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Suicide nanoplasmids coding for ribosome-inactivating proteins

Hardy Mitdank [a,1], Meike Tröger [a,1], Alexander Sonntag [a], Nima Amini Shirazi [a], Eric Woith [a], Hendrik Fuchs [b], Dennis Kobelt [c], Wolfgang Walther [c,d], Alexander Weng [a,*]

[a] Freie Universität Berlin, Institut für Pharmazie, Königin-Luise-Str. 2+4, 14195 Berlin, Germany
[b] Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Institute of Laboratory Medicine, Clinical Chemistry and Pathobiochemistry, Augustenburger Platz 1, 13353 Berlin, Germany
[c] Experimental Pharmacology & Oncology Berlin-Buch GmbH, Robert-Rössle-Str.10, 13125 Berlin, Germany
[d] Experimental and Clinical Research Center, Charité Universitätsmedizin Berlin and Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Str. 10, 13125 Berlin, Germany

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ABSTRACT

Conventional eukaryotic expression plasmids contain a DNA backbone that is dispensable for the cellular expression of the transgene. In order to reduce the vector size, minicircle DNA technology was introduced. A drawback of the minicircle technology are considerable production costs. Nanoplasmids are a relatively new class of mini-DNA constructs that are of tremendous potential for pharmaceutical applications. In this study we have designed novel suicide nanoplasmid constructs coding for plant derived ribosome-inactivating proteins. The suicide-nanoplasmids were formulated with a targeted K_{16}-lysine domain, analyzed for size, and characterized by electron microscopy. The anti-proliferative activity of the suicide-nanoplasmids was investigated *in vitro* by real time microscopy and the expression kinetic was determined using an enhanced green fluorescent protein nanoplasmid variant. In an aggressive *in vivo* neuroblastoma tumor model, treated mice showed a reduced tumor growth whereby the therapy was well tolerated.

1. Introduction

Conventional vectors (plasmids) contain genes for resistance factors that are necessary for the propagation in *Escherichia coli*. According to the European Medicines Agency (EMA), resistance genes should be avoided in the context of gene therapy (EMA, 2018), in order to exclude the transfer of an antibiotic resistance to the human microbial fauna. In addition, the bacterial backbone is being prone to induce an immune reaction since it is recognized as Pathogen Associated Molecular Pattern (PAMP). This effect is mediated by the activation of toll-like receptors (TLR9) (Klinman, 2004). With the introduction of minicircle technology (Kay et al., 2010) these factors were taken into account. Moreover, minicircle DNA is significantly smaller than the corresponding plasmid DNA, resulting in higher transfection efficacies (Hardee et al., 2017). However, a major drawback of this technology is the complex and cost-intensive production process starting with a parental plasmid from which the minicircle DNA is generated during propagation in genetically modified *E. coli*.

An advanced development in the field was the invention of the nanoplasmid technology (Luke et al., 2009; Luke et al., 2014). Nanoplasmids are small circular DNA constructs, which can be produced in large yields and without antibiotic resistance genes, while showing up to 10 times higher transgene expression compared to minicircle vectors (Lu et al., 2017). Only few studies have been published, mainly dealing with the development of nanoplasmid DNA vaccine vectors (Chowdhury et al., 2020; Suschak et al., 2020).

The concept of suicide vectors is based on the delivery of a DNA construct that encodes for a toxic factor (Navarro et al., 2016). Among the toxins the sequence of diphtheria toxin (DT) from *Corynebacterium diphtheriae* is one that is often used. DT targets the ribosomal protein machinery by ADP-ribosylation of elongation factor 2 (EF2) (Lin et al., 2015). Pseudomonas exotoxin A (PE) from *Pseudomonas aeruginosa* belongs to the same class of enzymes and acts identically via ADP-ribosylation of EF2. The DNA encoding PE has been used for the treatment of breast cancer stem cells (Moradian and Rahbarizadeh, 2019). The genetic information of further pore-forming toxins such as streptolysin O (SLO) from *Streptococcus pyogenes* (Yang et al., 2006) has been utilized, reviewed in (Pahle and Walther, 2016). A further class of

* Corresponding author
E-mail address: alexander.weng@fu-berlin.de (A. Weng).
[1] Authors contributed equally

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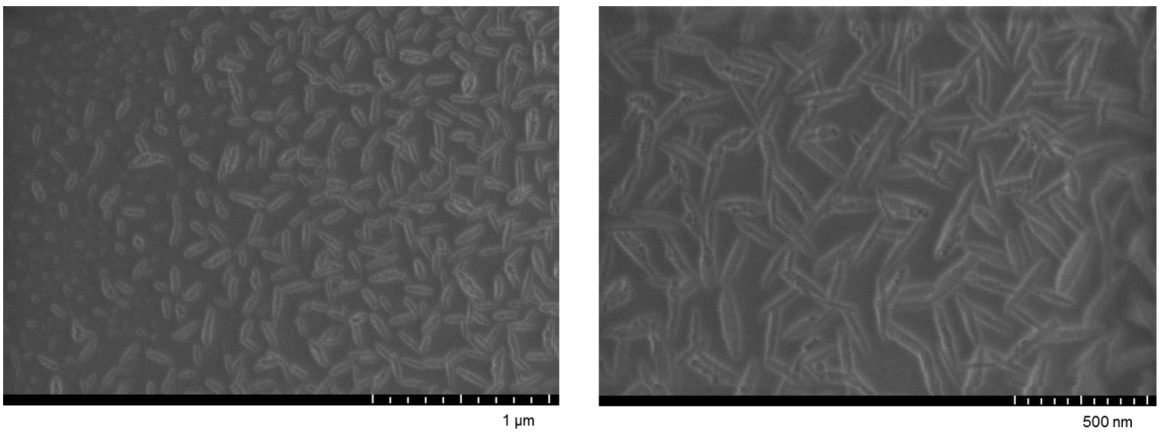

Fig. 1. SEM-images of targeted suicide nanoplexes (Sap-NP). Analysis revealed rod like structures.

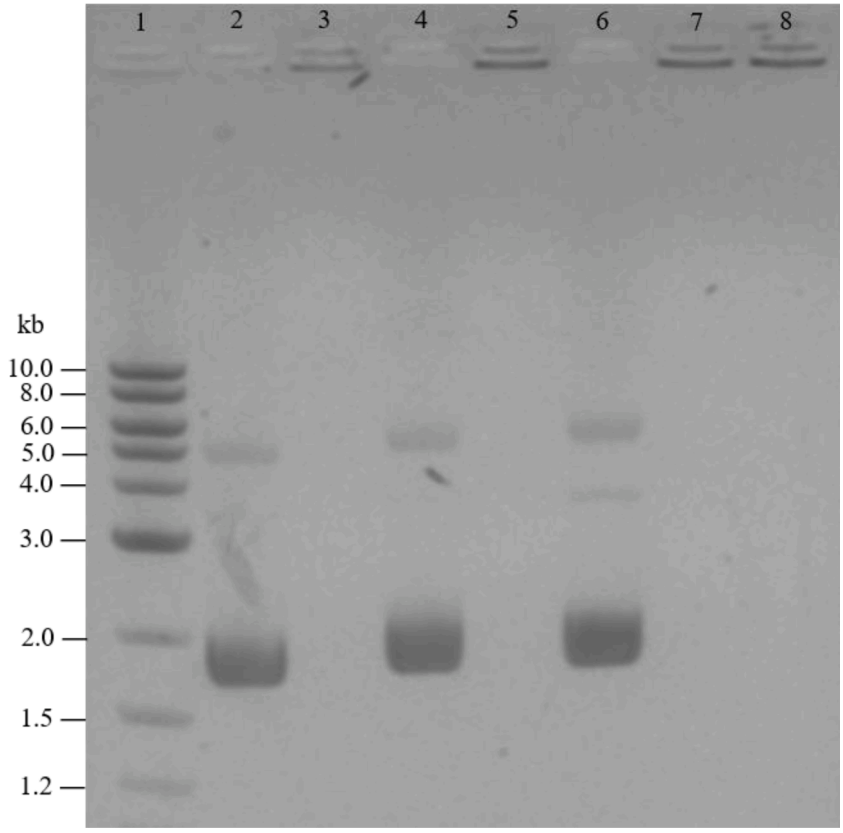

Fig. 2. Agarose gel retention analysis of formulated nanoplexes. Lane 1: DNA Ladder labeled with corresponding number of kilobases, lane 2: eGFP-Nanoplasmid, lane 3: eGFP-Nanoplex (4:1), lane 4: Gyp-Nanoplasmid, lane 5: Gyp-Nanoplex (7:1), lane 6: Sap-Nanoplasmid, lane 7: Sap-Nanoplex (4:1), lane 8: Sap-Nanoplex (7:1). In all lanes with formulated nanoplexes (3, 5, 7, and 8) no free DNA was detected, showing complete complexation of the used nanoplasmids by the targeted peptide.

Table 1
Hydrodynamic diameter (D_H), polydispersity index (PdI) and ζ-potential of formulated nanoplexes. Samples were measured in triplicate after 30 min incubation at room temperature, mean value ± standard deviation is reported. All tested formulations gave rise to cationic nanoparticles, with ζ-potentials between + 29 and + 37 mV and D_H between 46 and 61 nm, PdIs < 0.3.

	D_H [nm]		PdI		ζ-Potential [mV]	
eGFP-Nanoplex (4:1)	57.34	± 0.2	0.14	± 0.01	29.4	± 3
Sap-Nanoplex (4:1)	60.88	± 4	0.27	± 0.02	33.2	± 2
Sap-Nanoplex (7:1)	46.46	± 2	0.22	± 0.03	31.2	± 3
Gyp-Nanoplex (7:1)	53.01	± 0.7	0.221	± 0.003	36.6	± 1

toxins that have been investigated are type I ribosome-inactivating proteins (RIPs) from plants. Type I RIPs act as N-glycosylases (EC 3.2.2.22) and irreversibly inactivate protein synthesis by removing an essential adenine at position 4324 of the ribosomal rRNA (Endo, 1988). One advantage of type I RIPs for anti-tumoral applications is their high resistance against denaturation (Kokorin et al., 2019; Weise et al., 2020) and thus high stability. They show high enzymatic activity and in theory, one molecule of a RIP is sufficient to kill a cancer cell. It is therefore not surprising that RIPs have been used in suicide gene cancer studies (Sama et al., 2018a; Zarovni et al., 2009; Zarovni et al., 2007). However, in these studies conventional plasmid vectors were used to translocate the RIP genes into the nucleus of the target cells. To our knowledge,

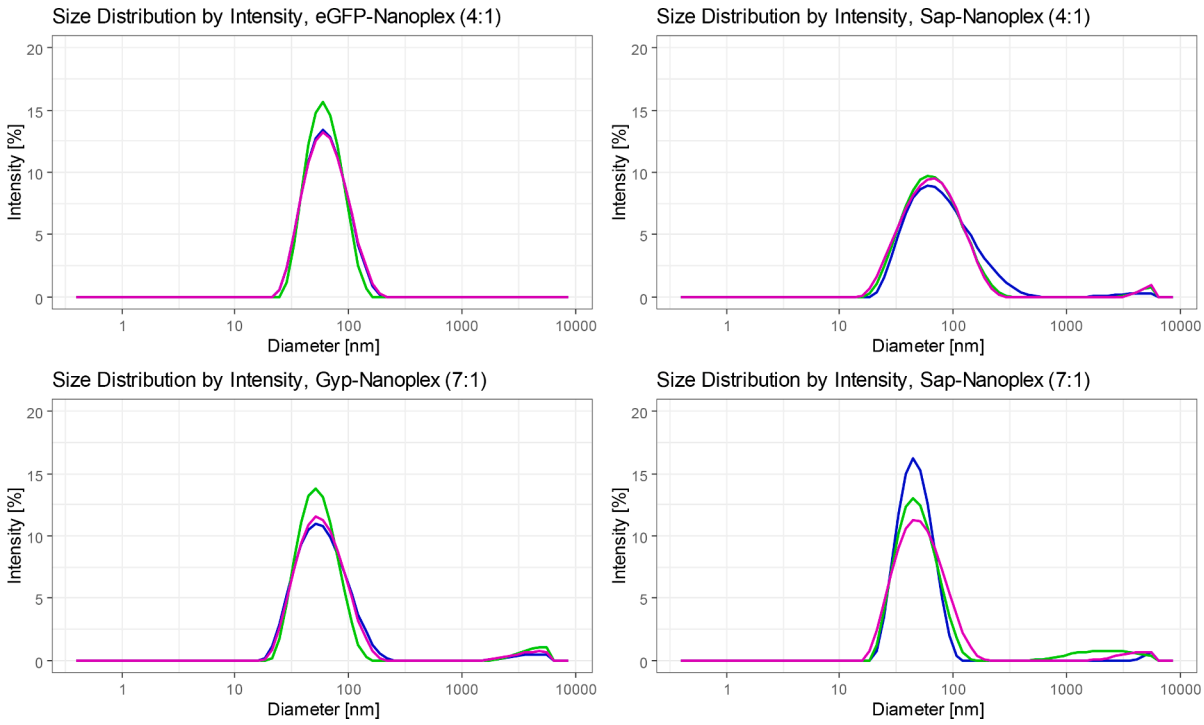

Fig. 3. Size Distribution of formulated nanoplexes. The different colors represent individual measurements of the same sample. All measured nanoplexes exhibit a relatively narrow size distribution with average hydrodynamic diameters < 100 nm. In the case of Sap- and Gyp-nanoplexes, a minor share with diameters > 1 μm is observed, probably due to aggregate formation.

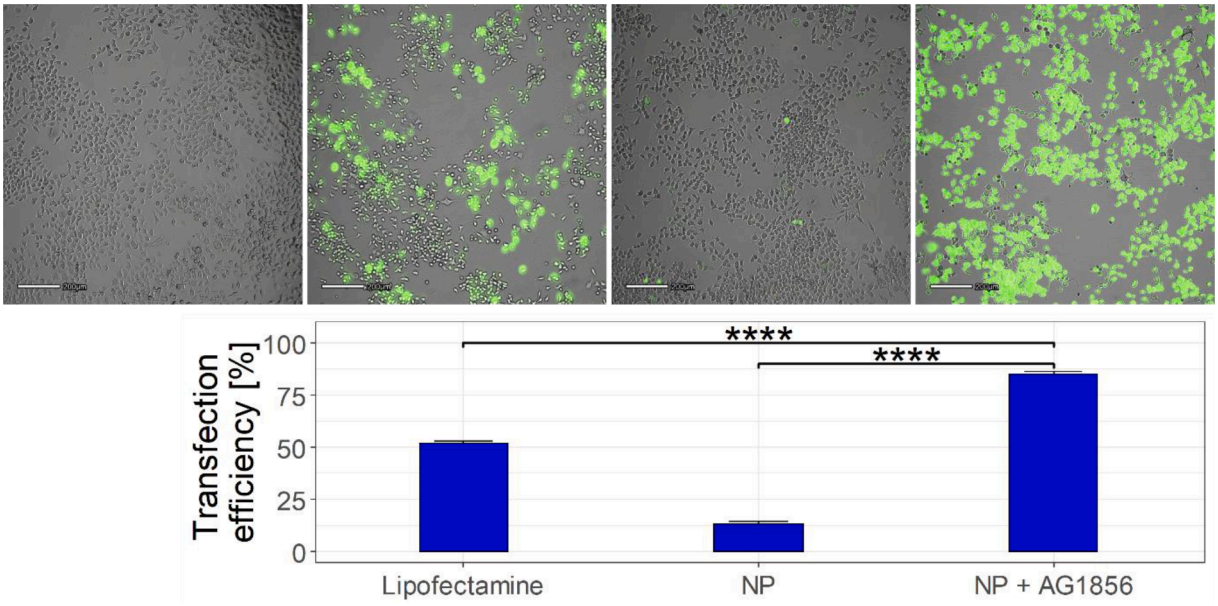

Fig. 4. Transfection efficacy of eGFP-nanoplasmids. Neuro2A cells were transfected with eGFP-nanoplasmids using peptide Y ± AG1856 (5 μg/ mL) and lipofectamine. After 48 h cells were analyzed by fluorescence microscopy (upper panel) and flow cytometry (lower panel). Upper panel, from left to right: Control cells, eGFP-nanoplasmid/ lipofectamine, eGFP-nanoplasmid/ peptide Y (NP), eGFP-nanoplasmid-peptide Y/AG1856 (NP + AG1856). Lower panel: Transfection efficacy. The formulation NP (nanoplasmid/ peptide Y) + AG1856 (5 μg/mL) showed the highest transfection efficacy. Results are expressed as mean ± SD, n=3. Two-sided t-test, ****$p \leq 0.0001$.

there are no published studies where a nanoplasmid vector was utilized for delivering a gene coding for a toxic factor.

In this study we have designed two suicide nanoplasmid vectors. Nano-Sap encodes for saporin, which is a type I RIP from the plant *Saponaria officinalis* L. and well known in the context of cancer therapies (Polito et al., 2013). *In vivo* anti-cancer studies with saporin have been performed in the past (Falini et al., 1992). The second suicide nanoplasmid vector (Nano-Gyp) was constructed with the cDNA of a newly discovered type I RIP, gypsophilin-S from the organism *Gypsophila elegans* M. Bieb. (Kokorin et al., 2019). Both suicide nanoplasmid vectors were formulated with a receptor-targeted K_{16}-lysine domain. *In vitro* studies were performed using neuroblastoma cells and the *in vivo*

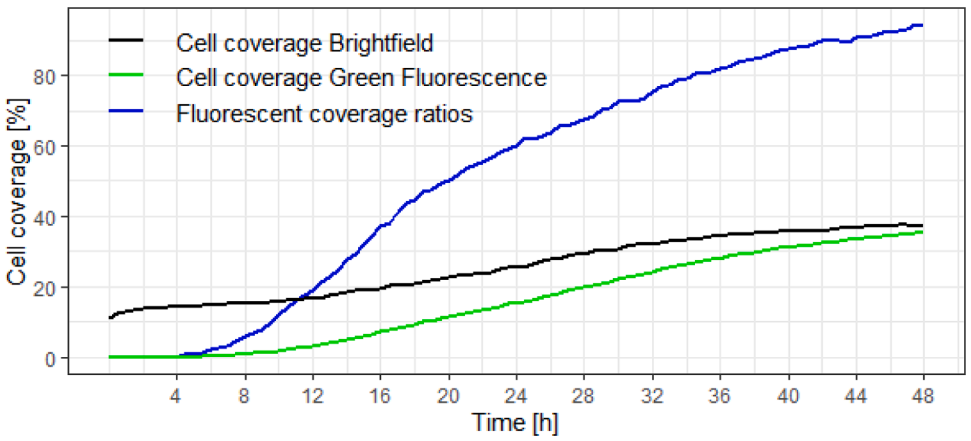

Fig. 5. Kinetics of eGFP expression in Neuro2A cells after transfection with eGFP-nanoplexes, supplemented with 5 µg/mL AG1856. Cell coverage in percent in both the brightfield and the green (excitation: 452 ± 45 nm) channel were determined by the CytoSMART® Cloud Service by means of image analysis. Fluorescent coverage ratio is determined by Cell coverage Green Fluorescence [%]/ Cell coverage Brightfield [%] * 100 % and is indicating the share of eGFP-expressing cells. The expression of eGFP started 6 h after transfection and the proportion of eGFP-expressing cells constantly increased during the 48-hour incubation period.

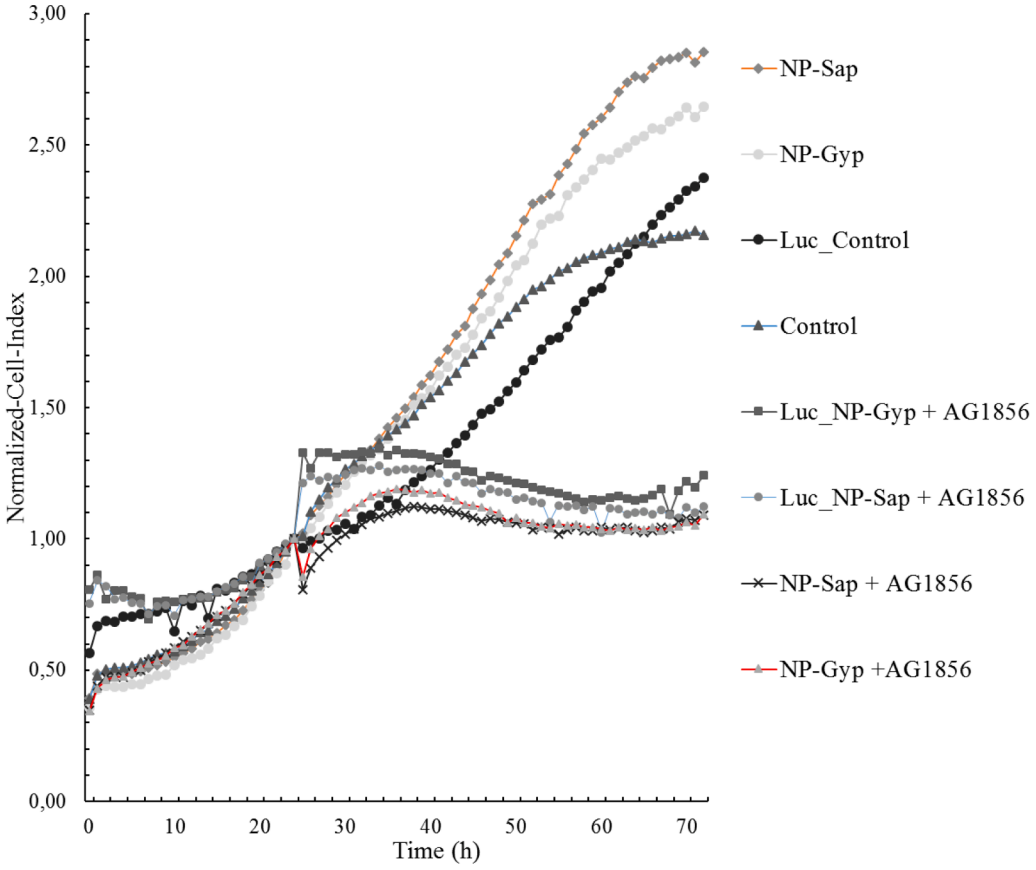

Fig. 6. Live cell imaging (CytoSMART® Omni) of the efficacy of targeted suicide nanoplexes in neuroblastoma cells. Neuro2A and Neuro2A_Luc cells were treated with targeted nanoplexes ± AG1856. Cells treated with both components showed strong inhibition of cell proliferation. Cell proliferation was normalized to the point of intervention (addition of compounds, t = 24 h). Both groups showed a significantly reduced growth in comparison to the negative control and the AG1856-free groups (U-Test, $p<0.05$).

efficiency was investigated in a corresponding tumor model with tumor bearing nu/nu NMRI mice. To increase the transfection efficacy of the complexed suicide nanoplasmid vectors, a transfection enhancer that we have recently identified in the organism *Agrostemma githago* L. was applied as well (Clochard et al., 2020).

2. Materials and methods

2.1. Construction of suicide nanoplasmids

Starting with the amino acid sequence of gypsophilin-S (Gyp) – a type I ribosome-inactivating protein from *Gypsophila elegans* M. Bieb. (Kokorin et al., 2019) – the cDNA was generated by gene synthesis and integrated in a mammalian gene expression plasmid vector (VectorBuilder, Neu-Isenburg, Germany). Subsequently, the gene of Gyp was flanked by cleavage sites for SalI/NheI via polymerase chain reaction (PCR). The resulting insert was isolated via agarose gel electrophoresis, extracted from the band cutout, and simply integrated by blunt end ligation into the pJET1.2 cloning vector (CloneJET PCR cloning kit, Thermo Fisher, Waltham, USA). This step enables propagation of plasmid DNA in *E. coli* in order to have sufficient amounts for restriction, extraction from agarose gel and ligation (briefly for the cloning into the destination vector). The gene of saporin (Sap) was taken from a pET11d bacterial expression vector and cloned into the pJET1.2 vector in the same manner. The resulting pJET1.2 plasmids were originally used for other purposes but coincidentally bear suitable cleavage sites (SalI/XbaI) for cloning the genes of interest into the nanoplasmid vector of Nature Technology Corporation (NTC).

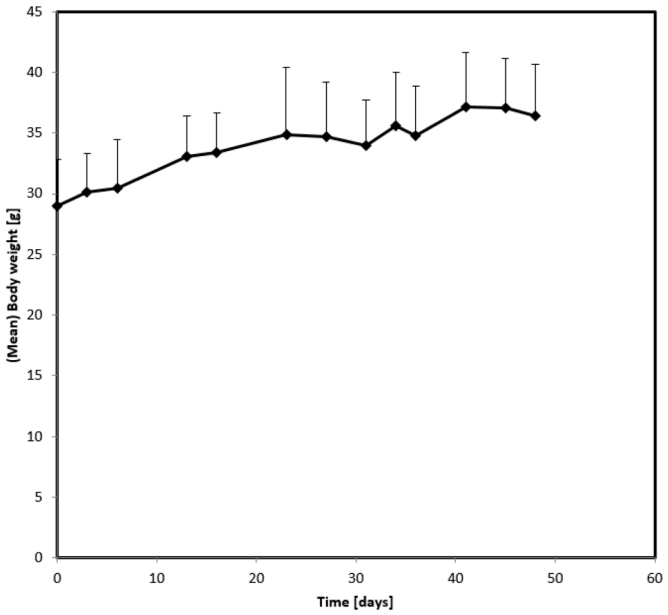

Fig. 7. Toxicity study of targeted suicide nanoplexes and AG1856. Two NMRI nu/nu mice received in total 6 applications of AG1856 (s.c.) and targeted suicide nanoplexes (i.v.). The body weight was determined regularly. No reduction of body weight was observed, and mice behaved normally.

The nanoplasmids NTC9385R-Gyp-BGH pA (2573 bp) and NTC9385R-Sap-BGH pA (2585 bp) as well as NTC9385R-EGFP-BGH pA (2487 bp), the corresponding nanoplasmid encoding for enhanced green fluorescent protein (eGFP), were produced by Nature Technology Corporation, Lincoln, USA. The transgenes were under control of a chimeric promotor consisting of CMV HTLV-I.

The functionality and the production process of nanoplasmids was summarized in a poster by (Aaron Carnes, 2016). In summary, the nanoplasmid construct has a size-reduced origin of replication (300 bp instead of 1000 bp) and works with RNA-OUT antibiotic-free selection. Special host strains are necessary for the nanoplasmid production. Successfully transformed bacteria are selected with sucrose instead of antibiotics, based on the accumulation of the polysaccharide levan in the periplasm, which is toxic to *E. coli.* Levan consists of fructose monomers and a terminal glucose and is formed by levansucrase while degrading the sucrose. Levansucrase is chromosomally coded in the host strain. Consequently, untransformed bacteria die, while bacteria carrying the nanoplasmid survive due to hybridization of the levansucrase coding mRNA (gene silencing). Furthermore, NTC has optimized the fermentation process and combined it with a patented lysis process to obtain remarkable yields. After a period of time with high biomass production and low plasmid amplification at lower temperature (30°C), the temperature is elevated to 42°C, which induce the expression of high copy replication proteins and therefore increase the plasmid propagation tremendously. Good manufacturing practice conform production is possible.

2.2. Isolation and characterization of transfection enhancer

The transfection enhancer agrostemmoside E (AG1856) was isolated from *Agrostemma githago* L. by a combination of size-exclusion chromatography and HPLC (Clochard et al., 2020). AG1856 was characterized by LC-MS (LC-MS-Triple Quadrupole 6400, Agilent Technologies®, Palo Alto, CA, USA). The structure of AG1856 was presented previously and is described elsewhere (Clochard et al., 2020). The transfection enhancer AG1856 exhibits excellent water solubility. The transfection process is improved by modulating the endosomal escape process of internalized nanoplexes (Weng et al., 2015). For all *in vitro* and *in vivo* experiments AG1856 was applied separately. It was not formulated into the nanoplexes.

2.3. Formulation of nanoplasmids

The receptor-targeted peptide (Sama et al., 2018a; Weng et al., 2015; Tagalakis et al., 2011; Hart et al., 1998) with the amino acid sequence $K_{(16)}$GACYGLPHKFCG (peptide Y) was produced by GeneCust (Boynes, France). In previous studies it was shown that the integrin like sequence GACYGLPHKFCG facilitates the efficient transfection of nanoplexes (Tagalakis et al., 2011). Nanoplexes were formulated by admixing same volumes of peptide Y and DNA solution in ultrapure water, followed by a 30-minute incubation period at room temperature. Due to the 16-fold lysine domain peptide Y is positively charged and the primary amino groups of the lysines are protonated at neutral pH-value. Due to the backbone containing each one phosphate groups per nucleotide, DNA is a poly-anionic molecule. Because of the electrostatic interaction a nano-suspension will be obtained following the mixing of peptide Y with DNA.

Sap- and Gyp-nanoplexes for *in vitro* experiments were formulated at a mass ratio of 7:1 (peptide: DNA) whereas a mass ratio of 4:1 was used for eGFP- and Sap-nanoplexes for the *in vivo* experiments. N/P ratios, defined as the molar ratio of positively charged amino groups in the peptide to negatively charged nucleic acid phosphate groups, were calculated to be 11.7 (for mass ratio 7:1) and 6.4 (for mass ratio 4:1), respectively.

2.4. Characterization of nanoplexes

The morphology of the targeted suicide nanoplexes was investigated by scanning electron microscopy (SEM) using a Hitachi SU 8030 scanning electron microscope (Hitachi, Tokyo, Japan) at 15 kV. The samples (5 µL) for SEM were taken from formulations that were produced as for the animal experiments. The sample was dried overnight on silicon waver at RT prior measurement.

The DNA complexation was analysed by agarose gel electrophoresis (Sama et al., 2017). Nanoplexes with 500 ng nanoplasmid per approach were formulated with peptide Y in a total volume of 30 µL as described above. Equivalent amounts of free nanoplasmid as control and the nanoplexes were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized under UV.

The hydrodynamic diameter (D_H) and the ζ-potential of all nanoplexes used within this study were measured by dynamic light scattering (DLS) and laser Doppler micro-electrophoresis using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, United Kingdom), equipped with a 4 mV HeNe laser, 633 nm, at a fixed scattering angle of 173°. For each nanoplex, 2.5 µg DNA were complexed in a total volume of 50 µL as described above. The nanoplex solution was incubated at room temperature for 30 minutes and transferred into a disposable UV-transparent micro cuvette for size measurements. Cumulants analysis, as defined in ISO 13321:1996 and ISO 22412:2017, was used for the analysis of the autocorrelation functions by the Zetasizer software, producing a mean value for the D_H (Z-Average) and a width parameter of the monomodal curve known as polydispersity index (PdI). Size distributions were obtained by the Zetasizer software using the CONTIN algorithm to fit the correlation function to the optimal number of exponentials. For ζ-potential measurements, the nanoplex solution was diluted with ultrapure water to a final volume of 800 µL and supplemented with NaCl to a final concentration of 10 mM before transferring the complete solution to a folded capillary cell. Each measurement was performed three times with a minimum number of 10 subruns per measurement.

2.5. Cell culture and live cell imaging

Murine neuroblastoma cells Neuro2A (ATCC® CCL-131™) and

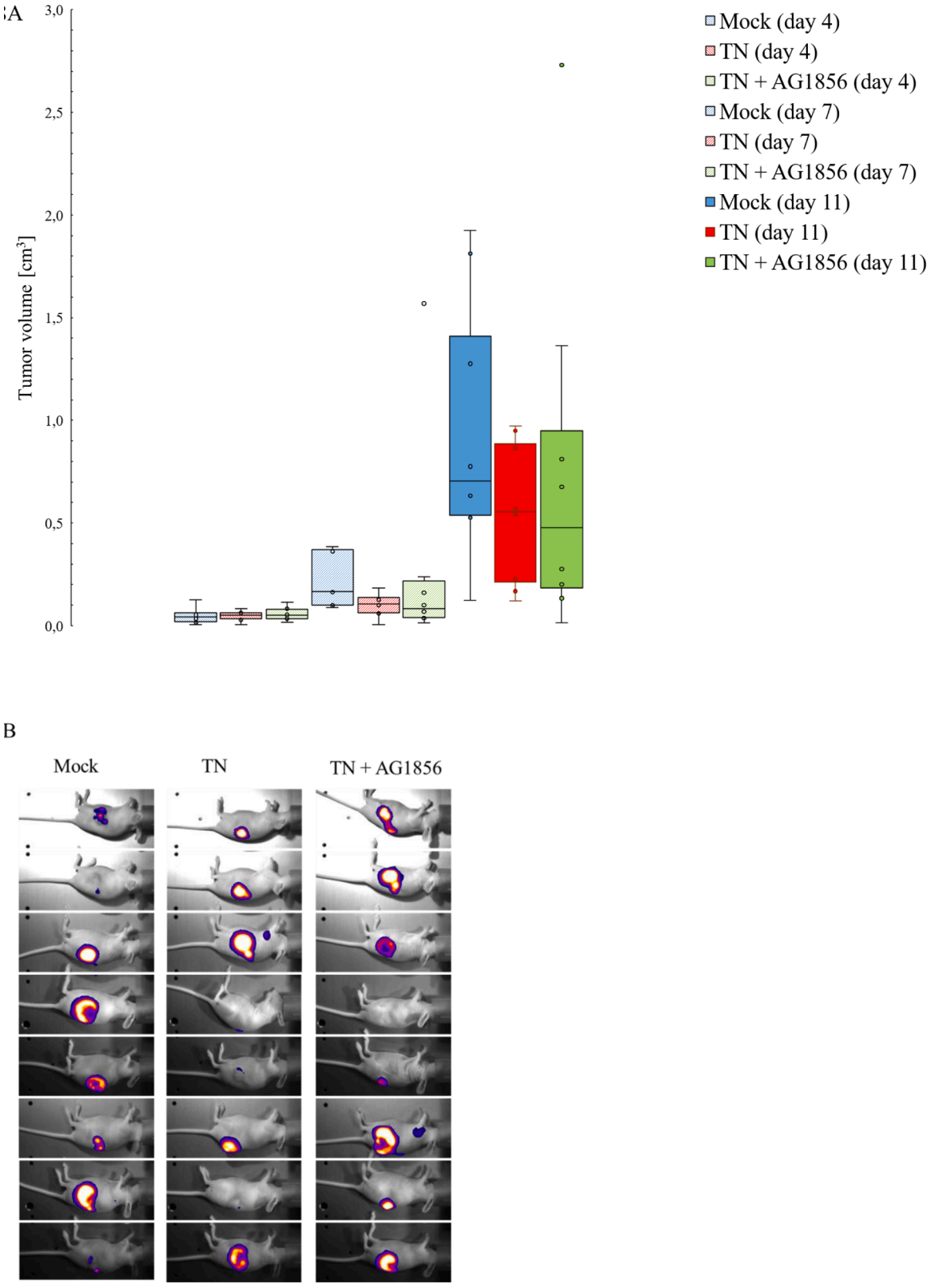

Fig. 8. (A) Box-plot diagram of anti-tumoral efficacy of targeted suicide nanoplexes (TN) in tumor bearing mice. Mice (NMRI nu/nu, 8/ group) obtained either PBS (mock) or TN (i.v.) with or without AG1856 (s.c.). Mice treated with TN ± AG1856 showed a slower tumor growth than mock mice. (B) Luciferase imaging of tumors applying luciferin (150 mg/kg) on day 11 of the therapy.

Neuro2A cells that were stable transfected with the luciferase gene (Sama et al., 2018b) (Neuro2A_Luc) were cultivated in BioWhittaker® Dulbecco's Modified Eagle's Medium (DMEM) (Lonza Group, Basel, Switzerland) supplemented with 10 % FBS and non-essential amino acids (Lonza Group, Basel, Switzerland) in a 5 % CO_2 atmosphere and 37°C. For *in vitro* transfections cells were seeded in clear well-plates and cultivated for 24 h. After this, the culture medium was exchanged with a mixture of freshly formulated nanoplexes (described above), culture medium (described above) and AG1856 (final concentration: 5 μg/ mL). Cells (4000 per well) were seeded in 96-well-plates and 100 ng DNA was formulated per well for *in vitro* transfections with suicide nanoplexes, the total culture volume per well was 200 μL. After the transfection, the cells were observed for 48 h. The cytotoxic effect was measured by a CytoSMART® Omni live cell imaging device (CytoSMART Technologies, Eindhoven, the Netherlands). Cell growth was monitored for 72 h. For *in vitro* transfections with eGFP-nanoplexes (500 ng per well), 15 000 cells per well were seeded in 24-well-plates in a culture volume of 400 μL. A CytoSMART® Lux3 FL, a small fluorescence live-cell imaging microscope, was used to monitor cell growth and eGFP expression during the 48-hour incubation period. The transfection efficiency was determined using flow cytometry. All cells exhibiting higher FITC-H signals than the untreated control cell population, were considered as transfected.

2.6. In vivo studies, toxicity, efficacy, and luciferase imaging

All animal studies were conducted in compliance with the United Kingdom Coordinated Committee on Cancer Research guidelines and have been authorized by the *Landesamt für Gesundheit und Soziales*, Berlin, Germany. All the studies were performed with Rj:NMRI-Foxn1$^{nu/nu}$ (NMRI-nu/nu) mice, obtained by Janvier Labs. To assess acute toxicity, two mice received 90 μg AG1856 (50 μL) subcutaneously. One hour later 100 μL nanoplex formulations (NP) were applied intravenously in the tail vein (30 μg DNA/ mouse). This was repeated on day 3 and 6. Mice were observed and the body weight was measured regularly. In order to perform the chronic toxicity study, the same mice received applications of 90 μg AG1856 (50 μL) subcutaneously and one hour later 100 μL NP on day 27, 31, 34 and 36. The mice were observed regularly and body weight was recorded until study termination on day 48. For the anti-tumoral effect, efficacy was investigated in the Neuro2A xenograft model. Neuro2A cells that have been stably transduced with the luciferase gene (Sama et al., 2018a) were injected subcutaneously in the left flank (10^7 cells per inoculation) in NMRI-nu/nu mice (6 – 8 weeks old). Then, mice were randomized to 8 mice/ group (in total 3 groups). The suicide gene therapy was started one day after the inoculation and was repeated on day 3, 5, 7 and 9. The application scheme and the study design was based on previous studies (Sama et al., 2018a). The tumor growth was determined by calliper measurements. The application scheme was as described above. One group received placebo (PBS), one group only targeted suicide NP (30 μg DNA) and the third group NP (30 μg DNA) + AG18156 (90 μg). From study day 11 on, bioluminescence imaging was performed by using the NightOWL LB 981 imaging system (Berthold Technologies, Bad Wildbad, Germany) as described elsewhere (Sama et al., 2018a).

3. Results and discussion

3.1. Targeted suicide nanoplexes

The morphology of the Sap-NP (4:1) that were used for the *in vivo* study, were analysed using scanning electron microscopy (SEM) (Fig. 1). The analysis revealed rod like structures with a width of roughly 50 nm and a length of roughly 200 nm. This is an interesting observation because it was shown that the shape of a nanoparticle has an impact on the biological effect, e.g. rode like structures exhibited higher anticancer activity than spherical particles (Scarpa et al., 2020). It is obvious that the shape might modulate the endocytosis of the nanoplex into a cell (Zhang et al., 2015).

3.2. Sizing and gel retention assay

The complexation of the DNA was assessed by agarose gel electrophoresis of the formulated nanoplexes. As shown in Fig. 2, no free DNA was detected in the nanoplex formulations, indicating complete complexation.

Nanoplexes were characterized regarding their size, PdI and ζ-potential by DLS and laser Doppler micro-electrophoresis. All measured nanoplex formulations resulted in the formation of cationic nanoparticles with positive ζ-potential (Table 1). The nanoplexes show a relatively narrow particle size distribution (PdI < 0.3) with mean hydrodynamic diameters between 45 and 61 nm (Fig. 3, Table 1).

3.3. Transfection efficacy of nanoplasmids

The transfection efficacy of the nanoplasmids was determined using an eGFP nanoplasmid construct formulated with peptide Y ± AG1856 and lipofectamine as positive control. As depicted in Fig. 4, nanoplexes alone carrying the eGFP-gene showed very low transfection efficacy whereas the combination with the transfection enhancer AG1856 caused a booster of the transfection efficacy to ~ 80 %. This value was far above the level of lipofectamine, which reached a transfection efficacy ~ 50%.

The eGFP expression profile was investigated in Neuro2A cells in a live cell imaging experiment over 48 h. The expression of eGFP started already 6 h after transfection. Compared to regular plasmid-DNA the nanoplasmids are reduced in size, which may contribute to a faster delivery into the nucleus. It was shown that the transfection efficiency depends on the size of the DNA-vector (Hornstein et al., 2016). The whole video sequence from which Fig. 5 was generated can be found in the supplementary information (videoS1).

3.4. In vitro efficacy of targeted suicide nanoplexes

After we had observed the delivery efficacy of the eGFP-nanoplexes in combination with AG1856, we aimed to transfer these promising results to a rather more therapeutically relevant scenario. For this reason, we designed suicide nanoplasmids by ligating the cDNAs of the ribosome-inactivating proteins saporin from *Saponaria officinalis* L. and gypsophilin-S from *Gypsophila elegans* M. Bieb. into the nanoplasmids. By the formulation with peptide Y, targeted suicide nanoplexes were generated. Cells that were only treated with targeted suicide nanoplexes but not with AG1856 showed no sign of an inhibition of proliferation. However, the proliferation of cells that were treated with targeted suicide nanoplexes + AG1856 was strongly inhibited shortly (~12 h) after addition of the compounds (Fig. 6) This was due to an expression of the ribosome-inactivating proteins saporin and gypsophilin-S and thus an irreversible inhibition of protein synthesis.

The results obtained from the *in vitro* experiments with the targeted suicide nanoplasmids confirmed the results from the experiments with eGFP-nanoplasmids and showed the excellent efficacy of the transfection enhancer AG1856. In the next step, we attempted to translate these results into *in vivo* conditions. It was envisaged to investigate the anti-tumoral efficacy of the targeted suicide nanoplexes in a neuroblastoma animal model using Neuro2A cells that were transduced with the luciferase gene. In advance to the therapy study, an *in vivo* toxicity study was performed.

3.5. In vivo toxicity studies

The toxicity of the gene therapy was investigated in NMRI-nu/nu mice. The transfection enhancer AG1856 was applied subcutaneously into the neck fold and one hour later, the targeted suicide nanoplasmids were injected into the tail vein. As depicted in Fig. 7, mice tolerated the

therapy very well. No decrease of bodyweight was observed, and no signs of any other irritations were noted.

3.6. In vivo anti-tumoral efficacy study

Next, we investigated the anti-tumoral efficacy of the targeted suicide nanoplexes (TN) *in vivo*. Based on our relevant experimental experience with Sap-plasmids (Sama et al., 2018a) and Sap-protein (Weng et al., 2012) we decided to use the Nano-Sap construct for the efficacy study. It should be noted that the selected tumor model is a very aggressive tumor model and tumors are growing with great speed. Against this background we obtained promising results showing a considerable decrease of tumor growth by the targeted suicide compared to mock treated mice (see Fig. 8). Investigation of liver enzyme activity in the serum did not show any significant differences between all groups indicating a well-tolerated treatment. In TN/ AG1856-treated mice an outlier, which was included in the statistical evaluation, was observed with a very big tumor. The data was statistically analysed by one-way-ANOVA, but significant differences were not detected. However, the median of TN/ AG1856-treated mice was still lower than the median of TN treated mice.

4. Discussion

In this study we have constructed targeted suicide nanoplexes. Our aim was to provide evidence whether such nanoplexes are suitable for anti-tumor treatment *in vivo*. It should be noted that this is the first study presenting suicide vectors based on nanoplasmids. The suicide nanoplexes were formulated by simple pipetting with targeted lysine peptide. The resulting morphology is intriguing since rod like nanoparticles were generated. A normal plasmid construct (~ 5000 bp) with the same suicide gene (saporin) formulated with the same targeting peptide as we used in this study resulted in the formation of normal spheres (Sama et al., 2018a). This is an interesting observation since it is known that the morphology does modulate the endocytosis (Zhang et al., 2015) and the membrane wrapping (Tang et al., 2018) of nanoparticles. The morphology is therefore an important factor for the transfection efficacy since endocytosis is the first prerequisite for a successful delivery. However, it is also obvious that an excellent endocytosis does not necessarily go hand in hand with an excellent transfection efficiency. What is necessary is the ability to escape from endosomes/ lysosomes inside the cell (Smith et al., 2019). We have recently identified a natural product (AG1856) from the plant *Agrostemma githago* L. (Clochard et al., 2020). It is composed of a hydrophobic triterpene backbone and two complex carbohydrate chains attached to this backbone. As shown in this study AG1856 greatly improved the transfection efficiency of the targeted suicide nanoplexes in Neuro2A cells *in vitro*. We have already shown that compounds such as AG1856 improve the endosomal escape process of gene-nanoplexes (Weng et al., 2015). However, a superiority of AG1856 is the extremely low self-toxicity (Clochard et al., 2020). This is confirmed by the *in vivo* toxicity results presented in this study. Here, we injected a high dose of 90 μg AG1856 per mice. Assuming a maximal blood volume of 1.5 mL and that all AG1856 is distributed in the blood stream, this corresponds to a theoretical concentration of 60 μg/ mL. This shows that AG1856 also exhibits a very good tolerance *in vivo*. In this study, AG1856 tremendously enhanced the transfection efficacy of eGFP- as well as of suicide nanoplasmids. In the *in vivo* experiments, this strong enhancement was not observed, which could be due to an inefficient transport of the nanoplexes to the tumor and a deficient penetration through the tumor tissue. Considering the fenestration of tumor vasculature an optimized formulation method, producing nanoplexes with optimized size and low polydispersity index could contribute to a better penetration (Tang et al., 2014). In principle it might be assumed that the suicide nanoplexes also affect other cells than tumor cells. As shown in this study no toxicity was observed in healthy cells, which might be due to the targeting effect, mediated by peptide y that was used for the formulation of the nanoplexes. Another explanation might be the much higher metabolic activity paralleled by a higher protein synthesis rate and transcription rate of fast growing tumor cells. It can be thus assumed that the neuroblastoma cells transcribe and translate the suicide genes much more efficient compared to slowly growing healthy cells.

Another important factor that needs to be investigated in future studies is an optimized application scheme for AG1856. In this study we followed an application scheme applying the compound s.c. (Sama et al., 2018a). Other applications need to be investigated in terms of an optimal flow into the tumor. A straightforward way would be an i.v. application together with the nanoplexes. To identify the perfect application scheme is therefore a balancing between unspecific toxicity and anti-tumoral efficacy.

4. Conclusion

In this study we have shown, that targeted suicide nanoplexes harboring novel nanoplasmids show high activity *in vitro* in neuroblastoma cells. We translated these *in vitro* results into an *in vivo* model. The suicide nanoplasmid formulations showed an anti-tumoral activity, however compared to the *in vitro* results the anti-tumoral efficacy was moderate. The efficacy of the nanoplexes might be improved by optimizing the formulation protocols, resulting in a reduced size variation and subsequent improved delivery of the nanoplexes to the tumor site.

Credit author statement

Conceptualization: Alexander Weng

Investigation: Hardy Mitdank, Meike Tröger, Nima Amini Shirazi, Eric Woith, Alexander Sonntag, Dennis Kobelt

Formal analysis: Alexander Weng, Dennis Kobelt, Hardy Mitdank, Meike Tröger

Writing - Original Draft: Alexander Weng, Hardy Mitdank, Meike Tröger

Supervision: Alexander Weng, Wolfgang Walther

Funding acquisition: Alexander Weng

Writing - Review & Editing: Hendrik Fuchs, Wolfgang Walther, Hardy Mitdank, Meike Tröger, Nima Amini Shirazi, Eric Woith, Alexander Sonntag, Dennis Kobelt

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Supplementary materials

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