RNA with chemotherapeutic base analogues as a dual-functional anti-cancer drug

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\textbf{ABSTRACT}

Nanoparticles of different sizes formulated with unmodified RNA and Protamine differentially engage Toll-like Receptors (TLRs) and activate innate immune responses \textit{in vitro}. Here, we report that similar differential immunostimulation that depends on the nanoparticle sizes is induced \textit{in vivo} in wild type as well as in immunized mice. In addition, we found that the schedule of injections strongly affects the magnitude of the immune response. Immunostimulating 130 nm nanoparticles composed of RNA and Protamine can promote lung metastasis clearance but provides no control of subcutaneous tumors in a CT26 tumor model. We further enhanced the therapeutic capacity of Protamine-RNA nanoparticles by incorporating chemotherapeutic base analogues in the RNA; we coined these \textit{immunochemotherapeutic RNAs} (icRNAs). Protamine-icRNA nanoparticles were successful at controlling established subcutaneous CT26 and B16 tumors as well as orthotopic glioblastoma. These data indicate that icRNAs are promising cancer therapies, which warrant their further validation for use in the clinic.

\textbf{INTRODUCTION}

RNA-based therapies hold great promise for cancer treatment as they can be used to block oncogenes (e.g. siRNA, antisense) or biological functions (i.e. aptamers), or express proteins of interest (antigens in vaccines or therapeutic proteins such as cytokines).\textsuperscript{1} RNA is also a natural danger signal recognized by several immune receptors and activating a broad range of cells including Dendritic cells (DCs), plasmacytid DCs (pDCs), B cells, monocytes and Natural Killer (NK) cells.\textsuperscript{2,3} These immunostimulatory properties of RNA (isRNA) also induce an IFN\textalpha response that enhances the recognition of tumor tissue by immune cells, by upregulating MHC molecules and enhancing antigen-presentation.\textsuperscript{4} As we have demonstrated previously, formulating RNA with the pharmaceutical compound Protamine, a natural cationic histone enables RNA to be protected against RNases and also facilitates its delivery into cells\textsuperscript{2,3,5,6} (reviewed in). Refining the formulation of Protamine-RNA permits the formation of nanoparticles of defined size for systemic administration, including intravenous injections.\textsuperscript{7} These are currently used in several clinical studies.\textsuperscript{9–12} The size of the particles can be precisely defined according to (i) the salt concentration in the solutions used to dilute Protamine and RNA, (ii) the ratio of Protamine to RNA and (iii) the concentration of both the Protamine and the RNA.\textsuperscript{8} Thus, we can generate particles from specifically 50–1,000 nm. Interestingly, the size of the particles impacts their innate immunostimulatory profile: in human peripheral blood nuclear cells (PBMCs) and in mouse splenocytes, particles less than 250 nm preferentially stimulate the production of Interferon-alpha (INF\textalpha) \textit{in vitro}, while larger particles stimulate the production of TNF-alpha (TNF\textgamma). At the weight ratios used here (more Protamine than RNA), the particles do not release the mRNA in the cytosol and thereby do not induce an adaptive immune response against the encoded antigen when administered \textit{in vivo} but solely an innate immune response through the triggering of innate immune sensors.\textsuperscript{13} We now evaluated the capacities of these particles to induce an immune response \textit{in vivo} that may control tumor growth in mice. We could find that \textit{in vivo} the particle size steer the type of induced innate immunity as seen \textit{in vitro} and that Protamine-RNA nanoparticles can be an efficacious anti-cancer reagent. However, this therapeutic activity was found only in restricted situations (lung metastasis and adequate application schedule). In order to improve the anti-cancer efficacy of Protamine-RNA nanoparticles, we took advantage of the nucleic acid nature of many anti-cancer drugs: the nucleotide analogs such as 5-fluorouracil (5FU), Gemcitabine, Pentostatin, Cladribine, AraC, Fludarabine and 6-mercaptopurine. We generated RNA containing such chemotherapy residues and could show here that this endowed RNA with dual biological activity: on top of innate immunostimulation, we bestowed them with cytotoxicity. We call these dual-purpose RNAs immunochemotherapeutic RNAs (icRNAs). The present manuscript discloses the immunostimulating activities of Protamine-RNA and Protamine-icRNA \textit{in vivo} and their potential as anti-cancer formulations.

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Materials and Methods:

Mice

6–8 week-old female and male animals were used throughout experiments. Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of the Zürich cantonal veterinary office and according to guidelines of the Swiss federal law on animal protection. All mice were kept in accordance with regulations from the Laboratory Animal Services Center (LASC) at the University Hospital of Zürich. Wild-type and Ifnar<sup>−/−</sup> mice were purchased from Envigo. SCID IL-2Rγ mice were purchased from Charles River.

Cell-lines

Murine colon carcinoma CT26 cells were stably transfected to express firefly luciferase and murine melanoma B16-F10 cells were maintained in RPMI medium (ThermoFisher Scientific) containing 10% fetal calf serum (FCS), 200 mM L-glutamine (Gibco) and 0.2% antimicrobial reagent Normocin (InvivoGen) (“Complete medium”). GL-261 glioma cell-lines were obtained from the National Cancer Institute (Frederick, MD) and cultured as described before. Cells were tested for pathogens and approved from LASC regulatory office for animal injections. In-house testing for mycoplasma was performed routinely using the PlasmoTest kit (Invivogen) as per the manufacturer’s instructions.

Oligonucleotides

Synthetic RNA oligonucleotides were obtained from Microsynth AG and from Dharmacon (Horizon Discovery Group Company). They were desalted after synthesis and sent to us lyophilized. Upon arrival in the laboratory, they were reconstituted at 5 mg/ml in RNase-free water and stored at −20°C. For experiments, the RNA stocks were dissolved in adequate buffers and formulated for utilization in vitro or in vivo.

Tumor models

Mice were inoculated subcutaneously with 1 × 10<sup>6</sup> CT26 or B16 tumor cells in 200 μl PBS. Tumor sizes were measured with a caliper every second day for calculating tumor volumes using the equation (a2 × b)/2 (a, width; b, length). Animals were sacrificed when exhibiting signs of impaired health or when tumor volume exceeded 1 cm<sup>3</sup>. For CT26 lung metastasis model, 1 × 10<sup>4</sup> tumor cells stably transfected to express firefly luciferase were injected intravenously. On days 3 and 10 post implantation, blocking was performed with protamine-RNA nanoparticles were injected intravenously as indicated.

Glioblastoma model: GL-261 cells (2 × 10<sup>4</sup>) were stereotactically implanted into the right striatum at day 0. Mice were observed daily and sacrificed as indicated or when developing neurologic symptoms. At day 5 and 10, mice received single intratumoral injections of reagents.

Protamine-RNA formulation:

Synthetic messenger RNA coding firefly luciferase was synthesized and purified as described previously, lyophilized and resuspended at 1 mg/ml in pure water. Protamine chloride (IPEX) was purchased from Meda and stored at 4°C. Protamine IPEX 5000 was diluted 14 times in pure water to provide a solution of protamine at approximately 1 mg/ml in low salt. One volume of RNA was mixed with two volumes of Protamine. Immediate and intensive mixing was performed and the formulation was left for 10 min at room temperature and was then further diluted with an excess (at least five volumes) of 5% glucose. In these conditions, the size of the Protamine-RNA particles is stable over time. Alternatively, 3 μl of a mixture containing 0.5 μg of RNA at 0.5 mg/ml in water and 1 μg of Protamine at 0.5 mg/ml in water (i.e. approximately 130 nm Protamine-RNA nanoparticles) was put in wells from a round bottom 96 well plate. 3 μl of non-salty (glucose 10%) or salty (PBS) solutions were added and incubated for 10 minutes at room temperature before addition of 200 μl of PBMCs.

ELISA

Blood was obtained from healthy donors, from which mononuclear cells were isolated using Ficoll- Paque™ Plus (GE Healthcare Life Sciences) method. A total number of one million cells per well were plated on 96-well plates and incubated overnight with appropriate RNA carrier containing 500 ng of RNA per well (200 μl cultures). Protamine-RNA particles were prepared as described previously. The next day supernatants were taken, and IFNα and TNFα concentrations were measured via ELISA by following the manufacturer’s protocol (Human IFNα; Mabtech, human TNFα; BioLegend). In mice, serum was taken 4 h after injection of Protamine/RNA particles, and measured for IFNα (MABTECH) and TNFα (BioLegend). The absorbance was measured at 450 nm with an ELISA reader (GloMax Discover and Explorer Detection System equipment, Promega) and cytokine concentrations were calculated according to a standard curve.

OT1 isolation and stimulation

C57BL/6-Tg(TcraTcrb)1100Mjb mice, also referred to as OT1 mice, were a generous gift from Pål Johansen (University Hospital Zurich). Spleen mononuclear cells from OT1 mice were isolated using the Ficoll-Paque™ Plus (GE Healthcare Life Sciences, Marlborough, MA) method. A total number of 150,000 cells per well (in 200 μl culture) were plated on 96-well plates and incubated for 24 h with 200 ng (1 μg /ml) of mRNA coding for Ovalbumin, formulated either in Lipofectamine MessenerMax (ThermoFisher) or in Protamine-RNA particles. After 24 h, supernatants were taken, and Interleukin-2 (IL-2) concentration was measured via ELISA, as per the manufacturer’s protocol (ELISA MAX Standard Set Mouse IL-2, Biolegend, San Diego, CA, USA). The absorbance was measured at 450 nm with an ELISA plate reader (GloMax Discover and Explorer Detection System equipment, Promega, Madison, WI, USA).
**C57Bl/6 Ova-vaccination**

Female C57Bl/6 mice of 4 to 8 weeks of age were injected subcutaneously in the neck with 2 μg of Ovalbumin-mRNA, formulated with either Lipid Nanoparticles or Protamine-RNA, with three total doses, each one week apart. One week after the third vaccination, mice were anaesthetized with Attane™ isoflurane ad. us. vet. (Minrad Inc., USA) and 200 μl blood was collected from the sublingual vein in EDTA treated BD Microtainer® tubes (BD Biosciences, USA) to perform flow cytometry analysis of SIINFEKL-specific CD8⁺ T-cells. Single cell suspension preparation and staining for flow cytometry

200 μl of whole blood was treated with 2 ml of eBioscience™ 1x RBC Lysis Buffer (Invitrogen, USA) and incubated for 5 min at room temperature (RT), vortexing every minute. Next, the cells were centrifuged at 500 g for 5 min, washed twice with 2 ml of PBS and resuspended in 50 μl PBS. Cells first underwent live-dead staining with Zombie NIR Fixable Viability Kit (Biolegend), according to the manufacturer’s protocol. Subsequently, murine lymphocytes were stained with 2.5 μl PE-conjugated SIINFEKL Tetramer H-2Kb (The Tetramer Shop, Kongens Lyngby, Denmark) and incubated for 15 min at RT in the dark. Without washing, 50 μl of FITC-conjugated anti-CD3 (BioLegend, San Diego CA, USA), and Pacific Blue-conjugated anti-CD8α KT15 (BioRad, Hercules CA, USA)antibodies, at a final dilution of 1:200 in PBS, were added and the cells were incubated for 1 h at 4°C in the dark. In a next step, cells were washed twice with 200 μl IX PBS and fixed with 1% paraformaldehyde (PFA) in PBS for 30 min at 4°C in the dark. Subsequently, the cells were washed twice with 200 μl PBS, resuspended in 200 μl PBS and stored at 4°C in the dark until sample acquisition with BD LSRFortessa™ (BD Biosciences, New Jersey, NJ, USA) and BD FACSDiva™ Software (BD Biosciences, New Jersey, NJ, USA). Compensation was performed by staining OneComp eBeads™ Compensation Beads (Invitrogen, USA) with the respective antibodies used for staining the T-cells. The obtained data were analyzed and plotted using FlowJo™ (BD Biosciences, New Jersey, NJ, USA).

**Size nanoparticles**

Protamine-RNA particles containing 10 μg of mRNA were suspended in one milliliter of 5% glucose solution in pure water, and the size distribution was characterized by dynamic light scattering with a ZetaSizer 3000HSA (Malvern Instruments, Worcestershire, UK). Data were analyzed with the built-in DTS software (Raleigh, NC).

**PEGylation of nanoparticles**

Protamine was diluted in HEPES 10 mM pH = 8 or PBS pH = 7.4 to concentration of 1 mg/mL. PEG-SPA and PEG-Folate (Nanocs) powder were diluted in DMSO to concentration of 10 mg/mL. PEGs were added to protamine with indicated molar excess (1–30x), mixed by pipetting and left for 2 h in room temperature. After that time the reaction was stopped by freezing the samples in −20°C. Samples were stored in −20°C until further use.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was carried out using 5–20% polyacrylamide gels. Ten micrograms of PEGylated Protamine solutions were mixed with 2x NuPAGE LDS sample buffer (Invitrogen, Carlsbad, MA) and 1 M dithiothreitol (DTT) solution at a ratio of 5:4:1 (sample in water:LDS:DTT). Samples were loaded on the gel and subjected to electrophoresis. After being separated, the proteins were visualized by staining with GelCode™ blue safe protein stain (Thermo Fisher Scientific, Waltham, MA).

**Cell survival assay**

Cells (5000 cells per well) were seeded in 300 μl of complete medium in a 48-well plate. One day later, supernatants were replaced by 200 μl of complete medium. Increasing concentrations of free 5FU or Protamine/RNA nanoparticles incorporating chemotherapeutic purine analogues Ribavirin, Nebularine or 5FU were added to the cells. Protamine/RNA was used as a controls. After three days cell cytotoxicity is measured using the Cell Counting Kit-8 (CCK-8, Sigma). Cells were incubated with CCK-8 solution and incubate at 37°C for 6 h. Absorbance is measured at OD 450 nm using the GloMax Discover and Explorer Detection System equipment (Promega).

**Bioluminescent in vivo imaging**

In vivo bioluminescence of tumor-bearing mice was determined using an IVIS Lumina (PerkinElmer) at specific time points as indicated. D-Luciferin (Synchem) dissolved in PBS (15 mg/ml stock) and sterile filtered, was injected (150 μg/g) intraperitoneally into mice. Emitted photons from live animals were quantified 10–20 minutes post Luciferin injections with an exposure time of 3 min. Regions of interest (ROI) were quantified for average radiance (photons s−1 cm−2 sr−1) (IVIS Living Image 4.0).

For biodistribution experiments with PEGylated Protamine particles, 5 μg of Cy5.5-labeled oligonucleotides (Microsynth) formulated in indicated particle types in 150 μl were injected intravenously into CT26 tumor-bearing BALB/c mice, 4 days post tumor implantation. In the process of injection and image acquisition, mice were anesthetized with isoflurane and body temperature was maintained by a thermostat-controlled thermal heating panel. Fluorescent signal localization was measured at 0, 20, 40, 60 and 120 min post injection, with exposure time of 120 s. A laser source of 660 nm was used to excite the fluorophore, and an emission filter of 710 nm was used for detecting fluorescence.

**Results**

**Immunostimulating capacities of Protamine-RNA nanoparticles in vivo**

As Protamine-RNA particles (where the RNA is chemically or enzymatically synthesized and contains A, C, G and U residues, Supplementary Figure 1) of different sizes display distinctive immuno-stimulating properties in vitro, we sought to investigate their capabilities to induce IFNα and anti-tumor responses in vivo. For in vivo experiments, it is necessary to formulate Protamine-RNA particles in isotonic buffer. However, we...
noticed that salty solutions such as PBS alter the size of Protamine-RNA nanoparticles, which became more enlarged over time (Figure 1a) and endowed them with immunostimulating capacities of large RNA particles (i.e. no induction of IFNa) (Figure 1b). In contrast, nanoparticles formulated in non-salty isotonic solutions such as 5% glucose retained their original particle size and consequently the immunostimulating features of RNA nanoparticles (Figure 1b). Thus, to preserve the size and immunostimulatory characteristics of Protamine-RNA particles, the isotonic formulation for injection should contain little or no salts, but for example polysaccharides.

To test the immunostimulatory properties of Protamine-RNA nanoparticles of different sizes in vivo, mice were injected with 20 μg of non-modified mRNA coding firefly luciferase formulated with 40 μg Protamine in conditions providing particles of expected size from 55 nm to 570 nm in average. Blood was taken 4 h post-injection and IFNa and TNFa serum levels were assessed. The smallest particles formulated in 5% glucose-induced IFNa and TNFa secretion, whereas largest particles generated a TNFa response but fail to induce IFNa secretion (Figure 1c). Similarly, in humanized SCID IL-2Ry-null mice, nanoparticles of 130 nm size formulated in 5% glucose induced a stronger human type I interferon response, while larger particles induced a stronger human TNFγ response (Figure 1d). Collectively, these data demonstrate that Protamine-RNA particles formulated in a 5% glucose isotonic buffer recapitulate in vivo the immune responses recorded in vitro for the different sizes of RNA particles. Previously, Bourquin and colleagues reported that the TLR7/8 agonist R848 induced a more potent IFNa response in vivo when administered in two doses with a 6 h interval. To determine whether a similar synergistic immune response could also be achieved with type I interferon-inducing Protamine-RNA nanoparticles (130 nm), two injections of these nanoparticles were given 2, 4, 5 or 6 h apart. 20 μg Protamine-RNA in a single injection induced a detectable IFNa response (as seen in Figure 1c with 123 nm particles), however, this was potently enhanced when the dose was administered in two separate injections of 10 μg each (Figure 2a “1 injection” versus “2 h delay” or “4 h delay”). Moreover, the optimal delay between injections was 2 h, which yielded the strongest IFNa response. This response was specific to type I interferons as the TNFa secretion achieved with a single injection was not enhanced (indeed it was even lower probably due to the dose of RNA given at a second injection being half of the dose of a single injection) when fractionated into two separate injections (Supplementary Fig. 2A). As this effect was specific for IFNa, we speculated that the first injection induced the type I Interferon response that upregulates TLR7 expression, as reported previously in B cells, leading to a greater IFNa response with the second injection. To test this, Interferon-alpha/beta receptor (Ifnar)-deficient mice were injected with 20 μg Protamine-RNA nanoparticles separated in two doses, given 2 h apart. Ifnar- mice failed to induce a potent IFNa in response to the double injection of Protamine-RNA compared to wild-type animals.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Protamine-RNA nanoparticles require formulation in isotonic sugar solution to retain immunostimulating anti-tumor properties in vivo. (a) Nanoparticle size in different solutions was determined by measuring Dynamic Light Scattering (DLS) using a Zetasizer (Malvern). (b) Human PBMCs were incubated with 130 nm Protamine-RNA nanoparticles formulated in 5% glucose or PBS for 24 h and IFNa and TNFa production were measured in supernatants by ELISA. (c) Age-matched and sex-matched BALB/c mice received an intravenous injection of 20 μg mRNA coding luciferase formulated with 40 μg Protamine formulated in increasing concentrations of NaCl. 4 h later blood was taken and serum levels of murine IFNa and TNFa were measured by ELISA (n = 6 mice per group). (d) Humanized SCID IL-2Ry-null mice were injected with Protamine-RNA nanoparticles of 130 and 250 nm size formulated in 5% glucose and PBS, respectively. 4 h later blood was taken and serum levels of human IFNa and TNFa were measured by ELISA. Data represent mean ± SEM (n = 3 mice per group) are representative of 2 independent experiments (B). Data were analyzed with 1-way ANOVA (A) or 2-way ANOVA (C and D) followed by Dunnett’s multiple-comparisons test. *P < .05; **P < .01; ***P < .001; ****P < .0001.
(Supplementary Fig. 2B; induction of TNFα by a double injection was also somewhat lower in Ifnar−/− mice than in wild type animals but the difference did not reach significance with a p value of 0.0577, Supplementary Fig. 2C). This confirms that a moderate IFNa response can trigger a transient state of enhanced sensitivity to danger signals, allowing the immune system to strongly respond to persisting, recurring or increasing innate stimulation as is the case when a pathogen is proliferating.

**Anti-cancer capacities of Protamine-RNA nanoparticles**

To determine the anti-tumor capacities of type I interferon-inducing 130 nm Protamine-RNA particles in vivo, BALB/c mice were implanted in the lungs with CT26-luciferase cells by intravenous injection. On Day 3 and 10 post tumor cell implantation, mice were administered 20 μg non-modified RNA encapsulated in Protamine (130 nm particle size). Protamine-RNA was administered in a single injection (20 μg RNA) or in two injections (10 μg RNA each) and luciferase expression was assessed by IVIS starting on day 3 (Figure 2b and 2c). Luciferase expression shows clear homing and growth of CT26 cells to the lung (Figure 2b). Mice receiving two injections (two h apart) of RNA in 130 nm Protamine particles, on day 3 and 10, show more efficient clearance of luciferase-expressing tumor cells than mice receiving the same total RNA dose in a single injection on both days (Figure 2c). Protamine-RNA nanoparticles containing high amounts (over half of the mass amount of RNA, in the present experiment Protamine mass amount is twice the one of RNA) do not induce T-cell responses against the encoded antigen, since the RNA is not efficiently released to the cytosol (and Supplementary Figure 3A, B). Thus, we attribute the anticancer effect of this approach to the activation of the innate immune response by the administration of Protamine-RNA nanoparticles. Still, T-cells may be induced secondary to the activation of the innate immune response and the death of tumor cells. This aspect is being studied in ongoing studies investigating the combination of anti-PD1 treatment and Protamine-RNA administrations. While Protamine-RNA nanoparticles were
effective at controlling progression of lung metastasis subsequent to intravenous injection of CT26 cells, the same approach failed to consistently curtail subcutaneous CT26 tumor growth (Figure 2d). These data demonstrate that two synergistic intravenous injections of Protamine-RNA, given two h apart, confer an enhanced IFNa response, which provides an anti-tumor effect in vivo, though this is not adequately efficacious against all locations of the tumors.

**RNA containing chemotherapeutic residues**

To augment anti-tumor effects of Protamine-RNA, we incorporated the chemotherapeutic pyrimidine 5-fluorouracil (5FU) into the RNA in place of uracil in a synthetic RNA oligonucleotide (RNA/5FU) with a random U-rich sequence (21-mer oligo containing 11 U residues) (Supplementary Fig. 4A). 5FU is a uracil analogue with a fluorine atom at the C-5 position. Metabolites of 5FU firstly, limit availability of nucleotides by inhibiting the enzymatic function of thymidylate synthase, which catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), and secondly are misincorporated into DNA and RNA. Both mechanisms lead to DNA damage and cell death in rapidly dividing cells. 5FU is used for the treatment of a number of cancers, in particular in colorectal cancer management. Proliferation of the mouse colon carcinoma CT26 cells was reduced in the presence of increasing concentrations of Protamine-RNA/5FU nanoparticles even more than in cells treated with identical amounts of the standard free 5FU (Figure 3a). This correlates to the observed higher chemotherapeutic activity of the modified ribonucleoside versus the free base (Supplementary Fig. 4B). When compared against other RNAs incorporating chemotherapeutic purine analogues Ribavirin (RBV) and Nebularine (Nebu), RNA oligonucleotides incorporating 5FU were the superior RNAs at deterring CT26 proliferation (Supplementary Fig. 4C). Protamine-RNA/5FU nanoparticles retained their immunostimulatory effects on human PBMCs and induced IFNa secretion (Figure 3b). Therefore, we termed these dual-purpose immunostimulatory and chemotherapeutic RNAs: immunochemotherapeutic RNAs (icRNAs).

Next, we assessed whether icRNA in Protamine nanoparticles could induce stronger anti-tumor effects in vivo than isRNA in Protamine nanoparticles. BALB/c mice were subjected to subcutaneous implantation of CT26 cells. On Day 4 and 11-post tumor cell implantation, mice were administered with two intravenous injections (10 μg each, two h apart) of Protamine-RNA/5FU (i.e. immunochemotherapeutic) or Protamine-RNA (mRNA coding luciferase, that is, immunostimulating only) 130 nm particles. Mice receiving Protamine-RNA/5FU showed a greater control of subcutaneous CT26 tumor volume compared to mice receiving Protamine-RNA (Figure 3c), which failed to give robust anti-tumor effects (also Figure 2d). This difference could be attributed to the cytotoxic effect of 5FU, as both nanoparticles induced similar levels of IFNa 4 h after i.v. injection (Figure 3d). Despite the increase in efficacy at clearing CT26 subcutaneous tumors, icRNA nanoparticles failed to control tumors in the subcutaneous B16 melanoma model (Supplementary Fig. 4D), even though 5FU is efficacious at inhibiting B16 melanoma cell proliferation in vitro (Supplementary Fig. 4E).

**Intra-tumor injection of Protamine-icRNA nanoparticles**

Decorating particles with PEG or PEG folate did not enhance their accumulation at the tumor site after intravenous injection (Supplementary Figure 5). Therefore, we proceeded with intra-tumoral injections for hard-to-treat tumors. To further investigate tumor clearance of subcutaneous B16 tumors, we used intra-tumor injection of Protamine-RNA/5FU nanoparticles for the first injection, followed by i.v. injection of the second dose, two h later. C57Bl/6 mice receiving Protamine-RNA/5FU nanoparticles showed significant reduction in tumor volume at Day 17 compared to mice treated with free 5FU or Protamine-RNA nanoparticles (Figure 3e). These potent anti-tumor effects were not restricted to subcutaneous tumors as treatment of established tumors in an orthotopic murine glioblastoma model with Protamine-RNA/5FU nanoparticles also significantly enhanced survival in this model (Figure 3f). In this model as in the B16 model, the intra-tumor injection of unmodified RNA in Protamine nanoparticles (PR Luc) did not induce an anti-cancer response.

**Discussion**

Here we describe dual-action Protamine-RNA nanoparticles that can induce the inhibition of tumor progression in vivo. Firstly, we report that the isotonic buffer is a crucial ingredient for Protamine-RNA nanoparticle formulations, as salt buffers, such as PBS, alter the desired nanoparticle size. This has a functional impact, as larger-sized nanoparticles induce preferably TNFα while smaller particles formulated in 5% glucose induce preferably IFNa secretion in vivo.

Previously, it was reported that repetitive stimulation with TLR7 agonists causes TLR tolerance, and completely blocks the induction of IFNa, IL-6 and TNFα production. This limitation is overcome by repetitive treatment at short intervals. Similarly, we observed that fractionated treatment of 130 nm Protamine-RNA particles in two intravenous injections with a 2 h interval gives the maximal type I interferon response, provoking augmented IFNa release compared to the same dose delivered in a single injection. This effect was dependent on IFNAR, suggesting that secretion of type I interferons induced by the first dose upregulates TLR7 signaling, which leads to a greater response to the subsequent dose. The same effect was not seen with TNFα and it highlights the substantial threat that the immune system considers viral infections to pose. The innate immune response anticipates a “signal of increasing danger” by upregulating pattern recognition receptor expression, thus an increasing viral threat will be met with a greater immune response. This has an important consequence for anticancer therapies based on administration of danger signals as it indicates a second injection within few hours would lead to a more robust priming of the immune response.

While isRNA nanoparticles induce a strong type I interferon response that can control lung metastasis in mice, the same approach was less successful against
subcutaneous tumors. This could be due to a greater accessibility of the lung tumor to immune cells due to increased vascularity of lung tissue. To circumvent these limitations we generated a more potent anti-cancer RNA. Significantly, we observed that substituting uracil with chemotherapeutic pyrimidine 5FU bestowed the RNA oligo with a dual mechanism of action; they retained their ability to stimulate the innate immune response but now also exerted potent cytotoxic effects against rapidly dividing cells. These immunotherapeutic RNA (icRNA) nanoparticles demonstrate more effective killing of tumor cells in vitro and in vivo in several tumor models employed here. Tumor homing of icRNAs particles is an aspect that might be further optimized using adequate ligands (although the tested modifications: PEG and Folate PEG so far did not enhance tumor homing of Protamine-RNA nanoparticles) or other particle formulations (lipoplexes, polyplexes or lipo-polyplexes), however, in their current form, icRNAs offer a novel, safe and effective approach that will expand and complement the current panoply of cancer immunotherapies.

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Disclosure statement

Steve Pascolo is the inventor on a patent "US10980875B2 Cytotoxic immunostimulating particles and uses thereof." The other authors report there are no competing interests to declare.

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Data availability statement

Data associated with this paper can all be found within the article and Supplemental online material. http://dx.doi.org/10.1080/2162402X.2022.2147665

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