kinases (MNKs) promote the expression of several oncogenic proteins and are overexpressed in several types of cancer. In human cells, there are four isoforms of MNKs. The truncated isoform MNK1b, first described in our laboratory, is a truncated isoform lacking the MAPK-binding motif present in the C-terminal region of MNK1a that arises from an alternatively spliced transcript of the MNK1 gene. MNK1b has a higher basal activity than MNK1a and its activity does not correlate with the phosphorylation of the activation loop residues, being independent of the upstream kinases (ERK1/2 and p38 MAPK). Much evidence supports the role of MNKs in oncogenic transformation, metastasis, and tumor progression. MNKs, via eIF4E phosphorylation, promote the expression of several oncogenic proteins, such as vascular endothelial growth factor and fibroblast growth factor, which induce angiogenesis; BCL2, MCL1, and XIAP, with an important role in cell mortality; metalloproteases (MMP) involved in invasion; and cMyc, cyclin D1, ornithine decarboxylase, and HDM2, which regulate cell growth. Both MNKs and phosphorylated eIF4E are overexpressed in different types of cancer such as glioblastoma, lymphoma,
breast, prostate, and ovarian cancers, as well as hepatocellular carcinoma, and its high levels of expression are associated with a worse prognosis.20,14–20 Furthermore, the fact that MNKs are not essential21 seems to be a good opportunity for pharmacological inhibitors directed against MNK to provide an effective anti-tumor strategy that is not harmful to non-tumor cells. Different MNK inhibitors have been developed in the last years,6,8,2,20 and two of them (BAY 1143269 from Bayer and eFT508 from eFFECTOR) are currently in clinical phase testing. However, many of these inhibitors are not very specific, producing multiple unwanted side effects and making the establishment of their mechanism of action difficult.

Aptamers are structured polynucleotide sequences (single-stranded DNA [ssDNA] and RNA) isolated from randomized oligonucleotide libraries by the systematic evolution of ligands by exponential enrichment technology, which selectively binds to target molecules with high affinity and specificity22,23 and that have dissociation constants in the nanomolar range. Furthermore, aptamers have several advantages over antibodies because of the nature of nucleic acids, such as significantly smaller, can be isolated rapidly and maintained an aptamer, named apMNK2F, that is smaller (only 29 nt), and has dissociation constants in the G-quadruplex structures predicted by mFold for the establishment of their mechanism of action difficult.

In our laboratory, we selected and characterized aptamers against MNK1b. Among them, the apMNK2F aptamer was able to inhibit proliferation, migration, and colony formation in MDA-MB-231 breast cancer cells.23 In this way, to enhance the therapeutic capacity of the aptamer and improve its stability and the possibility of reaching its target more easily, we have optimized the apMNK2F aptamer by eliminating dispensable regions of its sequence. In this way, we have obtained an aptamer, named apMNKQ2, that is smaller (only 29 nt), has greater stability and specificity, and maintains the activity of the parental aptamer. Thus, apMNKQ2 is able to inhibit molecular processes characteristic of tumor cells, both in terms of tumorigenic (proliferation, inhibition of apoptosis, and colony formation) and metastatic activity (migration and invasion) in breast tumor cells. Finally, in murine models of breast cancer, apMNKQ2 has demonstrated significant efficacy decreasing tumor growth and the number of metastases. In conclusion, apMNKQ2 could be used as an anti-tumor drug in the future.

RESULTS

apMNKQ2, a new optimized version of aptamer apMNK2F

The apMNK2F aptamer previously described in our laboratory31 has been modified to decrease its size and increase its stability and/or its resistance to nucleases. The sequence predicted by mFold for apMNK2F suggested that the first 18 nucleotides at the 5′ end and the last 14 nucleotides at the 3′ end were not involved in the secondary structure of the aptamer or in the G-quadruplex structures predicted with the QGRS Mapper program.31 Based on this, we designed a modified apMNK2F, named apMNK2FT, deleting those nucleotides not involved in the structure and adding five thymines in the 5′ end to separate the labeling molecule from the G-quadruplex structure and avoid any potential interference (Figure 1A). G-quadruplex formation in the new aptamer, which contains 48 nucleotides, was experimentally confirmed by 1H nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy (Figure 1B). The signals in the 11–12 parts per million region correspond with guanine imino protons involved in G-tetrad and are a clear indication of G-quadruplex formation. Moreover, the positive band around 260 nm and the negative one at 240 nm in the CD spectra are characteristic of a parallel G-quadruplex topology. These results are in agreement with the structural characteristics predicted by mFold (Figure 1A), which suggests a theoretical free energy slightly lower than the original aptamer (−2.64 vs. −3.08 for apMNK2F). Maintenance of the G-quadruplex structure is probably the cause for the new aptamer to keep its ability to bind MNK1 isoforms with high affinity (Figure 1C). However, apMNK2FT is much less effective than apMNK2F inhibiting tumor cell characteristics, such as proliferation or migration, but not colony formation, in the breast cancer cell line MDA-MB-231 (Figures S1A–S1D).

A detailed analysis using QGRS Mapper software predicted four different sequences from apMNK2FT, based on their capacity to form G-quadruplexes (Figure 2A). These sequences, named apMNKQ1, apMNKQ2, apMNKQ3, and apMNKQ4, have 18, 29, 13, and 22 nucleotides, respectively. Although with different stability, the four sequences exhibit a tendency to form G-quadruplexes as shown by NMR spectroscopy (Figure S2). The effect of the four sequences on the proliferation of three breast cancer cell lines, including two triple-negative cell lines (MDA-MB-231 and MDA-MB-468) and the Luminal A cell line MCF7, was analyzed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) activity assay (Figure 2B). For this, cells were transfected with the different aptamers at 250 nM and MTT activity was measured after 48 h. The results showed that the only aptamer able to inhibit MTT activity at this concentration in the three breast cancer cell lines studied was apMNKQ2. In addition, apMNKQ2 was also the most efficient to inhibit proliferation in other types of cancer cell lines, such as lung cancer (results not shown). For this reason, we have selected the apMNKQ2 aptamer for further characterization and anti-tumoral activity study.

First, we analyzed the stability and resistance to degradation by nucleases. For this, apMNKQ2FT and apMNKQ2 were incubated with several dilutions of DNase I endonuclease (Figure S3A) for 5 min. No degradation was observed at the different concentrations tested (stability 100%), in contrast with apMNK2FT, which was degraded by more than 90% (Figure S3B) and the parental aptamer apMNK2F that was completely degraded when incubated with 1 unit of DNase I.31 In addition, apMNKQ2 was incubated with human plasma for 6, 16, 48, and 72 h (Figure S3C), and no degradation was observed at the different times tested supporting the very high stability of this aptamer.

In conclusion, apMNKQ2 could be used as an anti-tumor drug in the future.
Altogether, these results reinforce the potential clinical use of apMNKQ2 in the treatment of cancer.

**apMNKQ2 has more affinity for MNK1b than for MNK1a**

To study the affinity of apMNKQ2 for both MNK1 isoforms, we made enzyme-linked oligonucleotide assay (ELONA) assays in which GST-MNK1a or GST-MNK1b proteins were incubated with increasing concentrations of biotin-labeled aptamers. A random sequence of 29 nucleotides (GCGGTCGACTTAAATGTCCATCTCAAACT), named p29, was used as a negative control (Figure 3A). The data obtained were analyzed using nonlinear regression showing that respond to a hyperbola whose equation is $y = (x \times B_{max})/(x + KD)$ where $B_{max}$ is the maximal binding and the dissociation constant (KD) is the concentration of ligand required to reach half-maximal binding. apMNKQ2 was capable of detecting MNK1a and MNK1b proteins in a concentration-dependent manner with KD of 66.13 ± 20.18 and 15.47 ± 2.36 nM, respectively, indicating that the aptamer has much more affinity for MNK1b than MNK1a (more of 4-fold).

In contrast, we have carried out apta-western blot assays using apMNKQ2 conjugated with biotin to confirm the affinity of apMNKQ2 for the two MNK1 isoforms. As shown in Figure 3B, apMNKQ2 is able to recognize the two isoforms of the protein in a similar extent under denaturing conditions; nevertheless, the apMNKQ2 aptamer is able to discriminate significantly between MNK1b and MNK1a under native conditions, confirming the previous result obtained by ELONA assays. These results indicate that the domain that recognizes the aptamer is less accessible in the MNK1a isoform.

In addition, we carried out ELONA assays using recombinant MNK2 and the two MNK-upstream MAPKs, p38MAPK and ERK1. As shown in Figure 3C, apMNKQ2 did not have an affinity for these kinases, indicating that is specific for MNK1 isoforms.

**apMNKQ2 inhibits cell proliferation and colony formation in breast cancer cells**

We first investigated the anti-proliferative effect of the aptamer apMNKQ2 in the three breast cancer cell lines. For this, cells were transfected with the apMNKQ2 or the unspecific p29 aptamer at different concentrations; cell viability was measured after 48 h by MTT reduction. The nonspecific p29 aptamer did not affect cell viability or only slightly (<20%). The apMNKQ2 aptamer did decrease cell viability in the three cell lines having the greatest
potency against MDA-MB-468 cells and less efficiency against MDA-MB-231 cells. The half maximal inhibitory concentration (IC50) values were 42, 144, and 19 nM for MCF7, MDA-MB-231, and MDA-MB-468, respectively (Figure 4A). This effect is not due to necrotic cell death, measured by lactate dehydrogenase (LDH) release assays (Figure S4A). We have also analyzed the number of cells alive by Trypan blue exclusion (Figure S4B) and the cell cycle distribution by propidium iodide staining and flow cytometry (Figure S4C) at concentrations around three times the IC50 values (150, 400, and 50 nM for MCF7, MDA-MB-231, and MDA-MB-468, respectively). We observed a significant decrease in the number of alive cells in MCF7 and MDA-MB-468 and an arrest in the phase S of the cycle in MDA-MB-468 cells. However, no significant changes were observed in alive cells and cell cycle distribution in MDA-MB-231 cells (Figures S4B and S4C).

To further determine whether the decrease in cell viability was due to apoptosis induction, cells were transfected with apMNKQ2 or p29 at the concentration indicated above. After 24 h, we analyzed the cleavage of two endogenous substrates of caspase 3, poly (ADP-ribose) polymerase (PARP), and α-spectrin by western blot. apMNKQ2 produced the increase of PARP (Figure 4B) and α-spectrin fragments (Figure S4D) cleaved by caspase 3 in MCF7 and MDA-MB-468 cells, but not in MDA-MB-231 cells.

Next, we analyzed MCL1 and XIAP protein levels, two anti-apoptotic proteins regulated by MNK1.10,17,32 It is interesting to highlight that apMNKQ2 significantly decreased the levels of MCL1 and XIAP in MCF7 and MDA-MB-231 cells, but not in MDA-MB-468 cells (Figures 4B and 4C).

Finally, we analyzed the effect of apMNKQ2 on the clonogenic capacity of the three breast cancer cell lines; the results showed that the aptamer decreased the number of colonies in all of them, being statistically significant for MCF7 and MDA-MB-231 (Figure 4D).

apMNKQ2 inhibits migration, invasion, and epithelial-mesenchymal transition in breast cancer cell lines

To evaluate the anti-metastatic effect of apMNKQ2, we performed transwell migration and invasion assays. As shown in Figure 5A, apMNKQ2 was able to inhibit migration in the three cancer cell lines, albeit statistically significantly in MCF7 and MDA-MB-231. In addition, the treatment with the aptamer produced a substantial decrease in invasion in MDA-MB-231 and MDA-MB-468 cells (Figure 5B). In invasion assays, MCF7 cells were not included because in preliminary experiments carried out in our laboratory under control conditions no invasion was observed after 48 h in this cell line (data not shown).

Epithelial-mesenchymal transition (EMT) is defined by the loss of epithelial characteristics and the acquisition of a mesenchymal phenotype. We have analyzed the levels of messenger RNAs (mRNAs) of occludin as epithelial marker and fibroblast-specific protein 1 (FSP1) as mesenchymal marker by quantitative polymerase chain reaction (qPCR). All three cell lines expressed occludin, with the expression being highest in epithelial-type MCF7 cells, whereas FSP1 expression was significantly higher in MDA-MB-231 cells (Figure S5A). The results shown in Figure 5C indicated that apMNKQ2 induced a significant increase in occludin expression in MDA-MB-231 cells. Accordingly, we observed a 1.8-fold increase in occludin protein levels in these cells (Figure S5B). FSP1 expression decreased in MCF7, but did not change in MDA-MB-231 and MDA-MB-468 cells (Figure 5D). These results suggest that apMNKQ2 induces EMT inhibition and, consequently, reduced migration and invasion.
Figure 3. apMNKQ2 is specific for MNK1 and have higher affinity for MNK1b than for MNK1a

(A) Analysis of the apMNKQ2 or p29 binding to MNK1b or apMNKQ2 binding to MNK1a was performed by ELONA. GST-MNK1a or GST-MNK1b was plated at 0.2 µg/well (3 pmol/well) and then incubated with apMNKQ2-Bio or p29-Bio at 0–500 nM concentrations. The points represent the mean ± standard error of the mean of three independent experiments. (B) Recombinant GST-MNK1a and GST-MNK1b were resolved in SDS-PAGE or native-PAGE and transferred to PVDF membranes. Apta-western blotting was performed with 50 nM apMNKQ2-Bio and streptavidin-horseradish peroxidase (HRP) and developed by ECL. The membranes were reprobed with anti-GST antibodies. The graph at the bottom shows the quantification of four independent experiments. p < 0.05 relative to MNK1a. (C) The specificity of apMNKQ2 was analyzed by ELONA. Recombinant proteins His-MNK2, p38x, ERK1 and His-MNK1b (6 pmole/well) were incubated with 40 nM apMNKQ2-Bio or streptavidin-HRP alone (Blank). Bars represent the mean ± standard error of the mean of three independent experiments.

apMNKQ2 decreases the levels of MNK1 isoforms and eIF4EP in the MDA-MB-231 breast cancer cell line

In our previous work, using the parental aptamer against MNK1, apMNK2F, we could not find an effect of the aptomer on eIF4E phosphorylation.31 Here, we have analyzed whether the optimized aptamer apMNKQ2 induced eIF4E dephosphorylation. In these assays, MDA-MB-231 cells were transfected with apMNKQ2 or with p29 control aptamer at 400 nM. After 24 h of transfection, protein levels were analyzed by western blot using specific antibodies. Our results showed that, at this time, apMNKQ2 induced a significant decrease in eIF4E phosphorylation (Figure 7A). In addition, the levels of both MNK1a and MNK1b isoforms, but not MNK2, were significantly decreased (Figure 7A). This decrease was not due to a decrease in mRNA levels (Figure 7B).

apMNKQ2 reduces tumor growth and metastasis number in vivo

To evaluate the potential therapeutic activity of apMNKQ2, an orthotopic xenograft mouse model of MDA-MB-231 cells was used. The results showed that 1.8 mg/kg apMNKQ2 decreased the relative tumor volume (RTV) significantly from day 7 of treatment reaching a tumor growth inhibition (TGI) of 26% at the end of treatment (18 days) (Figure 8A). Furthermore, there were no difference in weight between treated and untreated mice, suggesting that apMNKQ2 is safe at the dose administered (Figure 8B). The proliferation index, measured by immunohistochemistry with the proliferating cell nuclear antigen (PCNA) antibody, confirmed a significant 33% reduction in tumors from apMNKQ2-treated mice relative to control mice (Figure 8C). In addition, the PARP cleavage increased significantly in the tumors from apMNKQ2-treated mice, indicating apoptosis induction in more advanced stages.34 To study whether apMNKQ2 affects EMT through the inhibition of TGF-β-induced pathways, we used the immortalized human breast cell line MCF10A, which has been well characterized in TGF-β-induced EMT. MCF10A cells stimulated with TGF-β for 3 or 6 days acquire morphological mesenchymal phenotype (Figure 6A). The decrease in the levels of the epithelial marker E-cadherin and the increase in the mesenchymal marker N-cadherin can be observed after 3 days of treatment (Figure 6B). In addition, the levels of three transcription factors that play a key role in EMT; ZEB1, Slug, and Snail are also increased (Figure 6B). Then, MCF10A cells were transfected with apMNKQ2 (100 nM) and treated or not with TGF-β. After 48 h of treatment, transwell migration and invasion assays were performed. As shown in Figures 6A and 6B, apMNKQ2 significantly inhibited TGF-β-induced migration and invasion, while it did not affect untreated MCF10A. In addition, a significant decrease in zymography-analed MMP-2 activity was observed in both untreated and TGF-β-treated MCF10A cells transfected with apMNKQ2 (Figures 6C and S7A). In agreement with these results, apMNKQ2 produced a decrease in N-cadherin levels in both untreated and TGF-β-treated cells, but, surprisingly, a decrease in E-cadherin levels can be observed (Figures 6D and S7A). Finally, the levels of ZEB1, Slug and Snail were also analyzed by western blot. The aptamer apMNKQ2 reduced the ZEB1 and Slug levels, in both untreated and TGF-β-treated cells, whereas Snail levels increased after apMNKQ2 treatment (Figures 6D and S7A). An analysis of mRNA levels indicated that the decrease in ZEB1 and Slug is not mediated by transcription downregulation, whereas, probably, the increase of Snail is produced at the transcription level (Figure S7B). Similarly, apMNKQ2 induced the decrease in ZEB1 and an increase in snail protein levels, but did not affect Slug protein levels in MDA-MB-231 cells, while the levels of the mRNAs increased after apMNKQ2 treatment (Figure S7C).
Figure 4. apMNKQ2 inhibits cell viability, induces apoptosis by downregulating anti-apoptotic proteins, and inhibits colony formation in breast cancer cell lines.

(A) MCF7, MDA-MB-231, and MDA-MB-468 cells were seeded in 96-well plates at a density of $10^4$ cells/well. After 16–24 h, they were transfected with apMNKQ2 (blue line) or the control p29 (black line) at different concentrations (0–500 nM) for 48 h, and then MTT assay was performed. Bars represent the mean ± standard error of the mean of three independent experiments. (B) Lysates (20–30 μg) were subjected to SDS-PAGE at 10% for total PARP, cleaved PARP, MCL1, and XIAP and western blot was performed using specific antibodies (see Table S1). Actin was used as a control for homogeneity of loading. A representative blot is shown. (C) Quantification of bands of XIAP and MCL1 from western blot (B) was normalized with respect to actin and expressed as the percentage relative to the value in control cells. Values represent the mean ± standard error of the mean of three to six different experiments. (D) Cells transfected with apMNKQ2 or p29 at 3 times the IC50 were seeded at $1 \times 10^3$ cells/well in six-well plates. After 8–9 days, colonies were fixed, stained, and counted. Bars represent mean ± standard error of the mean of three independent experiments. *p < 0.001, **p < 0.01, *p < 0.05 relative to control cells; ***p < 0.001, **p < 0.01, *p < 0.05 relative to p29 control. See also Figure S4.
To validate whether the tumor reduction is specific to apMNKQ2 treatment, we measured MCL1 levels and occludin mRNA levels, two of the most significant changes observed when treating MDA-MB-231 cells in vitro (Figures 4C and 5C). There was a significant decrease in MCL1 levels (Figure 8E) and an increase in occludin mRNA levels (Figure 8F) in agreement with the results previously obtained in vitro. Occludin protein levels also increased, but were not statistically significant (Figure 8G). We repeated this assay with very similar results. As shown in Figure S8A, TGI was 27.5% after 18 days (p < 0.05) of treatment with apMNKQ2 2 mg/kg and 29.1% at

**Figure 5. apMNKQ2 inhibits migration, invasion, and EMT in breast cancer cell lines**

Cells transfected with apMNKQ2 or p29 at 3 times the IC50 were maintained in serum-deprived medium for 16 h, then collected, counted and seeded (4 × 10⁴ cells) for transwell assay without or with matrigel to measure migration (A) and invasion rate (B), respectively. Representative images are shown at the top of the figures. (A) Data are expressed as the percentage relative to the value of control cells and (B) the number invading cells and represent the mean ± standard error of the mean of three to four different experiments. (C) Quantitative reverse transcriptase PCR analysis of occludin and FSP1 (normalized with respect to actin expression) was performed as described in the Materials and Methods section. Bars represent the mean ± standard error of the mean of three to five different experiments. *p < 0.01, **p < 0.05 relative to control cells; **p < 0.01, *p < 0.05 relative to p29 control. See also Figure S8.
the end of treatment (28 days). In addition, one group of animals was treated with everolimus 2 mg/kg, a drug used clinically for the treatment of breast cancer, reaching a TGI of 47.5% at the end of treatment (Figure S8A). As in the previous assay, an increase in occludin mRNA levels (Figure S8B) and a decrease in MCL1 protein levels (Figure S8C) were also observed. An attempt was made to improve the efficacy of apMNKQ2 by increasing the doses; however, 4 mg/kg exceeds the maximum amount of nucleic acids that can be conjugated with in vivo Jet-Pei, being toxic to the animals (results not shown).

To also evaluate the potential anti-metastatic effect of apMNKQ2, we used a spontaneous metastasis model in BALBc mice bearing orthotopic 4T1 breast tumors. First, we studied the effect of apMNKQ2 on proliferation, migration, and invasion in this mouse breast cancer cell line. Surprisingly, apMNKQ2 did not produce a significant decrease in cell viability in 4T1 cells at the concentrations used (up to 1 µM) (Figure S9A), but was able to inhibit migration (as measured by scratch wound-healing and transwell migration assays) and invasion (Figures S9B and S9C). These results showed that 3.6 mg/kg apMNKQ2 did not decrease the RTV at any time point during treatment (18 days) (Figure S9D). Furthermore, there were no differences in weight between the treated and untreated mice, which also suggests that apMNKQ2 was safe at the dose administered in a syngeneic model (Figure S9E). The number and size of metastases in the lung were analyzed at the end of treatment using the inverse tincture method with Chinese ink. Metastases were separated according to their size down to 0.5, 1, 2, and more than 2 mm. As shown in Figure S9F, apMNKQ2 produced a 50% decrease in the total number of metastases (p = 0.06), and reduced the size of metastases, with significantly fewer metastases around 2 mm.

Altogether, these results indicate the potential use of apMNKQ2 as an anti-tumor and anti-metastatic drug in breast cancer.

**DISCUSSION**

Despite advances in prevention, diagnosis, and treatment and, therefore, the consequent increase in survival, breast cancer remains a complex and heterogeneous disease that, in many cases, has no cure and that leaves an average survival time of 18–24 months, depending on the extent of the tumor and its histopathological and molecular profiles.

In a previous article, we described that the aptamer apMNK2F, an aptamer selected in our laboratory against the MNK1b protein, was able to inhibit proliferation, migration, and colony formation in MDA-MB-231 breast cancer cells. Furthermore, by in silico studies, we
demonstrated that this aptamer could have the ability to form two G-quadruplex structures. G-quadruplex-forming aptamers have been described to have important advantages over unstructured sequences in that they are not immunogenic, are thermodynamically and chemically stable, and show both increased resistance to various serum nucleases and increased cellular uptake. In this regard, we first set out to optimize the apMNK2F aptamer by removing regions of the sequence that did not seem to be involved in the formation of the G-quadruplex structure and to determine whether this optimization would result in a smaller aptamer that retained its activity. The new shorter aptamer, apMNK2FT, retains the G-quadruplex structures and maintains its ability to bind MNK1 isoforms with high affinity. However, it is much less effective than apMNK2F in inhibiting processes characteristic of tumor cells, such as proliferation or migration, but not colony formation, in the breast cancer cell line MDA-MB-231. After a detailed analysis of the aptamer apMNK2FT using QGRS Mapper software four different sequences (apMNKQ1, apMNKQ2, apMNKQ3, and apMNKQ4) with the ability to form G-quadruplex structures were predicted (Figure 2A). Among them, the selected candidate, apMNKQ2, with only 29 nts, inhibits MTT activity in breast cancer cells and is resistant to degradation by DNAse I and by enzymes presented in human plasma supporting that G-quadruplex structures confer high stability.

One of the properties sought when identifying new anti-tumor drugs is their ability to inhibit processes involved in the development of the tumor itself, such as cell proliferation, apoptosis, and clonogenic activity. We have shown that apMNKQ2 is able to decrease cell viability in three human breast cancer cell lines (MCF7, MDA-MB-231, and MDA-MB-468) but not in 4T1 mouse breast cancer cell line. Although most MNK inhibitors have an anti-proliferative effect, in some cases it is not affected. Thus, the MNK-7g inhibitor does not affect the distribution of MDA-MB-231 cells in the cell cycle, indicating that it does not affect cell viability. However, it is able to exert its action by inhibiting migration, suggesting that MNK may be affecting metastasis-related processes.

Metastasis is a complex process that requires multiple events, including the EMT of epithelial cancer cells, induction of angiogenesis, and intravasation and extravasation of cancer cells, for the cells to regain epithelial features (MET) and eventually form a new colony in the appropriate distant microenvironment. There is much evidence that MNKs play an important role in metastasis-related events. For example, the inhibition of MNKs decreases protein expression of the EMT-activating transcription factor ZEB1, leading to the inhibition of EMT and reduction of migration and invasion of pancreatic cancer cells. In this work, we have demonstrated that apMNKQ2 is able to inhibit migration and invasion both in breast cancer cells and in an in vivo model. In contrast, apMNKQ2 acts by regulating EMT, affecting the expression of the main transcription factors involved in this process. EMT transcription factors are known to regulate each other in complex patterns that have not been established yet and that depend on tumor type and tissue context. In our TGF-β model, apMNKQ2-induced reduction of ZEB1 and Slug expression could lead to an increase of Snail at the mRNA and protein levels. Similar results were described in oral cancer cells and hepatocellular carcinoma cells using Slug and ZEB1 small interfering RNAs (siRNAs), respectively. Since Snail acts as a direct repressor of E-cadherin, the observed Snail overexpression may be associated with a low E-cadherin expression. However, apMNKQ2 inhibits...
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TGF-β-induced migration in breast cancer cells, as it is able to reduce N-cadherin levels. Consistent with our findings, N-cadherin knockdown MCF10A cells have lower cell motility in the presence of TGF-β, while E-cadherin depletion in MCF10A also results in slow migration. In addition, there is a decrease in MMP2 activity that was upregulated by TGF-β, which may be related with the inhibition of invasiveness.

The most widely used traditional inhibitors of MNKs (CGP57380 and cerdorsamide) had low selectivity and efficacy, exerting off-target effects. For this reason, efforts have been made to identify inhibitors that can be more specific. Most of the newly designed and synthesized MNK inhibitors present IC50 values in the micromolar or high nanomolar range and recognize both MNK1 and MNK2. This is probably because they have been obtained by searching for molecules that block the kinase activity, specifically because they have been obtained by searching for molecules that block the catalytic site of the enzyme, which is highly conserved in different kinases. Instead, a new family of inhibitors that acts as MNK "degraders" is being developed. To our knowledge, apMNKQ2 is the first specific MNK1 inhibitor developed and is able to significantly and specifically downregulate MNK1 isoforms through a mechanism that remains to be elucidated.

We have presented evidence implying that the aptamer apMNKQ2, developed against MNK1 kinase, is a potential therapeutic agent for the treatment of breast cancer and possibly other tumors in which MNK1 plays a relevant role. Additional efforts are currently underway to find the best delivery method, and to carry out in vivo studies that allow us to determine the optimal effective dose and then to perform the corresponding pharmacodynamics and pharmacokinetic studies.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

GST-tagged human MNK1a and MNK1b were subcloned into the BamHI and NotI sites of pGEX-4T3 and expressed in Escherichia coli Rosetta cells. Proteins were purified with glutathione-Sepharose (GenScript, Piscataway, NJ) according to the manufacturer’s instructions. Briefly, expression was induced with 1 mM IPTG for 2 h at 25°C. Cells were suspended in buffer containing 5 mM sodium phosphate, 150 mM NaCl, 1 mM EDTA (pH 7.4), and 1 mg/mL lysozyme, and incubated on ice for 30 min. Afterward, 0.5% Triton X-100 was added and bacteria were subjected to sonication. Cell debris was removed by centrifugation, and the supernatant was incubated with 1 mM MgCl2, 5 mM KCl, 1 mM MgCl2, 5 mM KCl. The modified apMNK2F aptamers (apMNK2FT and apMNKQ2), conjugated with biotin, were diluted in selection buffer at the concentrations indicated in the figures, denatured for 10 min at 95°C and cooled for 10 min on ice. Next, 100 μL of the diluted aptamers were added to each well, the plate was incubated at 37°C for 1 h and washed four times with selection buffer to remove unbound ssDNA. Afterward, 100 μL of a 1/1,000 dilution of horse-radish peroxidase-conjugated streptavidin (GE Healthcare, Madrid, Spain) were added to the individual wells. After 1 h incubation at 37°C on a shaking platform, the plates were washed four times and developed using ABTS solution (Roche, Madrid, Spain) according to the manufacturer’s instructions. OD 405 nm values were determined using a SpectraFluor microplate reader (TECAN, Barcelona, Spain).

Secondary structure prediction

Aptamer secondary structure prediction was obtained using mFold software (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) at 37°C, 150 mM [Na+] and 1 mM [Mg+++] (selection buffer conditions) and QGRS Mapper (https://bioinformatics.ramapo.edu/QGRS/index.php), a web-based server for predicting G-quadruplexes in nucleotide sequence.

NMR and CD

The aptamers were resuspended in H2O/D2O 9:1 in 10 mM potassium phosphate buffer, pH 7, [DNA] ~ 100 μM. NMR spectra were acquired in Bruker Avance spectrometer operating at 600 MHz, and processed with Topspin software. Water suppression was achieved by including a WATERGATE module in the pulse sequence for 2 h at 4°C. Finally, after extensive washes with the same buffer, the proteins were eluted with 10 mM glutathione in 50 mM Tris-HCl (pH 8).

His-MNK1b, cloned as described in was purified by affinity chromatography by ProAlt (Protein Alternatives S.L., Madrid, Spain).

ELONA

Recombinant proteins (200 ng) diluted in coating buffer (SeraCare, Milford, MA) were incubated in a 96-well microtiter plate (NUNC, Rochester, NY) overnight at 4°C. Then, the plate was blocked using 5% bovine serum albumin in phosphate-buffered saline (PBS) and incubated 1 h at room temperature, and subsequently washed four times in selection buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM MgCl2, 5 mM KCl). The modified apMNK2F aptamers (apMNK2FT and apMNKQ2), conjugated with biotin, were diluted in selection buffer at the concentrations indicated in the figures, denatured for 10 min at 95°C and cooled for 10 min on ice. Next, 100 μL of the diluted aptamers were added to each well, the plate was incubated at 37°C for 1 h and washed four times with selection buffer to remove unbound ssDNA. Afterward, 100 μL of a 1/1,000 dilution of horse-radish peroxidase-conjugated streptavidin (GE Healthcare, Madrid, Spain) were added to the individual wells. After 1 h incubation at 37°C on a shaking platform, the plates were washed four times and developed using ABTS solution (Roche, Madrid, Spain) according to the manufacturer’s instructions. OD 405 nm values were determined using a SpectraFluor microplate reader (TECAN, Barcelona, Spain).
prior to acquisition. CD spectra were recorded on a Jasco J-815 spectropolarimeter fitted with a thermostatted cell holder. Spectra were recorded at a DNA concentration of 50 μM and under the same buffer condition as the NMR experiments.

**Aptamer stability assays**

apMNKQ2 aptamer (1 μg) was incubated with 0.25, 0.5, or 1 U of DNAse I (Thermo Fisher Scientific, Waltham, MA) for 5 min at 37°C. Subsequently, the reaction was stopped by adding 1 μL EDTA 50 mM and heating the samples at 65°C for 10 min. The digested aptamer apMNKQ2 was resolved on a chip Experion DNA1K Analysis Kit (BioRad, Hercules, CA), following the manufacturer’s instructions.

In plasma, 2.4 μg apMNKQ2 plus 16 μL of human plasma was incubated at 37°C for up to 72 h. Aliquots of the mixture were removed at 0, 6, 24, 48, and 72 h and heated for 10 min at 65°C to inhibit the activity of nucleases present in the plasma, and cooled on ice for 10 min. They were kept at –20°C until all aliquots were obtained, and finally loaded onto a chip Experion DNA 1K Analysis Kit.

**Cell culture and protein and RNA extraction**

MCF7 and MDA-MB-231 cells, last authenticated in May 2019 and June 2021, respectively, using the GenePrint 10 System, were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/Ham F-12 medium (PAA, Pasching, Austria) (mixed 1:1) with 10% fetal calf serum (Gibco, Grand Island, NY) and 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin (Sigma, St. Luois, MO) in a humidified incubator with 5% CO2/95% air at 37°C. MDA-MB-468 cells, last authenticated in May 2019 using the GenePrint 10 System, were maintained in RPMI medium (PAA, Pasching, Austria) with 10% fetal calf serum (Gibco) and 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin (Sigma) in a humidified incubator with 5% CO2/95% air at 37°C. MCF10A cells (purchased from ATCC, Manassas, VA) were maintained in DMEM/Ham F-12 medium (PAA, Pasching, Austria) supplemented with 10 μg/mL insulin (Sigma), 20 ng/mL epidermal growth factor (Sigma-Aldrich), 0.5 μg/mL hydrocortisone (Sigma), 5% horse serum (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin (Sigma) in an incubator with 5% CO2/95% air at 37°C. To obtain lysates, cells were washed once with cold buffer A (20 mM Tris-HCl pH 7.6, 1 mM dithiothreitol, 1 mM ethylenediaminetetra-acetic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 mM sodium molybdate, 10 mM sodium β-glycerophosphate, 1 mM sodium orthovanadate, 120 mM potassium chloride, 10 μg/mL anti-pain, 1 μg/mL peptatin A and leupeptin), and were mechanically harvested and lysed in the same buffer containing 1% Triton X-100 (volume ratio 1:2) and centrifuged at 12,000 g for 10 min. For tissues, tumors were thawed, weighed, and protein lysates were obtained as described above. Subsequently, protein concentration was determined using the BCA Kit (Thermo Fisher Scientific) and supernatants were aliquoted and stored at –80°C until use.

Total RNA from cells and tissue lysates were lysed and homogenized in NucleoZOL (Macherey-Nagel, Düren, Germany) following the manufacturer’s protocol. The RNA pellet was resuspended in 50 μL of Rnase-free water, treated with 1 μL of DNAse I (Fermentas, Waltham, MA), quantified, and stored at –80°C.

**Western blot and apta-western assays**

Cell lysates (20–30 μg) were resolved on 12%, 15%, or 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under the conditions indicated in figure legends, and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with monoclonal or polyclonal antibodies overnight at 4°C. After washing, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibody (GE Healthcare) for 1 h at room temperature and developed with enhanced chemiluminescence’s kits (GE Healthcare) or Clarity Western ECL Substrate (BioRad). PageKuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) was used in all the experiments. The β-actin antibody (Sigma) was used as a control to monitor the homogeneity of loading. Antibodies used in this study are listed in Table S1.

For apta-western assays, 100 ng purified MNK1 proteins were resolved on gel electrophoresis under denaturing (SDS-PAGE) or native (PAGE, neither SDS nor β-mercaptoethanol were used) conditions and transferred to PVDF membranes. The membranes were incubated with 50 nM biotin-conjugated apMNKQ2. After washing, the membranes were incubated with streptavidin-peroxidase 1:5,000 (GE Healthcare) and developed as above.

**MTT and LDH assays**

MCF7, MDA-MB-231, or MDA-MB-468 cells were seeded at 10⁴ cells/well in 96-well plates. After 16–24 h, aptamers or unspecific p29 ssDNA were transfected at the concentrations indicated in the figure legends using Lipofectamine 2000 (Invitrogen, Waltham, MA) following manufacturer’s instructions for siRNA transfection. After 48 h, the medium was removed to add 100 μL MTT (1 mg/mL in culture medium) to each well, and the plates were incubated 3 h at 37°C. Then, 100 μL/well of lysis buffer (10% SDS and 10 mM HCl) was added and, after 24 h of incubation, the absorbance was read at 540 nm on a SpectraFluor microplate reader (TECAN, Männedorf, Switzerland).

To perform cell death (LDH) assays, 48 h after aptamer transfection, supernatants were collected for mixing with the cytotoxicity detection kit (LDH) reagent (Roche), following the manufacturer’s protocol. The reaction was incubated for 30 min at room temperature, and stopped by adding 1 M HCl. Finally, solubilized formazan was measured in an Infinite F200 spectrophotometer (TECAN, Männedorf, Switzerland) at 490 nm. To calculate the percentage of cytotoxicity, two controls were included: supernatants of untreated cells (LDHlow) and of lysate cells (LDHhigh) with 0.2% Triton X-100. Finally, the results were expressed as cytotoxicity (%) = (LDHtreated − LDHlow)/(LDHhigh − LDHlow) × 100, for each experimental point with respect to the control.
Trypan blue exclusion test of cell viability
To determine the number of viable cells after aptamer transfection, cells were collected from vials or culture plates suspended in their corresponding medium. Aliquots of 40 μL cells mixed with 10 μL trypan blue were counted using a TC20 counter (BioRad), with cells excluding the dye being considered live.

Flow cytometric analysis of cell cycle
Twenty-four hours after transfection, the cells were harvested and fixed in cold 70% ethanol for 30 min at −20°C. Afterward, cells were washed twice with ice-cold PBS and incubated with 50 μg/mL propidium iodide (Sigma) plus 100 μg/mL RNase A (Sigma) for 1 h at 37°C in darkness. The cell cycle was analyzed by flow cytometry (FACScalibur, BD Biosciences, Franklin Lakes, NJ) using selective gating to exclude cell doublets. Cytometry data were analyzed using Flowing software 2.5.1. (Turku Bioscience, Turku, Finland).

Colony-forming assays
After 16–24 h, transfected cells as described above were collected and alive cells were counted by trypan blue exclusion assay (Sigma) using TC20 counter (BioRad) and seeded at 1 × 103 or 5 × 103 cells/well in six-well plates for MCF7 and MDA-MB-231 or MDA-MB-468 respectively. Approximately 12–16 days later, colonies were fixed, stained for 30 min with 0.02% Giemsa (Sigma), and counted with an eCount Colony Counter Pen (Heathrow Scientific, Vernon Hills, IL) and a magnifying glass (×1.75) (Bel-Art Scienceware, Wayne, NJ).

Scratch wound-healing assays
MDA-MB-231 cells were plated in six-well plates at 5 × 105 cells/well in 2 mL growth medium. After 24 h, the cells were transfected with the aptamer at the concentration indicated in the legends, and treated with 1 μM mitomycin C for 2 h to ensure that the wounds were filled due to cell migration and not by cell proliferation. Subsequently, the monolayer was scraped with a P-200 pipette tip, washed with medium to remove floating cells, and photographed with an Olympus IX70 (time 0). The plates were then incubated at 37°C and images were taken after 24 h. Wound area was measured using ImageJ software and calculated as the percentage of closed wound.

Migration and invasion assays
Migration and invasion assays were carried out using transwell insert chambers (Corning, Corning, NY). For the migration assay, cells transfected as described above were harvested after 16 h of serum deprivation, collected, counted, and 4 × 104 cells were plated in the upper chamber in serum-free medium. Complete medium (10% FBS) was added to the lower chamber as chemoattractant. After 24 h, medium was removed from the top and bottom of the wells; cells in the insert were fixed with 4% formaldehyde for 2 min, followed by 20 min with 100% methanol. Cells were stained with 30 μM Hoechst 33342 for 15 min, protected from light, washed twice with PBS and cells on the top were removed with a cotton stick. At least five photographs were acquired for each sample using a fluorescence microscope (Olympus IX70). The number of cells was analyzed using ImageJ software.

For the invasion assay, after serum-starvation for 24 h, 4 × 104 cells were seeded as above in matrigel-sealed upper chambers (Corning) in serum-free medium. After incubation for 48 h at 37°C, non-invasive cells on the top surface of the filter were removed with cotton swabs. Invasive cells that migrated to the lower surface of the filter were fixed, stained and scored as described above.

For MCF10A cells, cells were transfected with apMNKQ2 and treated with 5 ng/mL TGF-β1 in medium with 0.5% horse serum for 48 h and 6 × 104 cells were seeded in upper chambers without or with matrigel (0.5 mg/mL) for migration or invasion assays respectively.

Zymography
Zymography was used to analyze MMP-2 and MMP-9 activity in the medium of MCF10A treated or untreated with TGF-β1 and apMNKQ2 after 48 h of incubation. Medium was collected and centrifuged at 210 g to remove cell debris. Twenty-five microliters of medium were mixed with loading buffer without mercaptoethanol and resolved on a 7.5% SDS-PAGE polyacrylamide gel co-polymerized with 1 mg/mL gelatin. Gels were incubated 30 min in 2.5% Triton X-100 at room temperature and overnight at 37°C in developing buffer (Tris 50 mM pH 7.5, NaCl 200 mM, CaCl2 5 mM, Tween 0.02%). Gels were stained with Coomassie blue for 15 min and destained until MMPs activity appeared as clear bands.

Quantitative real-time PCR
Total RNA prepared as described above was used for the obtention of first-strand cDNA using the SensiFAST cDNA Synthesis Kit (Bioline, Segovia, Spain). The reverse transcription reaction products were used for qPCR amplification, using AceQ qPCR SYBR Green Master Mix (Nanjing Vazyme Biotech, Nanjing, China) according to the manufacturer’s instructions on a StepOne Plus Real-Time PCR system (Applied Biosystem, Waltham, MA). Triplicate reactions of the target gene and housekeeping gene were performed simultaneously for each cDNA template. The $2^{-\Delta\Delta C_{T}}$ method was used to calculate the relative expression of each target gene.31 Thus, the data were normalized with respect to housekeeping mRNA (β-actin) levels and $\Delta C_{T}$ (Ct(target)−Ct(housekeeping)) values were determined. $\Delta\Delta C_{T}$ was calculated as $\Delta C_{T_{apMNKQ2/ experimental}} - \Delta C_{T_{control}}$. Finally, data are expressed as $2^{-\Delta\Delta C_{T}}$. Oligonucleotides used in this work are listed in Table S2.

Immunohistochemistry
Tumor sections (5 μm) were deparaffinized and rehydrated as described previously32 and antigen retrieval was achieved by heat treatment in a pressure cooker for 2 min in 10 mM citrate buffer (pH 6.5). Next, endogenous peroxidase was blocked, and the sections were incubated with anti-PCNA (Santa Cruz Biotechnology, Dallas, TX) 1:100 overnight at room temperature. Sections were incubated with Master Polymer Plus Detection System (Peroxidase) (Master Diagnostica, Granada, Spain) for 45 min and developed with the Immunoperoxidase DAB kit (Master Diagnostica) according to the manufacturer’s instructions. Sections were counterstained with hematoxylin. Positive cells were analyzed using ImageJ software.
Efficacy study in a triple-negative breast cancer orthotopic mouse model

Two independent studies were performed by Leitat (LEITAT Technological Center, Barcelona, Spain) and in our laboratory (Department of Biochemistry and Molecular Biology, Complutense University, Madrid, Spain) following a similar procedure. All animal procedures were performed with the approval of the Complutense University Animal Experimentation Committee and Madrid Regional Government, according to European official regulations. The strain of mice was Hsd: Athymic Nude-Foxn1nu. The animals were maintained in an air and temperature-controlled cages, with a regular supply of water and food. MDA-MB-231 cells were inoculated (2.5–5 × 10^6 cells/animal respectively) in 50 μL DMEM or PBS into the fourth mammary fat pad mice. Once tumors were established (tumor volume of approximately 100 mm³), tumor-bearing athymic mice were randomized (10 mice in each group) and treated as follows: (1) vehicle control (selection buffer: in vivo-jetPEI (Polypus, Illkirch, France), (2) 1.8–2 mg/kg apMNKQ2: in vivo-jetPEI (5% glucose containing 46 μg apMNKQ2 and 5.6 μL jetPEI transfectant [N/P ratio of 6]), and (3) 2 mg/kg everolimus (Sigma) three times per week by intravenous injection. Tumors were measured two or three times per week with calipers and the tumor volume was calculated by the formula: D × d/2. The RTV was calculated using the following formula: RTV = (tumor volume on measured day)/(tumor volume on day 0). At the end of the treatment, the TGI ratio (%) was calculated using the following formula: TGI (%) = [(1 − (RTV of the treated group)/ (RTV of the control group)) × 100 (%). Animals were also weighed twice weekly and monitored for general health status and signs of possible toxicity because of the treatments. The mice were sacrificed after the indicated periods of treatment and the tumors were excised. Tumors were divided and frozen in liquid nitrogen for western blot and qPCR analysis or placed in 10% buffered formalin for immunohistochemistry.

For the efficacy assay in a breast cancer orthotopic mice model with spontaneous metastasis, performed by Leitat, the BALB/c mouse strain AnNHsd was used. 4T1 cells were inoculated (2 × 10^6 cells/animal) in 50 μL DMEM into the fourth mammary fat pad of mice. Once tumors were established (average tumor size of 72.5 ± 4.7 mm³), BALB/c mice (10 mice in each group) were randomized and treated with 160 μL of vehicle control (selection buffer: in vivo-jetPEI) or 3.6 mg/Kg apMNKQ2:in vivo-jetPEI (73.6 μg apMNKQ2 and 7.4 μL jetPEI transfectant [N/P ratio of 5]) three times per week by intravenous injection. Tumor volume and animal weights were measured as above. At the end of treatment, tumors were excised and one-half frozen and one-half was fixed in formalin. In addition, the extraction of the lungs was performed for the assessment of the metastasis with the inverse tincture method with Chinese ink.

Statistical analyses

Data are presented as mean value ± standard error of the mean. To determine whether differences between the averages of the different experimental groups were significant, statistical analysis was performed by ANOVA followed by Tukey’s test, unpaired t test, or one-sample t test versus a control value with Graphpad Prism 8.0 (San Diego, CA). Significance was assumed at p < 0.05.

Data availability statement

Data are contained within the article or Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.11.009.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.P.-D., R.F.-M., V.M.G., and M.E.M.; methodology, C.P.-D., R.F.-M., R.C.-M., J.I.K.-M., M.G.-H., M.I.P.-M., S.S., M.B.-U., M.S.-V., I.T., S.B.-B., and I.G.-P.; investigation, C.P.-D., R.F.-M., E.P.-G., C.S.-C.G., V.M.G., and M.E.M.; writing—original draft preparation, C.P.-D., R.F.-M., V.M.G., and M.E.M.; writing—review and editing, C.P.-D., R.F.-M., V.M.G., and M.E.M. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTEREST

C.P.-D., R.F.-M., V.M.G., and M.E.M. declare that a patent application has been filed relating to this work.

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