Targeted suicide gene transfections reveal promising results in nu/nu mice with aggressive neuroblastoma

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Abstract

Neuroblastoma represents the third most common malign neoplasm occurring in children and the most common in newborn. Although mortality in childhood cancer declined in the last decade, high-risk patients have poor prospects, due to the aggressiveness of the cancer. In the recent past, we underlined the potential of sapofectosid as novel and efficient transfection enhancer, demonstrating non-toxic gene delivery, but its value in tumor therapies has yet to be elucidated. A suicide gene, coding for saporin, a ribosome inactivating protein type I, was incorporated into targeted, peptide-based nanoplexes. The nanoplexes were characterized for their size, zeta potential and appearance by electron microscopy. Gene delivery was observed via confocal imaging. *In vitro* transfections were conducted to monitor the real-time cell viability. After initial tolerability studies, NMRI nu/nu-mice bearing tumors from Neuro-2A-Luc-cells (murine neuroblastoma cells, transduced with a luciferase gene), were treated with targeted nanoplexes (30 µg saporin-DNA i.v./ treatment) and sapofectosid (30 µg s.c. treatment). The treatment was compared to a vehicle (PBS) control and treatment without

sapofectosid in terms of body weight, tumor growth and integrated density of tumor luminescence. The study revealed an anti-tumoral effect of the sapofectosid mediated gene therapy in the Neuro-2A-tumor model. The treatments were well tolerated by the animals indicating the applicability of this approach. With these results, we were able to proof the efficacy of a therapy, consisting of targeted suicide gene nanoplexes and sapofectosid, a novel and potent transfection enhancer. This study points out the enormous value for future targeted cancer and gene therapies.

Keywords: Sapofection

Introduction

Neuroblastoma is a malign cancerous disease counting among the most common diseases during childhood. Statistically, 10.7 out of 1,000,000 between 0-14 years suffer the illness [1] and approximately 700 new cases are registered yearly. The disease is characterized by defective maturation of nerve cells during fetal development with tumors appearing in the peripheral nervous tissue, usually around the adrenal glands. In the last decades, major improvements have been made in 5-year survival rates, although the mortality rates for patients in the high-risk group [2] remains low, with less than 40% survival due to the aggressive growth of cancer cells. Further, there remains a high risk of relapse and chronic health problems, after treatment by radiotherapy and chemotherapy [3,4]. Targeted tumor gene therapies may offer a promising alternative to established therapies. Sapofectosid, a plant-derived triterpenoid saponin, isolated from *Saponaria officinalis L.*, is an efficient transfection reagent with the potential to become part of targeted tumor treatments. Saponin as components of transfection formulations have the ability to interact with lysosomal membranes during the process of endocytosis and so induce the endosomal escape, releasing the nucleic acid cargo into the cytosol [5]. The plant *Saponaria officinalis L.* combines this mechanism of the saponins with that of the toxin saporin, a ribosome-inactivating protein, in order to deter herbivores [5]. Once saporin is released from the endosomes into the cytosol, its enzymatic N-glucosidase activity causes a cleavage of an adenine-residue from the ribosomal RNA, leading to cell death [6,7]. The saponin-mediated endosomal escape was used in a number of studies, in order to increase the toxicity of tumor targeting cell toxins [8,9].

A recent study showed, that a co-administration of gene-containing nanoplexes and sapofectosid enabled non-toxic delivery of nucleic acids (transfection), indicating the potential value of sapofectosid for gene therapies [10]. The principle of the combination of nanoplexes with saponins such as sapofectosid was termed sapofection.

As with liposomes and polymers, peptides are being used in formulations of nanoparticles in order to conduct a successful transfection. Based on electrical attraction of the negatively charged nucleic acid and the positively charged peptide, nanoplexes represent stable gene vehicles [11]. A further addition of specific amino acid-sequences to the poly-lysine peptide [12] leads to a targeting of integrin receptors, which are usually overexpressed in cancer cells [13].

The aim of our study was to test a novel and innovative approach against neuroblastoma. Integrin receptor targeting nanoplexes, carrying a suicide gene coding for saporin, were administered together with sapofectosid to murine neuroblastoma cells. The effect was initially observed (real-time) in *in vitro* experiments before investigating the anti-tumor treatment on Neuro-2A-Luc bearing *NMRI nu/nu – mice*. Results were evaluated by analyzing body weight, tumor growth and tumor luminescence.

Materials & Methods

1.1. Targeted Nanoplex Formulation

Positively charged oligo (16×)-lysine peptides (KKKKKKKKKKKKKKKK) with an integrin-receptor targeting amino acid-sequence (GACYGLPHKFCG) were synthesized by GeneCust (Dudelange, Luxembourg). The peptide (Y) was admixed thoroughly with the plasmid DNA of saporin or GFP in a mass ratio of 4:1. Nanoplexes were allowed to form and stabilize for 30 minutes at room temperature.

1.2. Nanoplex Characterization

The nanoplex suspension was diluted with purified water to 1 mL and transferred into a folded capillary cell cuvette. Size and zeta potential measurements were conducted for three days every 24 h in triplicates, in order to monitor the particle stability. Size and zeta potential distribution after the

formulation were analyzed using a Malvern Zetasizer Nano ZS. For scanning electron microscopy, 5 µl of each suspension (1µg/ml) have been transferred to silicon wafers and dried overnight. Measurements were performed using a Hitachi SU 8030 scanning electron microscope (Hitachi, Tokyo, Japan) at 15 kV.

1.3. Confocal Live-cell Imaging

Neuro-2A-cells (10,000 cells) were seeded in seeded in Ibidi µ-Dishes (35mm, low) in 2 ml culture medium and incubated at 37°C and 5% CO_2. FITC-labeled Y-nanoplexes were formulated as described above, diluted in sapofectosid (2.5 µg/ml) and deployed for transfection. Transfected cells were incubated for 24 h. Cell nuclei were stained for visualization with Hoechst 33242 (8 µg/dish) (Life Technology, Carlsbad, CA, USA) 30 minutes before imaging. After a three time washing step, 2 ml Live Cell Imaging solution (Life Technology, Carlsbad, CA, USA) with 20 mM D-glucose was applied to the cells. The transfected cells were imaged by confocal laser scanning microscopy and analyzed with the ZEN2010 as acquisition software.

1.4. *In Vitro* Impedance Measurements

8,000 Neuro-2A-Luc-cells were seeded in two 8-well-plates (OLS OMNI Life Science GmbH & Co. KG, Bremen, Germany), containing gold-coated electrodes for impedance measurements (iCelligence® system, ACEA Biosciences, Inc., San Diego, CA, USA). The cells were subsequently incubated for 24 h at 37°C and 5% CO_2. Targeted nanoplexes carrying the gene of saporin (YD) were added to the cells (2.5 µg DNA/Well) with and without a sapofectosid co-administration of 1.25 µg/mL or 2.5 µg/mL, respectively. Nanoplexes containing the p-EGFP-n3 plasmid were applied to the cells as control transfection.

1.5. Luciferase Expression Stability

In order to test the luciferase expression of the stably transduced Neuro-2A-Luc-cell line, 4,000 cells were seeded into three 24-well-plates. After 4, 7 and 11 days the luminescence was measured. For this purpose, the culture medium was removed and a 100µl luciferase assay solution, containing D-Luciferin (25 mM, Sigma Aldrich, St. Loius, MO, USA), Tricine (200 mM, MERCK, Darmstadt, Germany), $MgSO_4$ (50 mM, MERCK, Darmstadt, Germany), $MgCO_3$ (1.07 mM, VWR, Radnor, PA,

USA), DTT (500 mM, Thermo-Scientific, Waltham, MA, USA), ATP (25 mM, SERVA, Heidelberg, Germany) and EDTA (10 mM, FLUKA, St.Gallen, Switzerland) for luminescence development and Triton X (1%, Sigma, St. Louis, MO,USA) for cell lysis was added and mixed properly. The luminescence was measured 30 minutes after the addition by a TECAN F200 infinite microplate reader in triplicates (Männedorf, Switzerland).

1.6. *In Vivo* Tolerability Studies

1.6.1. Acute toxicity

All animal experiments were performed in accordance with the United Kingdom Co-ordinated Committee on Cancer Research (UKCCCR) guidelines and approved by the responsible local authorities (State Office of Health and Social Affairs, Berlin, Germany).

The acute toxicity study was performed to evaluate the tolerability of targeted nanoplexes at different dosing (15 µg, 30 µg and 55 µg total per animal, 2 mice per group). The compound was injected once i.v. (50 µl injection volume). The animals were observed for two weeks and the body weight was measured during the study.

1.6.2. Combinatorial Toxicity

The administration of nanoplexes together with sapofectosid was analyzed in terms of tolerability. For this, 4 female NMRI nu/nu mice were treated once with a fixed dose. The determined DNA amount of the acute toxicity (1.6.1.) was injected in form of YD-particles i.v. into the tail vein. Referred to previous *in vivo* studies [5,8], sapofectosid was injected one hour later in an amount of 30 µg s.c. into the nuchal fold. Body weight was determined up to 15 days after application.

1.7. *In Vivo* Anti-tumor Studies

The anti-tumoral activity of targeted nanoplexes in combination with sapofectosid was evaluated. The determined tolerable nanoplex and sapofectosid concentrations were applied to the actual therapeutic treatment. To test this approach, the Neuro-2A-Luc-cells were inoculated subcutaneously (s.c.) at cell count of 1 x 10^7 cells [14]. Mice (10) were randomized into vehicle (PBS), nanoplex alone and nanoplex + sapofectosid groups of n=5 animals/group. Each mouse received a treatment 1, 4 and 7

days after tumor injection. The treatment of the combination was done sequentially, sapofectosid was injected s.c. into the neck followed by i.v. injection of nanoplexes one hour later. During the study tumor volumes and body weight was measured. Furthermore, once per week bioluminescence (BLI) measurement was done to monitor tumor growth and the therapy effects *in vivo*. Bioluminescence imaging was performed by using NightOWL LB 981 imaging system (Berthold Technologies, Bad Wildbad, Germany). For bioluminescence imaging, mice were anesthetized with isoflurane gas and received intraperitoneally 150 mg/kg D-luciferin (Biosynth, Staad, Switzerland). Tumor growth and metastasis formation was imaged and quantified by WinLight (Berthold Technologies) and ImageJ 1.48v. The experimental endpoint was defined by ethical guidelines of animal care.

Results

2.1. Targeted Nanoplexes

The size and stability of the formulated YD – nanoplexes was investigated by dynamic light scattering (DLS). Size measurements revealed a mean size of 85 nm on day 0, steadily increasing to over 100 nm after day 3 of particle formulation. The size distribution showed a bell curve below 100 nm with minor accumulations at the size range of ~ 1,000 nm. The PDI index was 0.3. The zeta potential varied stably between +20-30 mV (Fig. 1).

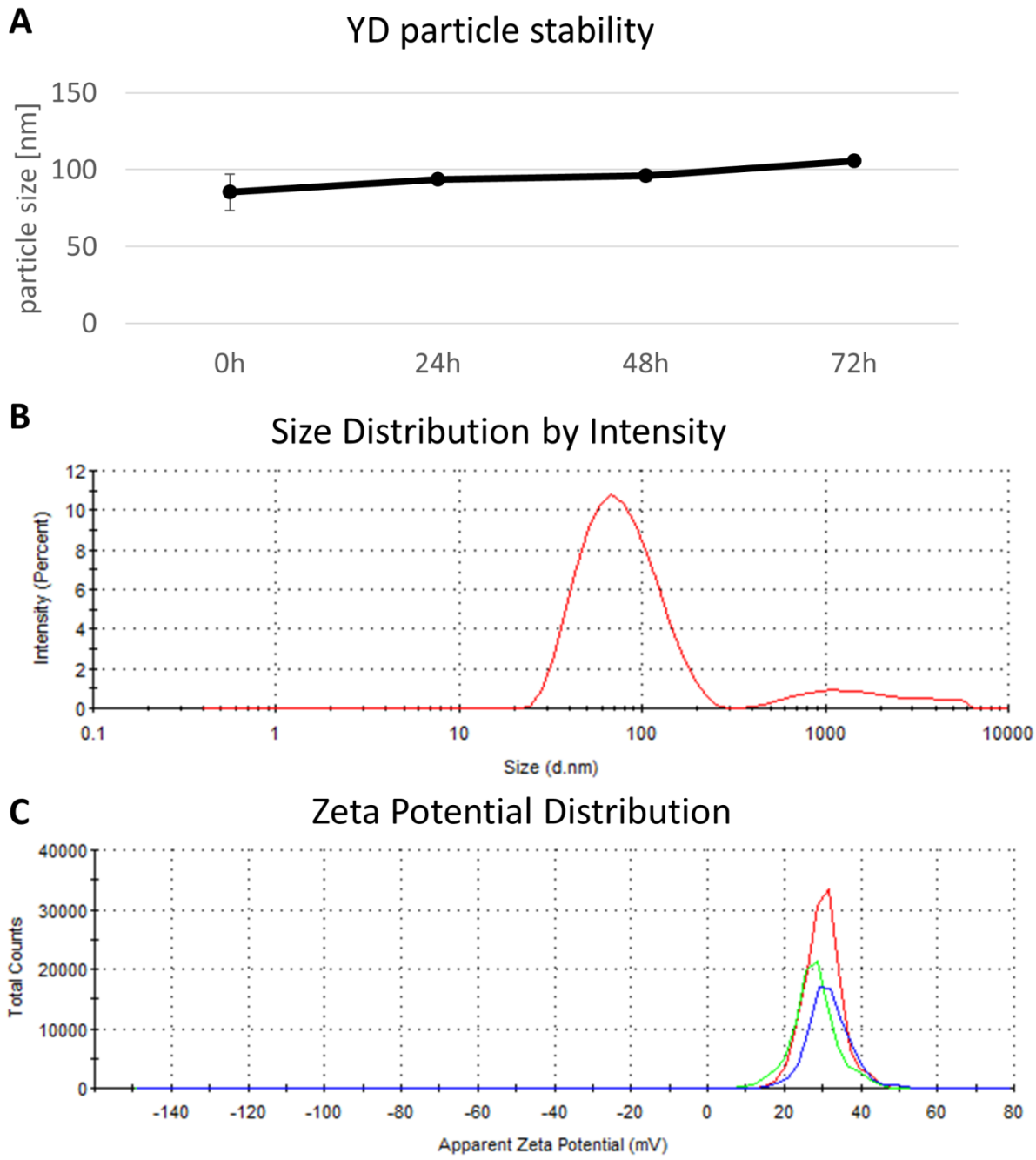

Fig 1. Particle stability and size distribution of formulated targeted nanoplexes (YD). The measurements revealed a particle size of 80-90 nm (B) and a zeta potential of ~ +30 mV (C). Formulated nanoplexes were stable up to 48 h (A).

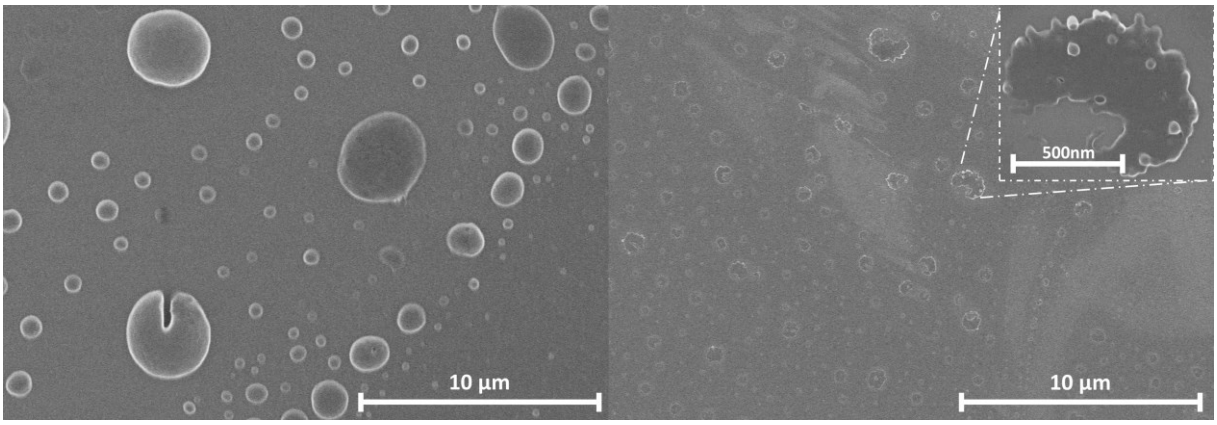

Fig. 2 Electron-microscopic images after YD-nanoplex formulation.
Left: YD-nanoplexes Right: YD-nanoplexes with the presence of sapofectosid (2.5 µg/ml). The administration of sapofectosid caused an alteration of the nanoplex surface, implying an interaction between both compounds.

2.2. *In Vitro* Real-time Toxicity Monitoring

The impact of sapofectosid on targeted nanoplex transfections was observed in terms of cell viability. The measured impedance at the bottom of the well, increased by the number of growing cells, could be directly related to the cell viability and hence toxic effects. The untreated negative control showed a rapid growth rate, particularly 75 h after the seeding. While YD-particles showed a similar viability as the negative control, an administration of sapofectosid (1.25 µg/ml and 2.5 µg/ml) led to a distinct impedance drop indicating severe toxicity (Fig. 3). YD-particles, containing the GFP-gene, were applied as a transfection control and showed no toxic effects with or without sapofectosid (2.5 µg/ml) (Fig. 4).

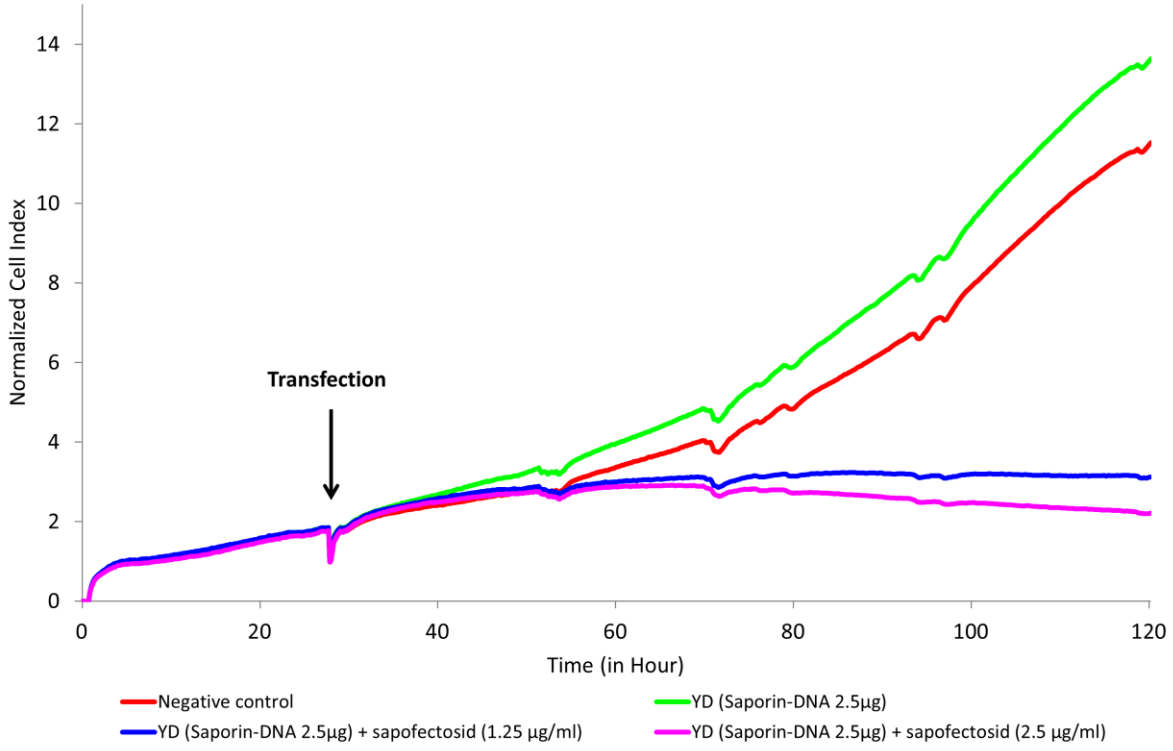

Fig. 3 Impedance/viability measurement after saporin-transfection. Neuro-2A-cell transfection was conducted after 24 h (arrow). The normalized cell index represents the impedance, normalized on the time point of transfection. The negative control and sole targeted nanoplexes showed no toxic effects. With presence of sapofectosid major toxic effects could be observed.

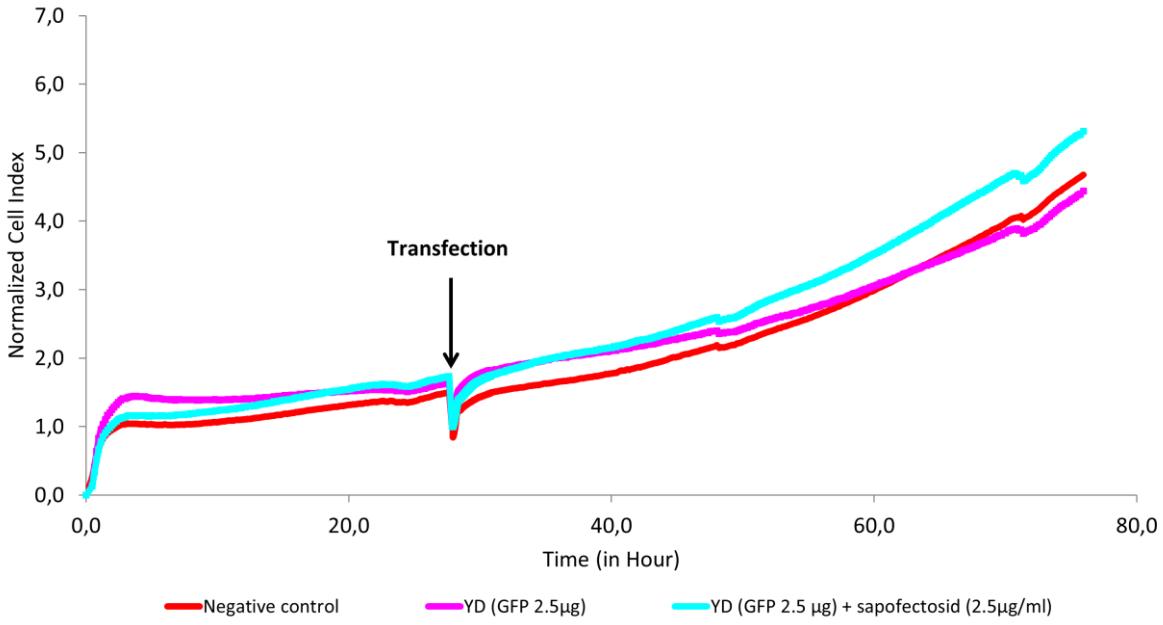

Fig. 4 Impedance/ viability measurement after GFP-transfection. Neuro-2A-cell transfections with GFP served as a control transfection. The transfection was conducted after 24 h (arrow). The normalized cell index represents the impedance, normalized on the time point of transfection. A transfection with nanoplexes, containing a reporter gene, caused no toxicity, as a similar viability to the negative control was noticed.

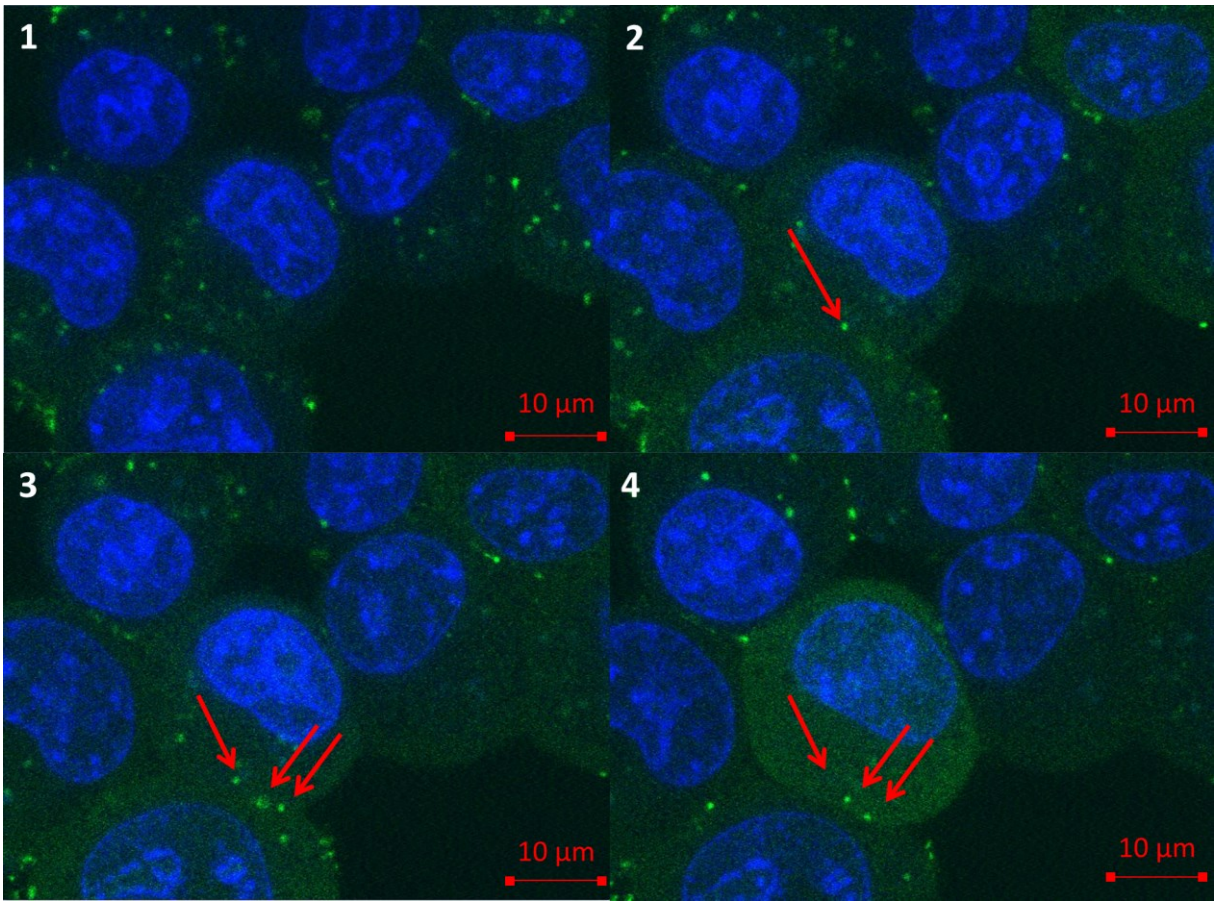

Fig. 5 Confocal live-cell imaging after transfection with FITC-labeled YD-nanoplexes

Image 1-4 are displayed in chronological order. Nuclei were stained in blue; FITC-YD-nanoplexes were shown as green dots. FITC-YD-nanoplexes accumulated at the cell membrane, before being endocytosed into the cell. A promptly increase of fluorescence was observed after nanoplex uptake, indicating a distribution in the cytosol.

2.3. Tolerability Studies To Nanoplex/Sapofectosid Treatment

Different amounts of DNA (15 µg – 55 µg) were administered to mice in 50 µl i.v., in order to determine the tolerability of the applied nanoparticles. Regular weight measurements up to 14 days after the administration revealed no significant weight change for all applied particle injections (Fig. 6). Due to precipitation after formulations with 55 µg DNA, further experiments were continued with 30 µg DNA. A combination of YD-particles (30 µg DNA) with sapofectosid (30 µg) did not show major alterations in terms of body weight, although a slight non-significant increase was observed, indicating good tolerability (Fig. 7).

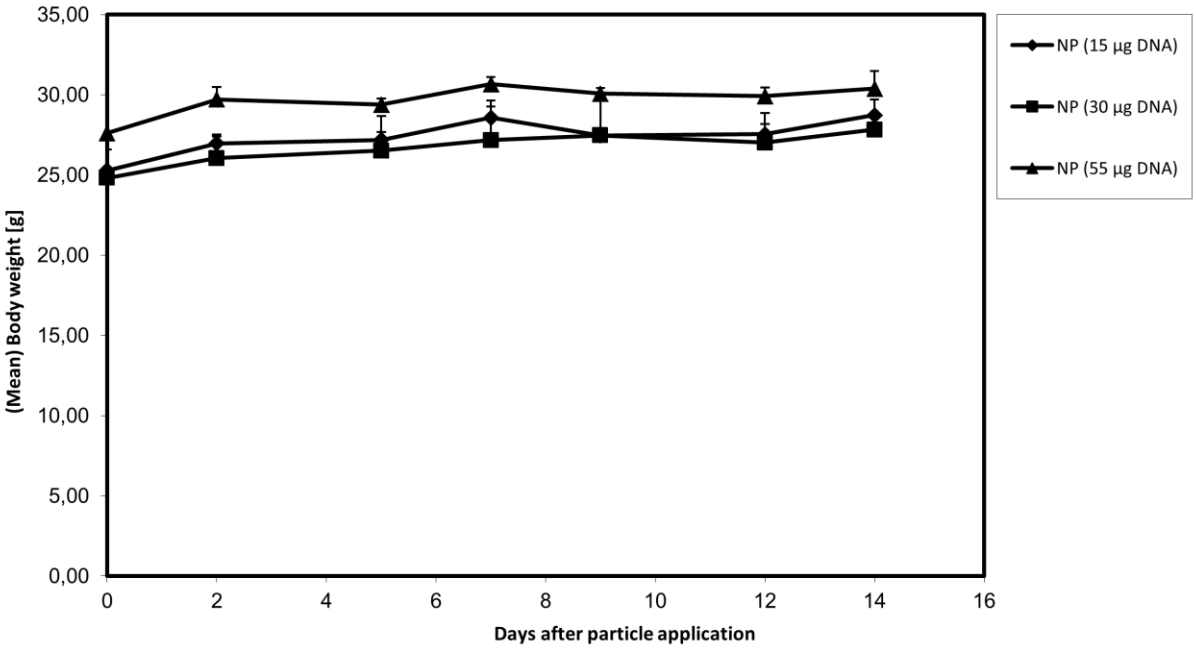

Fig. 6 Mean body weight - Acute toxicity study. Different amounts of DNA were formulated into YD-particles and applied to NMRI nu/nu mice. The body weight was observed for 14 days. No significant alterations in body weight or side effects were observed. NP: YD-nanoplexes. Deviations are given as S.D.

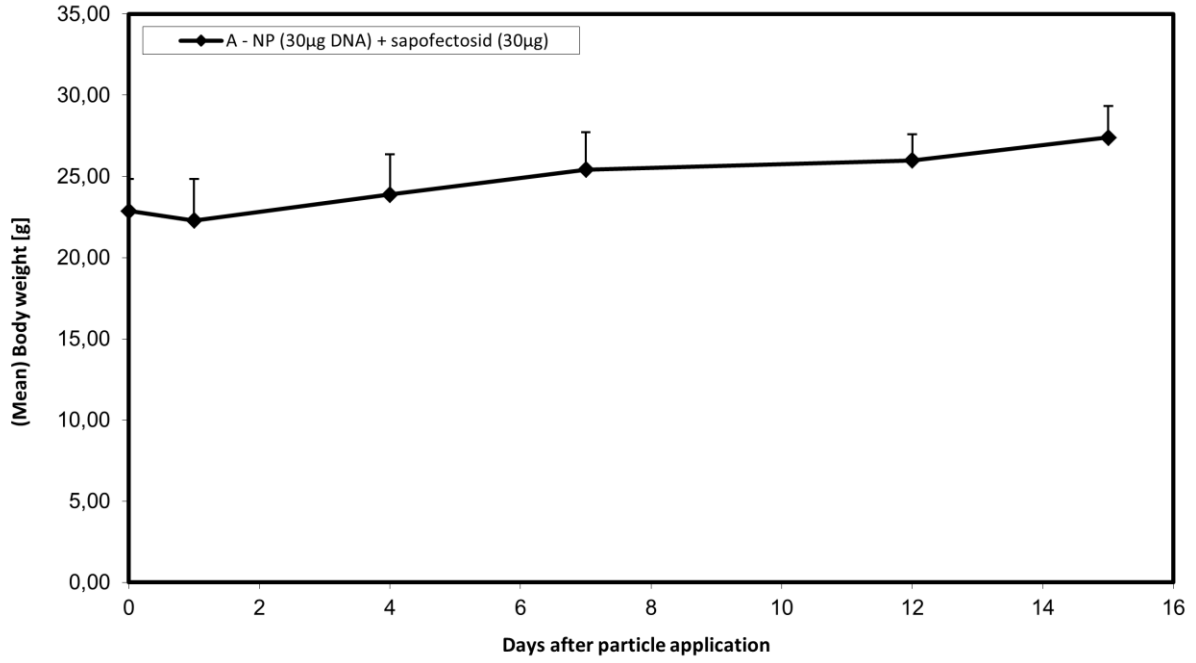

Fig. 7 Mean body weight – Combinatory toxicity study. Sapofectosid was applied s.c., 1 h after an i.v. nanoplex administration. Over two weeks the mean body weight was slightly increased. NP: YD-nanoplexes. Deviations are given as S.D.

2.4. *In Vivo* Efficacy Of Targeted Tumor Treatment

The combinatory treatment, tested in terms of tolerability (2.3.) was applied to NMRI nu/nu-mice after being implanted with the Neuro-2A-cells. Between tumor cell inoculation and end of therapy, the body weight was determined. All therapy groups showed a stable body weight of 23-32 g between days 4 and 11 after tumor cell inoculation (Fig. 8).

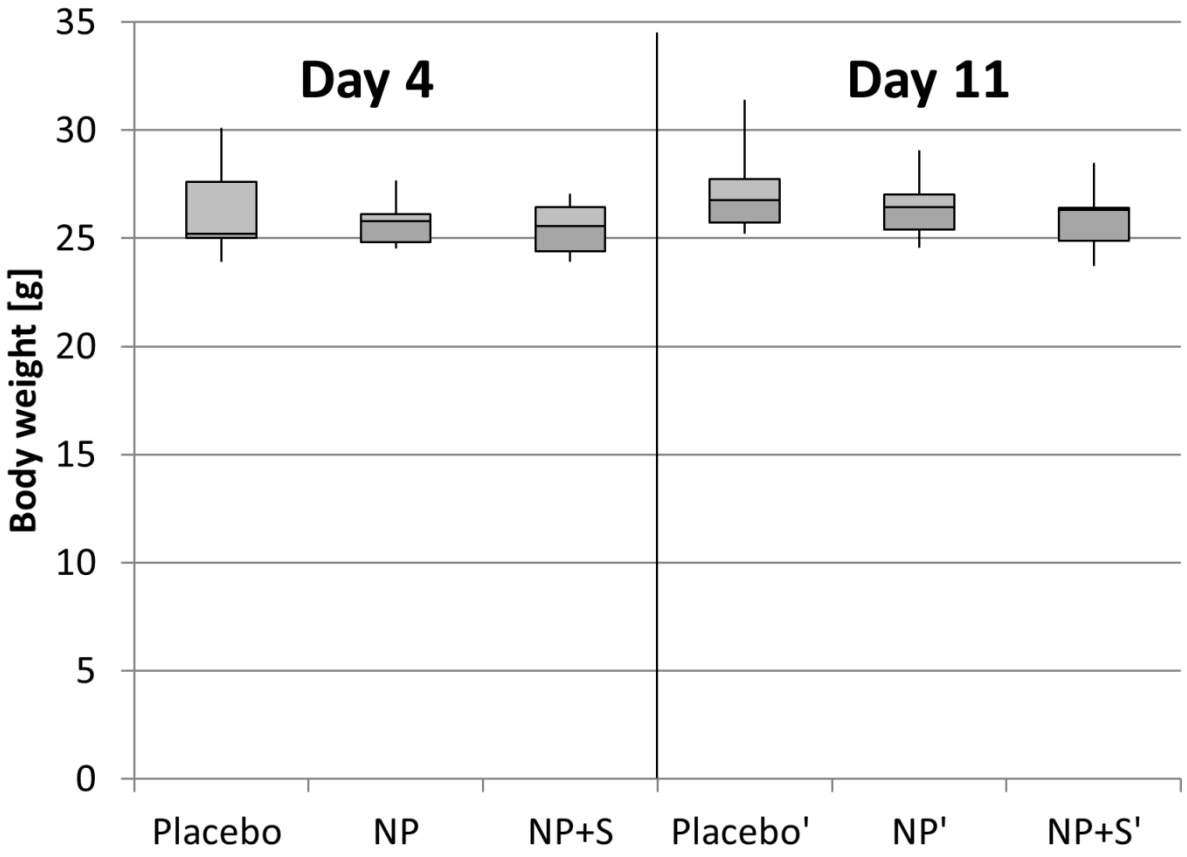

Fig. 8 Body weight during tumor treatment displayed as boxplot. No treatment group indicated differences or considerable changes in body weight. NP: YD-nanoplexes; NP+S: YD-nanoplexes + sapofectosid; ‘: treatment group after day 11

The tumor volume (given as cm^3) was measured 4 and 11 days after Neuro-2A cell inoculation. While after 4 days no difference was detected between the therapy groups, on day 11 considerable differences in terms of tumor growth were revealed by boxplot analyses (Fig. 9). All therapies exhibited a wide range of different tumor volumes from <0.5 cm^3 to >2 cm^3. Nevertheless, a noticeable shift of the boxplots, representing 50% of the obtained values, indicated a distinct difference in tumor growth suppression. The median of the vehicle group (1.25 cm^3) was significantly

higher than the NP- (0.53 cm³) and NP+S treated group (0.37 cm³). The T/C value (treated to control ratio; expressed in %), an indicator of anti-tumor effects, was assessed to evaluate the respective treatment efficacy. While the application of single nanoplexes achieved a T/C value of 69%, the co-administration of sapofectosid caused an additional 19% reduction in T/C values to 50% (Tab. 1). Statistical analysis revealed a significant difference between placebo treatment and combined nanoplex + sapofectosid therapy.

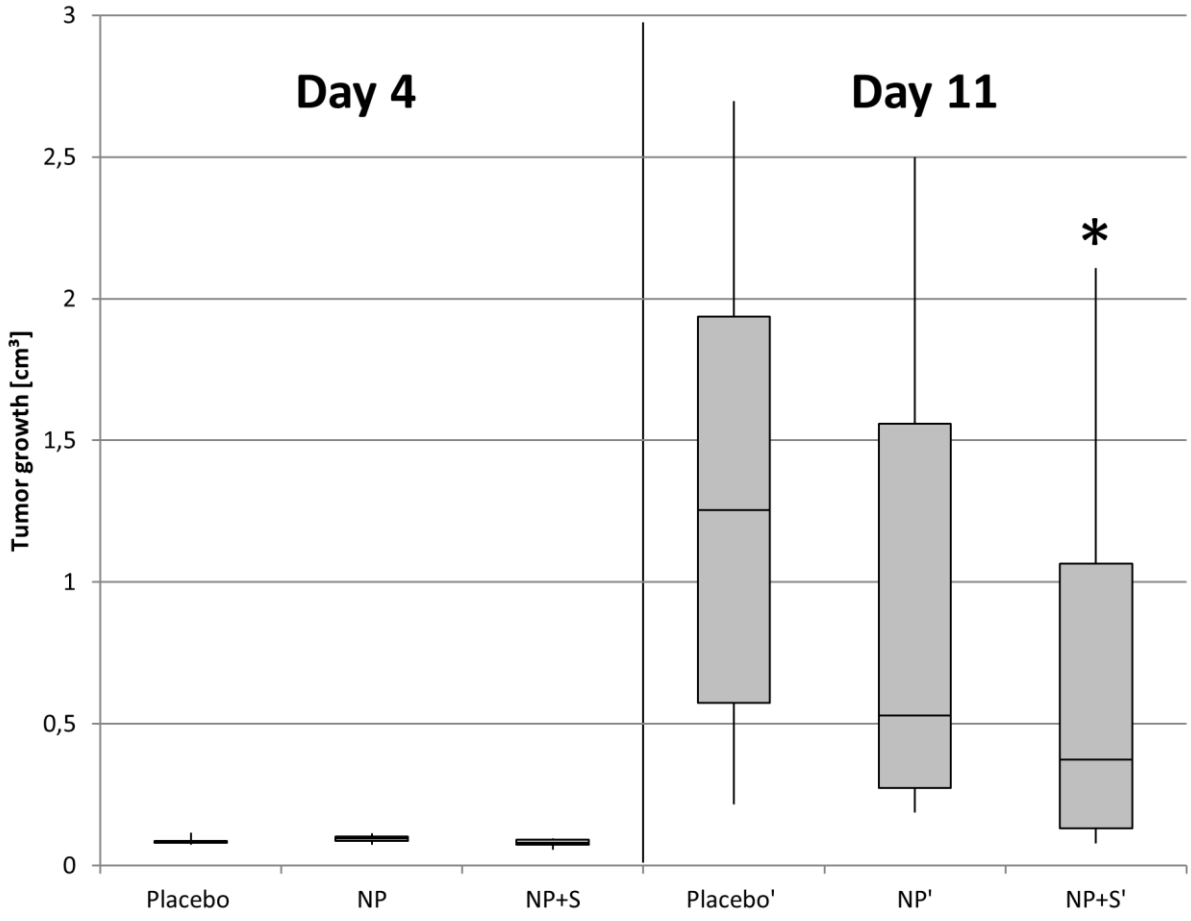

Fig. 9 Tumor volume of respective tumor treatment groups, displayed as boxplot. NP: YD-nanoplexes; NP+S: YD-nanoplexes + sapofectosid; ‘: treatment group after day 11; *: significantly different to the vehicle treatment, t-test with equal variances, $p<0.05$, $n\geq3$

Group	Day:		4	11
Placebo	(n)		10	10
	Tumor Volume	Median	0.083	1.255
	[cm³]	Mean	0.086	1.350
		[S.D.]	0.0118	0.9462
	RTV	Median	1.0000	12.5
		Mean	1.0000	15.4
NP	(n)		10	10
	Tumor Volume	Median	0.087	0.530
	[cm³]	Mean	0.087	0.922
		[S.D.]	0.0123	0.8305
	RTV	Median	1.0000	7.8
		Mean	1.0000	10.1
	T/C [%]		101.1	68.3
NP+S	(n)		10	10
	Tumor Volume	Median	0.080	0.374
	[cm³]	Mean	0.079	0.669
		[S.D.]	0.0144	0.7177
	RTV	Median	1.0000	5.1
		Mean	1.0000	7.9
	T/C [%]		92.4	49.6

Tab. 1 Statistical analysis of anti-tumoral efficacy during treatment (day 4 and 11 after tumor cell inoculation); NP: YD-nanoplexes; NP+S: YD-nanoplexes with sapofectosid; n: number of mice; S.D.: standard deviation RTV: relative tumor volume; T/C [%]: treated to control in %

Luminescence measurements were conducted in order to receive a more precise picture of tumor growth and treatment response. The luciferase expression of the injected tumor cells enabled a luminescence with an intensity proportional to the tumor size. Luminescence imaging showed the changes in terms of tumor volume between day 4 and 11. A reduction of tumor volume was observed after applying a nanoplex treatment. Among all groups a number of mice exhibited no tumor growth (Fig. 10). The quantification of luminescence was displayed as integrated density (IntDen, the sum of the values of the pixels in the image) for each treated mouse. Mice, treated with vehicle, showed a higher BLI intensity, implying an intensive tumor growth. The NP-group offered fewer tumor growth reflected by lower BLI intensity. An artificial trend line was placed at 4x10^8 IntDen in order to point the growth attenuation caused by the combinatorial therapy (Fig.11).

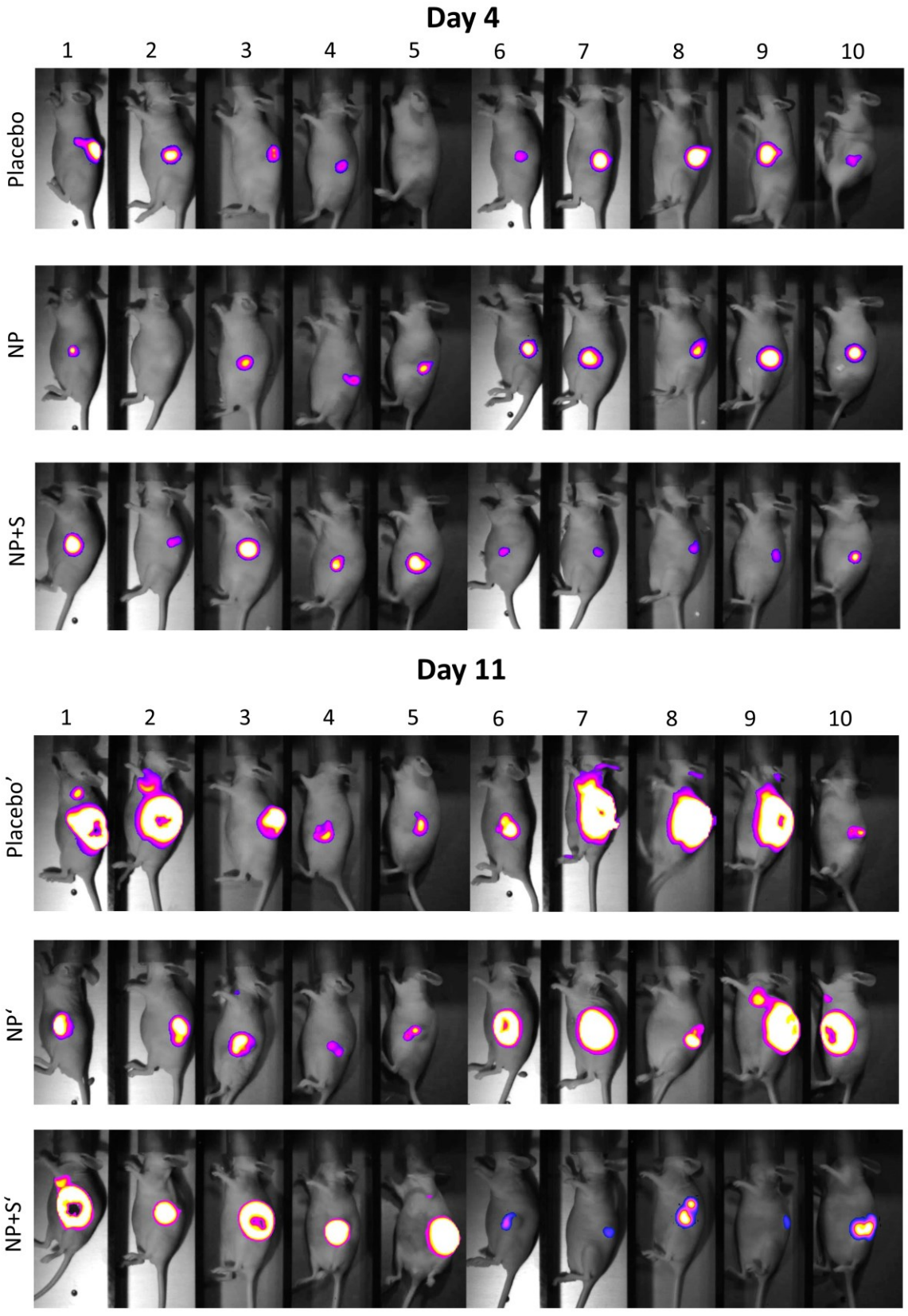
Day 4
1
2
3
4
5
6
7
8
9
10
Placebo
NP
NP+S
Day 11
1
2
3
4
5
6
7
8
9
10
Placebo′
NP′
NP+S′

Fig. 10 Luminescence images of treated NMRI nu/nu mice harboring Neuro-2A neuroblastoma s.c. tumors. At day 4 and 11, luciferin (150 mg/kg) was applied and images were obtained using a NightOWL LB 981 imaging system (Berthold Technologies, Bad Wildbad, Germany). After day 11 differences between the treatments were visible.

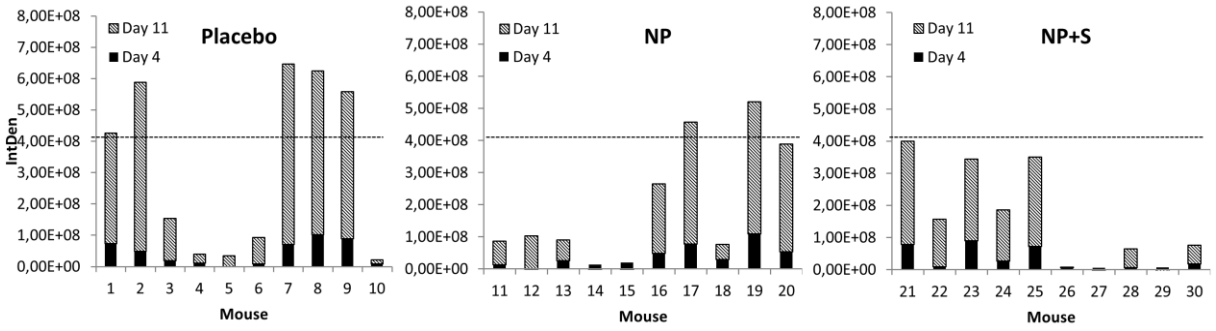

Fig. 11 Quantification of tumor luminescence by integrated density (IntDen). The tumor luminescence of day 4 and 11 was assessed and illustrated for each mouse. The application of NP led to a distinct suppression of tumor growth. Sapofectosid enhanced the attenuation. NP: YD-nanoplexes; NP+S: YD-nanoplexes + sapofectosid

Discussion

In this study, a targeted suicide gene therapy was developed in order to investigate the therapeutic potential of sapofectosid. Targeted nanoplexes, containing the suicide gene of a ribosome-inactivating protein (saporin) were formulated, characterized and applied together with sapofectosid, a plant-derived saponin, thought to be a transfection enhancer. Acting as a mediator of the endosomal release by interacting with the endosomal membrane, sapofectosid can change the amount of nucleic acids in the cytosol enormously. In advance to the therapy, *in vitro* studies were performed to optimize the scheduled treatments and to examine the properties and growth rate of the Neuro-2A-Luc cells. The characterization of targeted nanoplexes was necessary to ensure a reliable gene delivery. With a size of less than 100 nm and zeta potential around +30 mV as well as a particle stability of over 48 h after formulation, the YD-nanoplex formulation met obligatory conditions for consideration as a tumor therapy (Fig. 1). Electron-microscope images exhibited the shape of nanoplexes with and without sapofectosid. Due to shape alterations, an interaction between nanoplexes and sapofectosid can be assumed (Fig. 2). Confocal live cell-imaging illustrated the sapofectosid-mediated endosomal release of FITC-labeled nanoplexes and with that the incorporated genetic cargo (Fig. 5, supplementary data

Video 1). Neuro-2A-Luc cells showed a stable luciferase expression (supplementary data Figure 1). The viability of Neuro-2A-Luc was monitored after being transfected with YD-nanoplexes, carrying a suicide gene (saporin-plasmid). With consideration of the vector control (YD with GFP-plasmid ± sapofectosid) and the aggressive growth rate of the untreated negative control (Fig. 4), it can be concluded, that a distinct toxicity was caused by a sapofectosid-mediated saporin-gene delivery (Fig. 3). For *in vivo* experiments, NP and sapofectosid were investigated in terms of tolerability and confirmed for administration, since no toxic effects were observed. Slight gains of body weight could be explained with the mice's acclimation to their environment (Fig. 6-8). After tumor injection, a therapy with placebo, NP and NP+S was performed. Compared to the placebo group, a therapy with targeted saporin-nanoplexes achieved a considerable effect in tumor growth inhibition and was even improved with the addition of sapofectosid as transfection enhancer (Fig. 9). Statistical analysis (t-test) proved the benefit of sapofectosid by achieving statistical difference in terms of tumor growth by saponin co-administration. The anti-tumoral effect was confirmed by visual imaging of luminescent tumors. A stronger trend of tumor growth within the placebo group indicates a more aggressive growth, when no treatment was given (Fig. 10). The attenuation of tumor growth was clearly shown by individual analysis of luminescence increase 11 days after tumor injection (Fig. 11). A combinatorial therapy of nanoplexes and sapofectosid managed to suppress the integrated density of the luminescent tumor under a value of 4x10^8. No major tumor "breakouts" were observed as could be seen in the placebo group.

The combinatorial therapy of targeted nanoplexes together with sapofectosid has shown its major potential for future gene therapies. Simply formulated nanoplexes with a suicide gene yielded promising results against an aggressive and fast-growing tumor, which is difficult to treat. Selective therapy improvements such as improved nanoplex formulations and more selective targeting will allow greater therapy results. The i.v. application of nanoplexes and their associated biological distribution strengthens the potential of the conducted therapy enormously. Contrary to many other studies, where an injection was given vicinal to the tumoral, the performed i.v.-treatment represents a feasible option for clinical treatments.

In this study, we have entered a novel path in the field of gene and cancer therapy. By combining the process of sapofection with targeted suicide gene vehicles, we were able to create an innovative therapy, meeting obligatory conditions for cancer therapies of efficacy and tolerability. With further optimization in the future, we aim to establish a genuine alternative for established therapies.

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Conflicts of interest: none

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Supplementary Material:

1. Supplementary Data Figure 1 - Neuro-2A-cell Luciferase Expression
2. Supplementary Data Video 1 - Endosomal release by confocal live-cell-imaging