Exploring Cytotoxic mRNAs as a Novel Class of Anti-cancer Biotherapeutics

Kristin Hirschberger,1 Anita Jarzebinska,2 Eva Kessel,2 Verena Kretzschmann,1 Manish K. Aneja,1 Christian Dohmen,1 Annika Herrmann-Janson,1 Ernst Wagner,2 Christian Plank,1,3 and Carsten Rudolph1,4

New treatments to overcome the obstacles of conventional anti-cancer therapy are a permanent subject of investigation. One promising approach is the application of toxins linked to cell-specific ligands, so-called immunotoxins. Another attractive option is the employment of toxin-encoding plasmids. However, immunotoxins cause hepatotoxicity, and DNA therapeutics, among other disadvantages, bear the risk of insertional mutagenesis. As an alternative, this study examined chemically modified mRNAs coding for diphtheria toxin, subtilase cytotoxin, and abrin-a for their ability to reduce cancer cell growth both in vitro and in vivo. The plant toxin abrin-a was the most promising candidate among the three tested toxins and was further investigated. Its expression was demonstrated by western blot. Experiments with firefly luciferase in reticulocyte lysates and co-transfection experiments with EGFP demonstrated the capability of abrin-a to inhibit protein synthesis. Its cytotoxic effect was quantitated employing viability assays and propidium iodide staining. By studying caspase-3/7 activation, Annexin V-binding, and chromatin condensation with Hoechst33258 staining, apoptotic cell death could be confirmed. In mice, repeated intratumoral injections of complexed abrin-a mRNA resulted in a significant reduction (89%) of KB tumor size compared to a non-translatable control mRNA.

INTRODUCTION

Conventional anti-cancer therapy is often limited by chemoresistance,1 radiation resistance,2 and severe side effects like cardiotoxicity3 or neurocognitive deficits.1 Therefore, the need for innovative cancer therapeutic options is immense. For instance, administration of immunotoxins as anti-cancer therapeutics is being tested in clinical studies,4 with one drug gaining FDA approval.6 Immunotoxins are cell-specific ligands linked either to plant or bacterial toxins, leading to a selective killing of target cells.7,8 The advantage of this approach, compared to other tumor treatments, is high tumor specificity and hence less damage of healthy tissue. A further benefit is their applicability to both solid and non-solid tumors.7 Still, despite their cell specific toxicity in vitro, in some cases damage to healthy tissue, particularly to the liver, has been observed.9,10 Another disadvantage is the comparatively slow protein uptake from blood vessels by tumors due to their abnormal tissue architecture.11

An alternative that might avoid these deficiencies but shares many of the benefits of immunotoxins is the employment of nucleic acids coding for toxic proteins as suicide cancer therapy. Hochberg and co-workers12,13 have shown the potential of this approach by applying a plasmid coding for diphtheria toxin. Expression of diphtheria toxin is under the control of H19 promoter, which is highly active in a range of human cancers, ensuring that toxin production is confined to the tumor. Clinical trials (phase 2b) with this plasmid-based approach have been successful.12 Analogously, tumor growth was reduced after intratumoral delivery of a recombinant adeno-associated virus containing DNA coding for the toxin trichosanthin.14 Nevertheless, DNA-based gene therapeutics bear some risks and disadvantages, with potential genomic integration followed by mutation being one of the most important safety concerns.15 The employment of messenger RNA instead of DNA is an attractive approach to circumvent several deficiencies of plasmid-based transfection. The most important benefits of mRNA application versus DNA therapeutics are (1) no risk of insertional mutagenesis,13,15 (2) no need to enter the nucleus, therefore resulting in higher transfection efficiency and earlier onset of protein expression,16 (3) expression being self-limited because of short mRNA half-life,17,18 and (4) only the sequence of interest being introduced into the cell.16 Also, using the herpes simplex virus- thymidine kinase/ganciclovir (TK/GCV) suicide gene therapy system, Wang et al.19 observed that the reduction in tumor growth was significantly higher for mRNA than for plasmid DNA (pDNA).

In spite of the advantages of mRNA over pDNA as therapeutic agent, its presumably lower stability and high immunogenicity prevented its broad application until the last decade. Since Wulf et al.20 showed the first successful mRNA transfection in vivo in 1990, research has identified ways to evade these issues. Several studies have reported increased stability and decreased immunogenicity after incorporating...
In this study, we combine the advantages of immunotoxins and mRNA-based therapeutics. We investigated the cytotoxic potential of mRNA transcripts coding for three toxins that have been previously shown promising anti-tumor effects in pre-clinical or clinical studies. We focused on these three toxins since they represent an ideal proof-of-concept study demonstrating the efficacy of “killer RNAs” as promising anti-tumor agents. Further studies with different tumor models will be highly valuable in determining the true potential of such mRNA-based therapeutics.

RESULTS

Comparison of the Decrease in Cell Viability Caused by SubA, DTA, and AA cmRNA in HuH7 and KB Cells

Forty-eight hours post-transfection of KB and HuH7 cells with either of the cmRNAs SubA (subtilase cytotoxin, A-chain), DTA (diphtheria toxin, A-chain), AA (abrin-a, A-chain), or one of their stop cmRNAs, cell viability was assessed by quantifying the ATP content using the CellTiter-Glo Luminescence Viability Assay. Cell viability was proportional to the measured luminescence. 100 ng cmRNA were applied. Data are presented as mean in % ± SEM of cells transfected with the respective stop cmRNA (dotted line). Statistical significance for HuH7 and KB cells versus cells transfected with the respective stop cmRNA was assessed by Kruskal-Wallis test adjusted for multiple comparisons, with *p < 0.05, **p < 0.01, ***p < 0.001, and n = 3.

In this study, we compared the three cmRNAs regarding their potency to inhibit cell viability in two different tumor cell lines. The best-performer abrin-a, A-chain (AA) cmRNA was then investigated in detail to estimate its potential as new anti-tumor agent. In order to exclude any possible unspecific effects of mRNA transfection, a cmRNA (AAstop) was utilized that shows no translation. This was achieved by scrambling the Kozak element and mutating the start as well as all in frame downstream ATGs into TAGs. In in vitro experiments, the expression of AA was verified by western blot, and it was assessed for its capacity to decrease protein synthesis, its cytotoxicity, and the apoptotic characteristics of induced cell death. In vivo experiments in mice showed that intratumoral injection of AA cmRNA resulted in a significantly reduced tumor growth.

This is a first proof-of-concept study demonstrating the efficacy of “killer RNAs” as promising anti-tumor agents. Further studies with different tumor models will be highly valuable in determining the true potential of such mRNA-based therapeutics.
Inhibition of Protein Synthesis by AA cmRNA
The capability of AA to inhibit protein synthesis was assessed by cotreatment of rabbit reticulocyte lysate with 1 μg firefly luciferase (luc) cmRNA and co-transfection of KB cells with 50 ng EGFP cmRNA. Forty-five minutes after treatment of lysate with 0.1 μg AA cmRNA, luminescence was reduced significantly (p = 0.0079 in comparison to AAstop cmRNA) to 0.1% in comparison to lysate treated with luc cmRNA only (luc control, Figure 3A). Addition of AAstop cmRNA, however, did not result in decreased luc activity. KB cells transfected with AA cmRNA showed considerable EGFP fluorescence at 0.0005 ng, but no EGFP fluorescence was visible at 0.05 or 5 ng after 24 hr (Figure 3B). EGFP fluorescence in cells transfected with AAstop cmRNA was not affected at any of the tested concentrations. These microscopic observations were further confirmed by flow cytometry analysis, showing both the mean fluorescence intensity (MFI) of EGFP (Figure 3C) and the percentage of EGFP-positive cells (Figure 3D). Compared to cells transfected with EGFP cmRNA only (EGFP control), the MFI of EGFP was unchanged at 0.0005 ng AA cmRNA. At 0.05 and 5 ng AA cmRNA, the MFI of EGFP was decreased to 3.4% and 1.7%, respectively, compared to EGFP control cells. Similar to the results obtained with MFI, the number of EGFP-positive cells was unchanged at 0.0005 ng AA cmRNA (compared to EGFP control), but reduced by 77% at 0.05 ng or 100% at 5 ng. The differences between AA and AAstop cmRNA were significant for 0.05 and 5 ng (p < 0.0001). These results demonstrate that production of abrin-a after treatment of reticulocyte lysate or of KB cells with AA cmRNA was active and led to inhibition of protein synthesis of co-applied luc or EGFP cmRNA.

Toxicity of AA cmRNA on KB Cells
The toxic effect of AA cmRNA on KB cells in comparison to AAstop cmRNA was evident in microscopic images taken 48 hr after transfection (Figure 4A). Cell viability measurements (Figure 4B) showed reduction at all tested concentrations of AA cmRNA compared to UTs. Toxicity at 10 ng was low with 9.2% decrease in viability but increased considerably at 50 ng and 100 ng with 60.2% and 77.6% reduction of cell viability compared to UT cells. The percentage of dead cells, determined by staining with propidium iodide (PI), was in agreement with these findings. At 10 ng AA cmRNA, the number of PI-positive cells was twice as high as for UT. For 50 and 100 ng, the percentage of dead cells increased to 45%–51% of all cells. With the exception of PI staining at 10 ng dose, the differences between AA and AAstop cmRNA were statistically significant (10 ng, p = 0.0002; others, p < 0.0001). It could be shown that AA cmRNA not only distinctly decreases cell viability but also induces cell death.

Decrease in Cell Viability by AA-LF132 In Vitro and Inhibition of Tumor Growth In Vivo
Prior to performing the in vivo experiment, the Ethris’ proprietary cationic lipid formulation LF132 was tested in vitro on KB cells for its effectiveness. Forty-eight hours after transfection, very high toxicity of AA-LF132 but no toxic effect of AAstop-LF132 or 2% sucrose (vehicle control) was observed (Figure 6A). This was further confirmed by assessing cell viability (Figure 6B). For 10, 50, or 100 ng AA cmRNA, cell viability was reduced by 56%, 99%, or 100% compared to UTs, respectively. Accordingly, in comparison to AA-Lipofectamine 2000 (cf. Figure 4B), potency of inducing toxicity of AA-LF132 was considerably higher. AAstop-LF132 also
showed some toxicity at higher concentrations, but far less compared to AA-LF132. The reduction in luminescence by AA-LF132 was statistically significant in comparison to AAstop-LF132 for all tested doses (p < 0.0001).

To test the anti-tumor activity of AA-LF132 in vivo, 5 x 10^6 KB cells were injected into the flank of immuno-deficient Naval Medical Research Institute-nude (NMRI-nu) mice. In a small pre-experiment, the expression characteristics of intratumorally injected RNA were investigated. Therefore, 10 μg lipid nanoparticle formulated cmRNA coding for firefly luciferase was injected three times. Twenty-four hours after the third application, inhibition of protein synthesis was observed by fluorescence microscopy (B) and flow cytometry (C and D). Representative fluorescence images are shown (B). Mean fluorescence intensity (MFI) (C) and percentage of EGFP-positive cells (D) are depicted. Data are presented as mean in % ± SEM of control cells transfected only with EGFP cmRNA (EGFP ctrl, dotted line). Statistical significance versus AAstop cmRNA was assessed by two-way ANOVA adjusted for multiple comparisons, with ****p < 0.0001 and n = 3.

DISCUSSION

Employment of mRNA coding for different cellular and viral proteins for anti-cancer therapies has been investigated in previous studies.
A rabbit reticulocyte lysate system assessed that AA almost completely inhibits protein synthesis of co-applied luc cmRNA. Moreover, fluorescence inhibition of co-transfected EGFP cmRNA confirmed that transfected AA cmRNA was translated into an active toxin. For 0.05 ng dose, only one-fourth of transfected cells were EGFP positive with a weak EGFP signal (low MFI). In those cells, the comparably low amount of AA presumably was not able to inactivate all ribosomes before enough EGFP molecules for fluorescence detection had been synthesized. In contrast, none of the cells transfected with 5 ng AA cmRNA showed detectable EGFP fluorescence. This dose dependency of translational inhibition by abrin has been shown before. The results demonstrate that AA exerts its influence shortly after the start of translation. As AA directly inhibits protein synthesis by cleaving an adenine from the rRNA, thereby immediately blocking translation, such immediate effects on translation are conceivable. Also, Hung et al. detected depurination of

Van der Jeught et al., for instance, delivered mRNA coding for interferon β fused to the transforming growth factor β (TGF-β) receptor II intratumorally to enhance tumor-specific immunity. Also, an mRNA encoding the TK/GCV suicide system was applied intravenously to suppress tumor growth. In both studies, reduction of tumor growth was observed. The present study aimed to investigate whether protein toxins are suitable for employment as mRNA cancer therapeutics. For this purpose, cmRNAs coding for the catalytic A-chain of three AB-toxins, namely subtilase cytotoxin (SubA), diphtheria toxin (DTA) and abrin-a (AA), were employed.

A cell viability assay performed on the cancer cell lines HuH7 and KB revealed AA as the most promising candidate for further investigation. Concerning HuH7 cells, the decline in viability 48 hr after transfection was comparably high for all three toxins. While AA and DTA cmRNA showed a similar effect on KB cells, SubA cmRNA showed reduced toxicity compared to HuH7 cells. Because of differences in cell number and cell size, a direct comparison between the two cell lines regarding their sensitivity to toxin mediated cell death is limited. As DTA inactivates EF-2 by ADP-ribosylation and AA blocks the binding of EF-2 to the ribosome by cleaving an adenine from the rRNA, the two toxins show a similar and irreversible mode of action. The activation of unfolded protein response (UPR), as cause for cell death by SubA, distinguishes it clearly from the other toxins. As the induction of the UPR also increases the expression of GRP78, the substrate of SubA, it seems possible that UPR-induced apoptosis can be evaded. This might account for the cell-dependent differences in effectiveness observed when comparing SubA with DTA and AA. As the molecular weight of AA compared to DTA cmRNA is higher (AA, 1.0 kb, versus DTA, 0.8 kb), AA was more effective than DTA when considering molecular toxicity. As one challenge of successful mRNA-based therapy is transfection efficiency, high effectiveness per mRNA molecule is desirable. Moreover, with high molecular toxicity, comparably lower doses of AA are sufficient, thereby reducing potential toxic side effects of mRNA delivery.

To ensure that the various effects caused by transfection of AA were specific, a control cmRNA (AAstop) was applied in this study. AAstop displays the same sequence as AA but has a scrambled Kozak element, and the start as well as all in-frame downstream ATGs mutated to TAGs. Prior to translation, the 40 S ribosome scans the mRNA sequence, starting at the 5’ end, and initiates translation when it reaches the first AUG codon. Binding to this first AUG by the ribosome is strongly supported by a consensus sequence (Kozak element) directly upstream. Consequently, as could be asserted in this study, the introduced alterations in AAstop cmRNA prevented its translation.

The successful expression of abrin-a protein after transfection of KB cells with AA cmRNA was demonstrated by western blot analysis. At the lower dose of 40 ng cmRNA, protein production was reduced compared to 80 ng cmRNA.

Figure 4. Toxicity of AA cmRNA on KB Cells
Forty-eight hours post-transfection of KB cells with AA (abrin-a, A-chain) or AAstop cmRNA. (A) Representative pictures were taken of cells transfected with 100 ng cmRNA or of untransfected cells (UT). (B) Cell viability was determined by measuring ATP content with the CellTiter-Glo Luminescence Viability Assay. Cell viability was proportional to the measured luminescence. Data is presented as mean in % ± SEM. Statistical significance versus AAstop cmRNA was assessed by two-way ANOVA adjusted for multiple comparisons, with ***p < 0.001, ****p < 0.0001, and n = 3.

A cell viability assay performed on the cancer cell lines HuH7 and KB showed similar effect on KB cells, SubA cmRNA
isolated rat liver ribosomes already 15 min after treatment with the A-chain of the protein abrin-a. Previous studies demonstrated that inhibition of protein synthesis is the main reason for abrin-a-induced cell death.43

At low doses (9,100 molecules AA cmRNA per cell), translation of EGFP cmRNA was inhibited substantially, while cell death was only detected at higher doses. This discrepancy indicates that higher amounts of AA are needed to disturb protein metabolism to such an extent that it results in cell death. Furthermore, it has been assumed that abrin-a can induce cell death independent of inhibition of protein synthesis.45,46 Potentially, higher concentrations of abrin-a are necessary for this toxicity-enhancing effect. In accordance with what has been reported by other groups,47,50 after staining with Hoechst, chromatin condensation has been observed in previous studies.45 While it is established that cells treated with abrin-a undergo apoptosis, different pathways have been proposed. Qu and Qing45 suggest that the inhibition of protein synthesis and mitochondrial membrane damage after reactive oxygen species (ROS) production present two independent pathways. The intrinsic mitochondrial pathway has been confirmed by various groups.47,50 Saxena et al.48 showed the involvement of the Fas ligand and thereby of the extrinsic pathway after exposure to abrin.

In this study, besides toxicity in vitro, tumor growth in mice could be diminished considerably by four intratumoral injections of 10 μg formulated AA cmRNA. Twelve days after start of the treatment, the volume of tumors treated with AA-LF132 was significantly reduced by 89% compared to tumors that had been injected with AAstop-LF132. In comparison, a previous study using plasmid DNA coding for the A-chain of diphtheria toxin under a target-cell-specific

---

**Figure 5. Apoptotic Cell Death after Transfecting KB Cells with AA cmRNA**

At 24 hr (A–C) or 48 hr (D and E) post-transfection of KB cells with AA (abrin-a, A-chain) or AAstop cmRNA, cells were examined for apoptosis. (A and B) Annexin V-AF488 and propidium iodide (PI) double staining flow cytometry assay. (A) Percentages of cells in early apoptotic stage (Annexin V-AF488 positive, PI negative). Data is shown as mean in % ± SEM and is compared to untransfected control cells (UT, dotted line). (B) Representative dot plots of cells transfected with 100 ng cmRNA. The percentages of the different populations are given in the respective corners. (C) Caspase-Glo 3/7 assay; activity was proportional to luminescence. Data is represented as mean in % ± SEM of untransfected control cells (UT, dotted line). (D and E) Hoechst33258 staining. (D) Representative phase contrast and fluorescence images of cells transfected with 100 ng cmRNA are displayed. Arrows depict cells showing “membrane blebbing” or nuclear fragmentation. (E) Number of Hoechst33258-positive cells. Data (mean ± SEM) is presented as multiple of untransfected control cells (UT, dotted line). Statistical significance versus AAstop cmRNA was assessed by two-way ANOVA adjusted for multiple comparisons, with "p < 0.01, """p < 0.001, and n = 3.
promoter could diminish tumor size by 68% compared to the luc-expressing control plasmid group. To achieve these effects, three injections, each with 25 μg plasmid, were employed. Ramnath et al. showed a reduction in tumor volume in mice up to 62% compared to the control group after five intraleisional treatments with the protein abrin-a. Another group could show that by single injection of an immunotoxin containing the A-chain of abrin-a, tumor growth in mice could be delayed by 7 to 10 days.
For clinical application, a neoadjuvant therapy can be envisaged which comprises intratumoral co-administration of a toxin-encoding mRNA with an immune-stimulatory agent analogous to the employment of armed oncolytic viruses.\textsuperscript{53,55} Their anti-tumor potency could be extensively enhanced by inclusion of chemokines, cytokines or tumor-associated antigens.\textsuperscript{56} Due to its immunological properties, the mRNA molecule itself might serve as adjuvant.\textsuperscript{55} Conceivably, this approach could be pursued prior to surgical removal of a primary tumor with the aim of inducing tumor shrinkage and a systemic immune response to disseminated tumor cells. Besides direct activation of the immune system, blockage of molecules inhibiting T-cell differentiation or function, e.g. PD-1, displays a promising tool for stimulation of the immune system.\textsuperscript{56}

The present study clearly showed that cmRNA encoding for the A-chain of abrin-a toxin displays effective anti-tumor properties. By repeated injections of complexed cmRNA into tumors in mice, tumor growth could be constrained in a manner comparable to previous in vivo studies applying abrin-a or toxin-encoding plasmids. The employment of mRNA is very attractive, as it shows various safety-relevant benefits compared to pDNA and limited toxicity has been associated with immunotoxins. The promising results obtained with AA prompt further studies using different tumor models to fully appreciate the anti-tumor efficacies of toxin encoding cmRNAs.

MATERIALS AND METHODS

Plasmid Preparation

The toxin (SubA, DTA, AA) and control (SubAstop, DTAsstop, AAstop) sequences were cloned at the KpnI site (Thermo Fisher, Waltham, MA) into the backbone pVAX1-A120.\textsuperscript{24} Toxin and control sequences codon optimized for expression in Homo sapiens were produced by GeneArt in two parts. Sequences were retrieved from NCBI GenBank (SubA, AF399991.3; DTA, K01722.1; AA, AY458627.1). Only the A-chain of the toxins was utilized. Subsequent sub-cloning into pVAX1-A120 was performed using the GeneArt Seamless Cloning and Assembly Enzyme Mix (Invitrogen, Darmstadt, Germany) and One Shot TOP10 Chemically Competent E. coli (Invitrogen, Darmstadt, Germany).

Generation of cmRNA

DNA plasmids were linearized downstream of the poly(A) tail with the restriction enzyme NotI (Thermo Fisher, Waltham, MA), and purification was performed by chloroform extraction and ethanol precipitation. The linearized plasmids were used as template for in vitro transcription (IVT). IVT-mix was as follows: 0.1 μg/μL plasmid, transcription buffer 1 (Ethris, Planegg, Germany), 1 U/μL Ribolock Rnase Inhibitor (Thermo Fisher, Waltham, MA), 0.015 U/μL Inorganic Pyrophosphatase 1 (Thermo Fisher, Waltham, MA), 2 U/μL T7 Polymerase (Thermo Fisher, Waltham, MA), 7.5 mM rATP, 7.5 mM rGTP, 5.6 mM rCTP, 5.6 mM rUTP, 1.9 mM 5’-methyl-rCTP, and 1.9 mM 2’-thio-rUTP. The nucleotides were purchased from Jena Biosciences (Jena, Germany). The complete IVT-mix was incubated at 37°C for 4.5 hr, following which 1 U/μL DNase I (Thermo Fisher, Waltham, MA) was added to remove the plasmid template and the reaction incubated an additional 25 min at 37°C. RNA was precipitated with ammonium acetate at a final concentration of 2.5 mM and washed twice with 70% ethanol before it was re-suspended in aqua ad injectabilia. RNA concentration was measured on a spectrophotometric device (Nanodrop) and its correct size and purity determined with 1% agarose gel and RibogRuler High Range RNA Ladder (Thermo Fisher, Waltham, MA). For subsequent capping, cmRNA was employed at a final concentration of 1 mg/mL and denaturated in advance at 65°C for 15 min. The capping reaction mix contained 1× capping buffer (NEB, Frankfurt, Germany), 0.5 mM GTP (NEB, Frankfurt, Germany), 0.2 mM S-Methyladenosine (NEB, Frankfurt, Germany), 0.5 U/μL Vaccinia Virus Capping Enzyme (NEB, Frankfurt, Germany), 2.5 U/μL mRNA Cap 2’-o-Methyltransferase (NEB, Frankfurt, Germany), and 1 U/μL Ribolock RNase Inhibitor (Thermo Fisher, Waltham, MA). Reaction mix was incubated at 37°C for 60 min before the RNA was precipitated with ammonium acetate at a final concentration of 2.5 mM and washed twice with 70% ethanol before it was re-suspended in aqua ad injectabilia.

Cell-free Protein Synthesis System

In order to determine protein synthesis of luciferase firefly from luc cmRNA (kindly provided by Ethris, Planegg, Germany) in a cell-free system, the Rabbit Reticuloocyte Lysate System (Promega, Madison, WI) was applied according to the manufacturer’s instructions. Per reaction of 35 μL lysate, 1 μg luc cmRNA and either 0.1 μg AA or 0.1 μg AAstop cmRNA were employed simultaneously. After an incubation of 45 min at 30°C, luminescence was measured using Tecan InfiniteR 200 PRO (Tecan, Männedorf, Switzerland).

Cell Culture

Cells were maintained in a humidified incubator at 37°C and 5% CO₂. HuH7 cells (CSC-C9441L, Creative Bioarray, Shirley, NY) was applied according to the manufacturer’s instructions. For subsequent capping, cmRNA was employed at a final concentration of 1 mg/mL and denaturated in advance at 65°C for 15 min. The capping reaction mix contained 1× capping buffer (NEB, Frankfurt, Germany), 0.5 mM GTP (NEB, Frankfurt, Germany), 0.2 mM S-Methyladenosine (NEB, Frankfurt, Germany), 0.5 U/μL Vaccinia Virus Capping Enzyme (NEB, Frankfurt, Germany), 2.5 U/μL mRNA Cap 2’-o-Methyltransferase (NEB, Frankfurt, Germany), and 1 U/μL Ribolock RNase Inhibitor (Thermo Fisher, Waltham, MA). Reaction mix was incubated at 37°C for 60 min before the RNA was precipitated with ammonium acetate at a final concentration of 2.5 mM and washed twice with 70% ethanol before it was re-suspended in aqua ad injectabilia.

Transfections in Cell Culture

If not stated otherwise, transfections were performed with Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions using 2 μL Lipofectamine 2000 per μg mRNA in medium without additives. Transfections were performed in 96-well plates and 100 μL final volume of medium per well, unless specified differently. 1 × 10⁴ KB cells and 3 × 10⁴ Huh7 cells per 100 μL medium were used in all in vitro experiments but for the western blot, where 2 × 10⁴ cells per 100 μL medium were applied. For co-transfections, 50 ng/100 μL of EGFP cmRNA (kindly provided by Ethris, Planegg, Germany) and either 5, 0.05, or 0.0005 ng/100 μL of AA or AAstop cmRNAs were transfected. For western blot detection, 40 and 80 ng/100 μL of AA or AAstop cmRNA were used for transfection. In all other experiments, 10 ng, 50 ng, or 100 ng of mRNA were applied.
in a final volume of 100 μL. At 4 hr after transfection, medium was discarded, and supplemented cell culture medium was added. For in vitro purposes, cmRNAs (AA and AAstop) were formulated using Ethris’ (Planegg, Germany) proprietary cationic lipid formulation LF132, which was based on Jarzebińska et al. In order to examine their effectiveness in vitro, cells were transfected with 10,50, and 100 ng/100 μL of AA-LF132 or AAstop-LF132. As vehicle control, 2% sucrose (diluted accordingly) was applied. Just like with transfections using Lipofectamine 2000, at 4 hr after transfection medium was discarded and supplemented cell culture medium added.

**Cell Culture Assays**

For all assays, luminescence was measured using Tecan InfiniteR 200 PRO (Tecan, Männedorf, Switzerland). The Attune Nxt (Thermo Fisher, Waltham, MA) was employed for flow cytometry analysis. Microscopic images were taken with Leica DMi8 (Leica, Wetzlar, Germany). Except for the western blot analysis (two independent technical replicates), all cell culture assays were performed in three independent biological replicates, each treatment in triplicates.

**SDS-PAGE and Western Blot**

For western blot detection, cells were seeded in 6-well plates. The assay was conducted 24 hr post-transfection. After washing the cells with cold PBS, they were detached from the well using cell scrapers and lysed in lysis buffer (25 mM Tris-HCl, 0.1% Triton X-100 [pH: 7.8], 1 × complete protease inhibitor [Sigma Aldrich, Steinheim, Germany], 150 U/mL DNase I [Thermo Fisher, Waltham, MA]) for 30 min on ice. Total protein content was determined by the bicinchoninic acid (BCA) assay, following the manufacturer’s instructions (Thermo Fisher, Waltham, MA). Cell lysates (45 μg of total protein per sample) were separated on 4–12% polyacrylamide gels (Thermo Fisher, Waltham, MA) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in Western Breeze blocking solution (Thermo Fisher, Waltham, MA) and probed with antibodies against abrin-a (6.7 μg/mL, Tetracore, Rockville, MD) and vinculin (1:10,000, Abcam, Cambridge, UK). For protein detection, horseradish peroxidase (HRP)-conjugated anti-rabbit (1:10,000, sc2004; Santa Cruz Biotechnology, Dallas, TX) antibodies were added. For signal detection, Luminata Western HRP substrate was applied according to the manufacturer’s protocol (Merck Chemicals, Darmstadt, Germany).

**Protein Synthesis Inhibition**

To determine EGFP expression 24 hr post-transfection, culture medium was put aside, cells trypsinized with TrypLE Express Enzyme (Thermo Fisher, Waltham, MA), re-suspended in the cell culture medium, and analyzed by flow cytometry. Analysis was performed on singlet cells.

**Cell Viability/Toxicity Assays**

Assays were conducted 48 hr post-transfection. To determine cell viability, the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) was performed according to the manufacturer’s instruction. Before addition of the substrate, the plate was centrifuged for 5 min at 1,100 rpm, the medium discarded, and fresh supplemented medium added. For dead cell staining, culture medium was put aside, and cells were trypsinized with TrypLE Express Enzyme (Thermo Fisher, Waltham, MA) and re-suspended in the cell culture medium. PBS containing 5 μg/mL PI (Sigma Aldrich, Steinheim, Germany) was added at 1:5 (v/v) to the cell suspension before analysis with flow cytometry. Analysis was performed on singlet cells.

**Apoptotic Assays**

Annexin V-PI double staining was performed 24 hr post-transfection. Annexin V, Alexa Fluor 488 conjugate (Thermo Fisher, Waltham, MA) was used according to the manufacturer’s instruction, and PI solution (Sigma Aldrich, Steinheim Germany) was applied at 1 μg/mL. Analysis was performed on singlet cells. For this assay, 24-well plates, containing 4 × 10⁴ cells per well in a final volume of 400 μL medium, were used. Activity of caspases-3 and -7 was assessed 24 hr post-transfection with the Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to the manufacturer’s instruction. At 48 hr post-transfection, Hoechst33258 (Thermo Fisher, Waltham, MA) was employed at 10 μg/mL in supplemented cell culture medium and cells incubated for 15 min at room temperature. Before examination, Hoechst33258 was discarded, and fresh supplemented cell culture medium was added. Of each replicate, nine images at 20× magnification were taken, and the number of Hoechst33258-positive cells was counted.

**Expression of Firefly Luciferase in the Tumor and Reduction of Tumor Growth in Mice**

5 × 10⁶ KB cells were injected into the flank of female, 6-week-old NMRI-nu mice (RjOrl:NMRI-Foxn1nu /Foxn1nu, Janvier Labs, Saint Berthevin Cedex, France). For the pre-experiment, 10 μg cmRNA coding for firefly luciferase (kindly provided by Ethris, Planegg, Germany) in 50 μL 2% sucrose was injected into the formed tumors on days 9, 11, and 13 after injection of tumor cells. Twenty-four hours after the third application, luminescence was measured using an IVIS In Vivo Imaging System with Living Image software 3.2 (Caliper Life Sciences, Mountain View, CA). 100 μl luciferin (60 mg/mL in PBS) were injected intraperitoneally 15 min prior to imaging. For the reduction of tumor growth, the cmRNAs (AA and AAstop) were formulated in a proprietary lipid formulation (LF132) by Ethris (Planegg, Germany) in 2% sucrose (Sigma Aldrich, Steinheim, Germany). The cationic lipid formulation LF132 was based on Jarzebińska et al. Treatment was started as soon as tumors had reached a sufficient size (100 mm³, day 9). 50 μL of solution containing either 10 μg of AA-LF132, 10 μg of AAstop-LF132 or 2% sucrose were injected intratumorally on days 9, 11, 13, and 18 post-injection of tumor cells. Throughout the experiment, tumor volume was determined with a caliper using the formula \(a \times b \times \frac{1}{2}\), with \(a\) indicating the length of the tumor and \(b\) the width. On day 21 after injection of tumor cells, mice were euthanized by cervical dislocation. Tumor was explanted, and tumor size was measured again using the formula \(a \times b \times \frac{1}{2}\). The protocols for animal experiments were approved by the animal ethics committee and the government of Oberbayern (May 26, 2014; Permit Number, Az. 55.2-1-54-2531-53-09), and experiments were executed in accordance with the German Animal Welfare Law.
Data Analysis
Flow cytometry data was analyzed by FlowJo v.10.0.8 (FlowJo, Ashland, OR). GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was utilized for statistical analysis.

AUTHOR CONTRIBUTIONS
K.H. performed the experiments and wrote the manuscript. C.D. developed the LF132 formulation and contributed together with A.H.-J. to the planning of the in vivo experiment. A.J. formulated the RNAs and contributed to the cultivation of the cells for the in vivo experiment. E.W. contributed to the planning of the in vivo experiment and supervised the in vivo experiment. E.K. contributed to the planning of the in vivo experiment and performed the in vivo experiment. V.K. supervised the project and together with M.K.A. provided intellectual input and contributed to the manuscript. C.R. together with C.P. supervised the work, provided input on the experimental design, and contributed to and edited the manuscript.

CONFLICTS OF INTEREST
C.P. and C.R. are founders and shareholders of Ethris GmbH, a company that develops mRNA therapeutics. K.H., C.D., A.H.-J., V.K., and M.K.A. are employees of Ethris GmbH.

ACKNOWLEDGMENTS
Financial support by Ethris GmbH and the German Research Foundation, Excellence Cluster “Nanosystems Initiative Munich (NIM),” is gratefully acknowledged. We thank Dr. Daniela Emrich and Sarah Kern for their valuable contribution to the in vivo experiment.

REFERENCES
1. Sharom, F.J. (2008). ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics 9, 105–127.
2. Rich, J.N. (2007). Cancer stem cells in radiation resistance. Cancer Res. 67, 8980–8984.
3. Monsuez, J.J., Charniot, J.C., Vignat, N., and Artigou, J.Y. (2010). Cardiac side-effects of cancer chemotherapy. Int. J. Cardiol. 144, 3–15.
4. Monje, M., and Dietrich, J. (2012). Cognitive side effects of cancer therapy demonstrate a functional role for adult neurogenesis. Behav. Brain Res. 227, 376–379.
5. Kreitman, R.J., Stetler-Stevenson, M., Margulies, I., Noel, P., Fitzgerald, D.J., Wilson, W.H., and Pastan, I. (2009). Phase II trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with hairy cell leukemia. J. Clin. Oncol. 27, 2983–2990.
6. Eklund, J.W., and Kuzel, T.M. (2005). Denileukin diftitox: a concise clinical review. Expert Rev. Anticancer Ther. 5, 33–38.
7. Kreitman, R.J. (2006). Immunotoxins for targeted cancer therapy. AAPS J. 8, E532–E551.
8. Pastan, I. (1997). Targeted therapy of cancer with recombinant immunotoxins. Biochim. Biophys. Acta 1333, C1–C6.
9. Potala, S., Sahoo, S.K., and Verma, R.S. (2008). Targeted therapy of cancer using diphtheria toxin-derived immunotoxins. Drug Discov. Today 13, 807–815.
10. Pai-Scherf, L.H., Villa, J., Pearson, D., Watson, T., Liu, E., Willingham, M.C., and Pastan, I. (1999). Hepatotoxicity in cancer patients receiving erb-38, a recombinant immunotoxin that targets the erbB2 receptor. Clin. Cancer Res. 5, 2311–2315.
11. Pastan, I., Hassan, R., FitzGerald, D.J., and Kreitman, R.J. (2007). Immunotoxin treatment of cancer. Annu. Rev. Med. 58, 221–237.
12. Gofrit, O.N., Benjamin, S., Halachmi, S., Leibovitch, I., Dotan, Z., Lamm, D.L., Ehrlich, N., Yutkin, V., Ben-Am, M., and Hochberg, A. (2014). DNA based therapy with diphtheria toxin-A BC-819: a phase 2b marker lesion trial in patients with intermediate risk nonmuscle invasive bladder cancer. J. Urol. 191, 1697–1702.
13. Mizrahi, A., Czerniak, A., Levy, T., Amiur, S., Gallula, J., Matouk, I., Abu-lail, R., Sorin, V., Birman, T., de Groot, N., et al. (2009). Development of targeted therapy for ovarian cancer mediated by a plasmid expressing diphtheria toxin under the control of H19 regulatory sequences. J. Transl. Med. 7, 69.
14. Zhang, Y.H., Wang, Y., Yusufali, A.H., Ashby, F., Zhang, D., Yin, Z.F., Aslanidi, G.V., Srivastava, A., Ling, C.Q., and Ling, C. (2014). Cytotoxic genes from traditional Chinese medicine inhibit tumor growth both in vitro and in vivo. J. Integr. Med. 12, 483–494.
15. Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulfraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawluk, R., Morillon, E., et al. (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302, 415–419.
16. Rejman, J., Tavernier, G., Bavarsad, N., Demeester, J., and De Smedt, S.C. (2010). mRNA transfection of cervical carcinoma and mesenchymal stem cells mediated by cationic carriers. J. Control. Release 147, 385–391.
17. Yamamoto, A., Kornmann, M., Rosenacker, J., and Rudolph, C. (2009). Current prospects for mRNA gene delivery. Eur. J. Pharm. Biopharm. 71, 484–489.
18. Sahin, U., Kariko, K., and Tureci, O. (2014). mRNA-based therapeutics—developing a new class of drugs. Nat. Rev. Drug Discov. 13, 759–780.
19. Wang, Y., Su, H.H., Yang, Y., Hu, Y., Zhang, L., Blancafort, P., and Huang, L. (2013). Systemic delivery of modified mRNA encoding herpes simplex virus 1 thymidine kinase for targeted cancer gene therapy. Mol. Ther. 21, 358–367.
20. Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acadi, G., Jani, A., and Felgner, P.L. (1990). Direct gene transfer into mouse muscle in vivo. Science 247, 1465–1468.
21. Kariko, K., Muramatsu, H., Welsh, E.A., Ludwig, J., Kato, H., Akira, S., and Weissman, D. (2008). Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol. Ther. 16, 1833–1840.
22. Naylor, R., Ho, N.W., and Gilham, P.T. (1965). Selective chemical modifications of uridine and pseudouridine in polynucleotides and their effect on the specificities of ribonuclease and phosphodiesterases. J. Am. Chem. Soc. 87, 4209–4210.
23. Anderson, B.R., Muramatsu, H., Jha, B.K., Silverman, R.H., Weisman, D., and Kariko, K. (2011). Nucleoside modifications in RNA limit activation of 2′-5′-oligoadenylate synthetase and increase resistance to cleavage by RNAS L. Nucleic Acids Res. 39, 9329–9338.
24. Kornmann, M.S., Hasenpusch, G., Anjea, M.K., Nica, G., Flemmer, A.W., Herber-Jonat, S., Huppmann, M., Mays, L.E., Illenyi, M., Schams, A., et al. (2011). Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nat. Biotechnol. 29, 154–157.
25. Nallagatta, S.R., and Bevilaqua, P.C. (2008). Nucleoside modifications modulate activation of the protein kinase PKR in an RNA structure-specific manner. RNA 14, 1201–1213.
26. Backer, J.M., Kri沃shein, A.V., Hamby, C.V., Pizzonia, J., Gilbert, K.S., Ray, Y.S., Bradh, H., Paton, A.W., Paton, J.C., and Backer, M.V. (2009). Chaperone-targeting cytotoxin and endoplasmic reticulum stress-inducing drug synergize to kill cancer cells. Neoplasia 11, 1165–1173.
27. Gadadhar, S., and Karande, A.A. (2013). Abrin immunotoxin: targeted cytotoxicity and intracellular trafficking pathway. PLoS ONE 8, e58304.
28. Wawrzenzczak, E.J., Zangemeister-Wittke, U., Wabel, R., Henry, R.V., Parnell, G.D., Cumber, A.J., Jones, M., and Stahl, R.A. (1992). Molecular and biological properties of an abrin A chain immunotoxin designed for therapy of human small cell lung cancer. Br. J. Cancer 66, 361–366.
29. Pappenheimer, A.M., Jr. (1977). Diphtheria toxin. Annu. Rev. Biochem. 46, 69–94.
30. Paton, A.W., Sirmanote, P., Talbot, U.M., Wang, H., and Paton, J.C. (2004). A new family of potent AH5 cytotoxins produced by Shiga toxigenic Escherichia coli. J. Exp. Med. 200, 35–46.
31. Lin, J.Y., Lee, T.C., Hu, S.T., and Tung, T.C. (1981). Isolation of four isotopic proteins and one agglutinin from jequiriti bean (Abras precatorius). Toxicol. 19, 41–51.
32. Naglich, J.G., Metherall, J.E., Russell, D.W., and Eidel, L. (1992). Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. Cell 69, 1051–1061.

33. Bagaria, A., Surendranath, K., Ramagopal, U.A., Ramakumar, S., and Karande, A.A. (2006). Structure-function analysis and insights into the reduced toxicity of Abrus precatorius agglutinins I in relation to abrin. J. Biol. Chem. 281, 34465–34474.

34. Gill, D.M. (1978). Seven Toxic Peptides That Cross Cell Membranes (Academic Press), pp. 291–322.

35. Frankel, A.E., Rossi, P., Kuzel, T.M., and Foss, F. (2002). Diphtheria fusion protein therapy of chemoresistant malignancies. Curr. Cancer Drug Targets 2, 19–36.

36. Paton, A.W., Beddow, T., Thorpe, C.M., Whiststock, J.C., Wilce, M.C., Rossjohn, J., Talbot, U.M., and Paton, J.C. (2006). ABS subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. Nature 443, 548–552.

37. Endo, Y., and Tsurugi, K. (1987). RNA N-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. J. Biol. Chem. 262, 8128–8130.

38. Frankel, A.E., Powell, B.L., Hall, P.D., Case, L.D., and Kreitman, R.J. (2002). Phase I trial of a novel diphtheria toxin/granulocyte macrophage colony-stimulating factor fusion protein (DT388GMCSF) for refractory or relapsed acute myeloid leukemia. Clin. Cancer Res. 8, 1004–1013.

39. Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. Gene 234, 187–208.

40. Van der Jeught, K., Joe, P.T., Bialkowski, L., Heirman, C., Daszkiewicz, L., Talbot, U., van Oers, M.H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84, 1415–1420.

41. Endo, Y., Glück, A., and Wool, I.G. (1991). Ribosomal RNA identity elements for the main of the TGF-β receptor II: a novel TGF-β receptor II antagonist. Nature 353, 265–272.

42. Ye, J., Rawson, R.B., Komuro, R., Chen, X., Davé, U.P., Prywes, R., Brown, M.S., and Goldstein, J.L. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBP. Mol. Cell 6, 1355–1364.

43. Mishra, R., Kumar, M.S., and Karande, A.A. (2015). Inhibition of protein synthesis leading to unfolded protein response is the major event in abrin-mediated apoptosis. Mol. Cell. Biochem. 403, 255–265.

44. Hung, C.H., Lee, M.C., Chen, J.K., and Lin, I.Y. (1994). Cloning and expression of three abrin A-chains and their mutants derived by site-specific mutagenesis in Escherichia coli. Eur. J. Biochem. 219, 83–87.

45. Qiu, X., and Qing, L. (2004). Abrin induces HeLa cell apoptosis by cytochrome c release and caspase activation. J. Biochem. Mol. Biol. 37, 445–453.

46. Narayanan, S., Surendranath, K., Bora, N., Surolia, A., and Karande, A.A. (2005). Ribosome inactivating proteins and apoptosis. FEBs Lett. 579, 1324–1331.

47. Narayanan, S., Surolia, A., and Karande, A.A. (2004). Ribosome-inactivating protein and apoptosis: abrin causes cell death via mitochondrial pathway in Jurkat cells. Biochem. J. 377, 233–240.

48. Saxena, N., Yadav, P., and Kumar, O. (2013). The Fas/Fas ligand apoptotic pathway is involved in abrin-induced apoptosis. Toxicol. Sci. 135, 103–118.

49. Kooiman, G., Reutelingsperger, C.P., Kuijten, G.A., van Oers, M.H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84, 1415–1420.

50. Bhutta, S.K., Mallick, S.K., Maiti, S., Mishra, D., and Maiti, T.K. (2009). Abrus abrin derived peptides induce apoptosis by targeting mitochondria in HeLa cells. Cell Biol. Int. 33, 720–727.

51. Amit, D., Tamir, S., Birman, T., Gofrit, O.N., and Hochberg, A. (2011). Development of targeted therapy for bladder cancer mediated by a double promoter plasmid expressing diphtheria toxin under the control of IGF2-P3 and IGF2-P4 regulatory sequences. Int. J. Clin. Exp. Med. 4, 91–102.

52. Ramnath, V., Kuttan, G., and Kuttan, R. (2002). Antitumour effect of abrin on transplanted tumours in mice. Indian J. Physiol. Pharmacol. 46, 69–77.

53. Cattaneo, R., Miest, T., Shashkova, E.V., and Barry, M.A. (2008). Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. Nat. Rev. Microbiol. 6, 529–540.

54. Guo, Z.S., Liu, Z., and Bartlett, D.L. (2014). Oncolytic immunotherapy: dying the right way is a key to eliciting potent antitumor immunity. Front. Oncol. 4, 74.

55. Tavernier, G., Andries, O., De Smedt, S.C., and Rejman, K. (2009). The basis of oncoimmunology. Cell 135, 1355–1364.

56. Palucka, A.K., and Coussens, L.M. (2016). The basis of oncoimmunology. Cell 164, 1233–1247.

57. Jarzbietska, A., Pasewalt, T., Lambrecht, J., Mykhailyk, O., Kümmelring, L., Beck, P., Hasłupus, G., Rudolph, C., Plank, C., and Dohmen, C. (2016). A single methylene group in oligoalkylamine-based cationic polymers and lipids promotes enhanced mRNA delivery. Angew. Chem. Int. Ed. Engl. 55, 9591–9595.