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Development of a novel nanotechnological approach for the treatment of medulloblastoma

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1. INTRODUCTION	6
1.1 Pediatric brain tumors	6
1.1.1 Medulloblastoma.....	8
1.1.2 SHH-Medulloblastoma	10
1.1.3 SHH-MB mouse model	13
1.1.4 Conventional SHH-MB treatment	15
1.2 New therapeutic approaches for the treatment of SHH-MB	16
1.2.1 SHH inhibitors	16
1.2.2 Gene therapy.....	18
1.2.3 Immunotherapy.....	18
1.3 Nanotechnology in nanomedicine	20
1.3.1 Different type of NPs	23
1.3.2 Lipid- based NPs	24
1.3.3 Polymeric NPs.....	24
1.3.4 Inorganic NPs.....	25
1.3.5 Protein-based NPs	26
1.4 Plant Molecular Farming (PMF)	26
1.4.1 Virus Nanoparticles as delivery systems.....	30
1.4.2 Plant Virus Nanoparticles.....	31
1.5 Plant virus modification	35
1.5.1 Genetic engineering	36
1.5.2 Chemical conjugation.....	37
1.5.3 Plant VNPs and VLPs loading	39
1.6 Tomato Bushy Stunt Virus (TBSV)	41
References are listed in Appendix I.....	45
2. AIMS AND SCOPE	46
3. TOMATO BUSHY STUNT VIRUS NANOPARTICLES AS A PLATFORM FOR DRUG DELIVERY TO SHH-DEPENDENT MEDULLOBLASTOMA	48
3.1 INTRODUCTION.....	48
3.2 MATERIALS AND METHODS.....	50
3.2.1 TBSV genetic engineering, production in plants, purification and characterization	50
3.2.2 Mouse model.....	52

3.2.3 Primary cell cultures and TBSV NPs internalization	52
3.2.4 RNA Isolation and Real Time qPCR analysis	53
3.2.5 Immunofluorescence analysis	53
3.2.6 NRP-1 and H-FABBP molecular docking and molecular dynamics simulations.....	53
3.2.7 Chimeric TBSV molecular docking and molecular dynamics simulations.....	54
3.2.8 Transmission Electron Microscopy	55
3.2.9 Dynamic light scattering and ζ -potential analyses.....	56
3.2.10 Production of DOX loaded TBSV NPs	56
3.2.11 Cell viability assay	57
3.2.12 Absolute quantification of chimeric TBSV in target tissues after intravenous injection.....	57
3.2.13 Statistical Analysis	58
3.3 RESULTS.....	59
3.3.1 Construction, production and purification of WT and chimeric TBSV NPs.....	59
3.3.2 In vitro validation of chimeric TBSV uptake.....	62
3.3.3 Immunolocalization of the interaction partners.....	63
3.3.4 Docking and MD simulation of the tLyp1-NRP-1 and Coop-FABP3 complexes	64
3.3.5 TBSV-tLyp1 and TBSV-Coop characterization and loading with DOX	71
3.3.6 Chimeric TBSV-mediated delivery of DOX to Shh-MB cells	75
3.3.7 ChimericTBSV NPs targeting to Shh-MB in vivo.....	76
3.4 DISCUSSION	78
3.5 CONCLUSIONS	81
References are listed in Appendix II	81
4. PEPTIDE-FUNCTIONALIZED AND DRUG-LOADED TOMATO BUSHY STUNT VIRUS NANOPARTICLES COUNTERACT TUMOR GROWTH IN A MOUSE MODEL OF SHH-DEPENDENT MEDULLOBLASTOMA.....	82
4.1 INTRODUCTION.....	82
4.2 MATERIALS AND METHODS.....	84
4.2.1 Mice treatment.....	84
4.2.2 Histology, morphometry and immunohistochemistry	85
4.2.3 RNA extraction and Real Time qPCR	86
4.2.4 Western blot.....	86
4.2.5 Immunofluorescence.....	87
4.2.6 Enzyme-Linked Immunosorbent Assay (ELISA) for antibodies titration.....	87

4.2.7 Statistical Analysis.....	88
4.3 RESULTS.....	89
4.3.1 DOX-loaded TBSV-CooP nanoparticles inhibit progression of MB PNLs.....	89
4.3.2 Effects of DOX-loaded TBSV-CooP nanoparticles on full-blown tumors	94
4.3.3 Antibody titration.....	97
4.4 DISCUSSION	98
4.5 CONCLUSIONS	100
References are listed in Appendix III	101
5. TARGETING OF TOMATO BUSHY STUNT VIRUS WITH A GENETICALLY FUSED C-END RULE PEPTIDE	102
5.1 INTRODUCTION.....	102
5.2 MATERIALS AND METHODS.....	104
5.2.1 Genetic engineering of TBSV and its production in plants.....	104
5.2.2 Chemical conjugation of CF555 dye on TBSV NPs	104
5.2.3 Enzyme-Linked Immunosorbent Assay (ELISA)	105
5.2.4 Flow cytometry.....	105
5.2.5 Confocal imaging of cultured cells	106
5.2.6 Doxorubicin loading of TBSV NPs	107
5.2.7 Cell viability assay	108
5.2.8 <i>In vivo</i> biodistribution analysis of TBSV	108
5.2.9 Statistical Analysis	109
5.3 RESULTS.....	110
5.3.1 Construction, Production and Purification of wild type (WT) and Chimeric TBSV NPs.....	110
5.3.2 Fluorophore labeling of TBSV NPs	111
5.3.3 <i>In vitro</i> binding of TBSV-RPAR to NRP-1	114
5.3.4 TBSV-RPAR Loading with DOX and cytotoxicity test <i>in vitro</i>	117
5.3.5 Systemic TBSV-RPAR NPs home to pulmonary tissue.....	119
5.4 DISCUSSION	120
5.5 CONCLUSIONS	122
References are listed in Appendix IV	122
6. GENERAL CONCLUSIONS AND PERSPECTIVES	123
Appendix I.....	125
References of Chapter 1.....	125

Appendix II.....	154
References of Chapter 3	154
Appendix III	160
References of Chapter 4	160
Appendix IV	163
References of Chapter 5	163

1. INTRODUCTION

1.1 Pediatric brain tumors

Pediatric brain tumors (PBTs) are a heterogeneous group of neoplasms that affect the central nervous system (CNS) of children. They are considered a major public health problem due to their high incidence, morbidity, and mortality [1]. The incidence of PBTs varies worldwide, with higher rates reported in developed countries, such as the United States, Europe, and Australia, compared to developing countries [2]. Several factors may contribute to the development of PBTs, including genetic predisposition [3], exposure to environmental toxins [4], and viral infections [5]; however, the exact cause remains unknown. There are different types of PBTs, and they can be classified based on the type of cells they originate from. The most common are gliomas and medulloblastomas (MB) [6] (Figure 1).

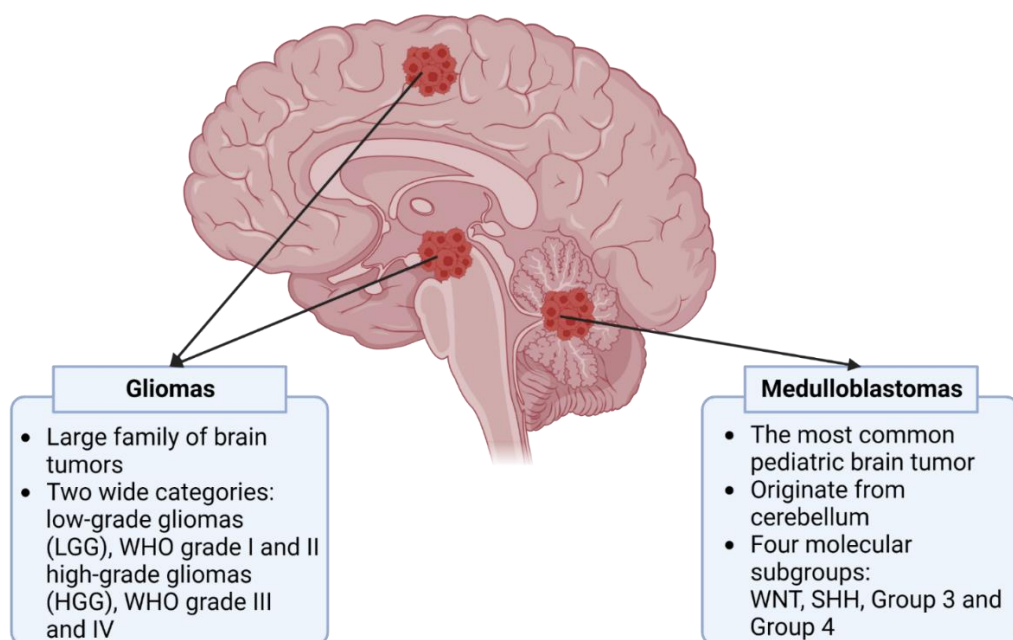


Figure 1. Classification of the main pediatric brain tumors

Gliomas are tumors that originate from glial cells, which are the supportive cells of the nervous system, and are classified into different subtypes based on the type of glial cell

involved, with astrocytomas being the most common type [7]. MBs are tumors that originate from cerebellar neural progenitor cells. They are the most common type of brain tumors in children aged 0-14 years [8]. The World Health Organization (WHO) classification system is widely used to categorize brain tumors based on their histological and molecular features, malignancy, or the degree to which they are cancerous. This system is important for establishing a diagnosis, guiding treatment, and predicting prognosis. The WHO classifies pediatric brain tumors into four grades (Table 1), from I to IV, with I being the least malignant and IV being the most malignant [9–12].

Table 1. Clinical profile of the most common pediatric brain tumors.

	Grade	Location	Treatment	Prognosis
Low Grade Glioma	I-II	Mostly cerebellum or optic pathway, but can occur in any region of the brain or spine	Surgery, craniospinal irradiation (CSI) and chemotherapy when complete resection is not possible	Largely dependent on tumor location, as this affects the probability of complete surgical resection
High Grade Glioma	III-IV	Cerebellum and brainstem	Surgery, craniospinal irradiation (CSI) and chemotherapy (for children over 3 years)	Poor
Medulloblastoma	IV	Posterior fossa	Surgery, craniospinal irradiation (CSI) and chemotherapy (for children over 3 years)	Average, but dependent on genomic subgroup of MB

Grade I tumors are considered benign, meaning they do not spread to other parts of the body and are not likely to invade surrounding tissue. These tumors are usually slow-growing and can be surgically removed with a good chance of a full recovery [13]. Grade II tumors are considered low-grade malignant, they are able to grow and invade surrounding tissue but not to spread to other parts of the body. These tumors are also typ-

ically slow-growing and can be surgically removed, but may require additional treatment such as radiation therapy or chemotherapy [14,15]. Grade III tumors are considered high-grade malignant, they invade surrounding tissue and spread to other parts of the body. These tumors are typically more aggressive and require more extensive treatment, including surgery, radiation therapy, and chemotherapy [16]. Finally, grade IV tumors are considered the most malignant, they grow quickly and spread rapidly to other parts of the body. These tumors are known to invade surrounding tissue and require aggressive treatment, including surgery, radiation therapy, and chemotherapy [17].

1.1.1 Medulloblastoma

MB is a type of brain cancer that affects both children and adults, although it is the most common type of malignant brain tumor in children and accounts for about 20% of all childhood brain tumors, being slightly more common in males than in females [18][19]. MB originates from the embryonic cells that form the cerebellum, the part of the brain responsible for coordinating movement and balance. The cells from which MB originates are called granule cells precursor (GCPs) and are located in the outer layer of the cerebellum. These cells are involved in the formation of new neural connections and are essential for normal brain development. When these cells become cancerous, they grow rapidly and spread to spinal cord through the cerebrospinal fluid [20]. The worldwide incidence of MB varies, but it is estimated to occur in about 1 in every 100,000 children [21]. Most of the cases are diagnosed in children between the ages of 3 and 10, but it can also occur in infants and older individuals [22]. In 2016, with the advances in genomics, the WHO consensus on the MB subgroup divided it into 4 major subgroups: Wingless (WNT), Sonic Hedgehog (SHH), Group 3, and Group 4 [23] (Figure 2). These subgroups are thought to originate from different types of cells in the cerebellum [24] and have different molecular profiles, growth patterns [25] and responses to treatment [26].

Subgroup		WNT	SHH	Group 3	Group 4
Clinical Characteristics	% of Cases	10	30	25	35
	Age at Diagnosis	 	  	 	
	Gender Ratio (M:F)	1:1	1:1	2:1	3:1
	Anatomic Location				
	Histology	Classic, Rarely LCA	Desmoplastic, Classic, LCA	Classic, LCA	Classic, LCA
	Metastasis at Diagnosis (%)	5-10	15-20	40-45	35-40
	Recurrence Pattern	Rare; Local or metastatic	Local	Metastatic	Metastatic
	Prognosis	Very good	Infants good, others intermediate	Poor	Intermediate
Molecular Characteristics	Proposed Cell of Origin	Progenitor cells in the lower rhombic lip	Granule precursors of the external granule layer	Neural stem cells	Unipolar brush cells
	Recurrent Gene Amplifications	-	<i>MYCN</i> <i>GLI1</i> or <i>GLI2</i>	<i>MYC</i> <i>MYCN</i> <i>OTX2</i>	<i>SNCAIP</i> <i>MYCN</i> <i>OTX2</i> <i>CDK6</i>
	Recurrent SNVs	<i>CTNNB1</i> <i>DDX3X</i> <i>SMARCA4</i> <i>TP53</i>	<i>PTCH1</i> <i>TERT</i> <i>SUFU</i> <i>SMO</i> <i>TP53</i>	<i>SMARCA4</i> <i>KBTBD4</i> <i>CTDNEP1</i> <i>KMT2D</i>	<i>KDM6A</i> <i>ZMYM3</i> <i>KTM2C</i> <i>KBTBD4</i>
	Cytogenetic Events  Gain  Loss	6	3q, 9p 9q, 10q, 17p	1q, 7, 18 8, 10q, 11, 16q i17q	7, 18q 8, 11p, X i17q
	Other Recurrent Genetic Events	-	-	<i>GFI1</i> and <i>GFI1B</i> enhancer hijacking	<i>PRDM6</i> , <i>GFI1</i> , and <i>GFI1B</i> enhancer hijacking

Age:  Infant  Child  Adult

Figure 2. Molecular subgroups of medulloblastoma. LCA= large cell/anaplastic; SNV= single nucleotide variant [27].

The WNT is the best known MB subgroup, comprising around 10–15% of the cases. It can occur at any age; however, it is most commonly encountered in children 6–12 years of age (peak incidence at 10–12 years) with slight female predominance [28]. It is characterized by activation of the WNT/ β -catenin signaling pathway, total of 85–90% of WNT MB cases harbor mutations in *CTNNB1*, the gene encoding for the protein β -catenin, and is associated with a better prognosis compared to the other subgroups [29].

The WNT subgroup is associated with an excellent prognosis (> 95% survival at 5 years in pediatric patients), is rarely metastatic at diagnosis (5%-10% of cases), and rarely recurs [5].

The SHH-MB is the most common malignant brain tumor of childhood, constitutes 28–30% of all MBs, has a bimodal age distribution (infants<4 years and adults>16 years old), and is characterized by the aberrant activation of the SHH signaling pathway [28]. The overall outcome is intermediate depending on the age group. In general, the 5-years overall survival rate in patients with SHH-MB is about 75%. However, this outcome probably reflects the heterogeneity within the subgroup; more-detailed analyses of risk factors have documented that outcomes differ according to patient age, histological subtype, presence of metastasis at diagnosis, and underlying molecular abnormalities [30]. Group 3 accounts for 25–28% of all MBs [29]; it occurs predominantly in males (2:1 male to female ratio) [28] and is characterized by alterations in the MYC gene [31]. This subtype has a more aggressive course and is associated with a higher risk of recurrence and lower survival rate with high rates of metastasis and the worst survival outcomes of any subgroups (under 60% at 5 years) [32]. Group 4 rarely arises in infants and occurs much more frequently in males than in females (3:1 ratio) across all age groups (median age = 9 years). It accounts for 35-40% of childhood MBs and is characterized by alterations in the *KDM6A* gene, a histone H3 Lys27 (H3K27) demethylase; nonsense mutation of which are found in 13% of patients [33]. This subgroup is associated with intermediate prognosis, is frequently metastatic (35%–40% at diagnosis) and recurrences tend to occur late [34].

1.1.2 SHH-Medulloblastoma

SHH-MB subgroup is known for its specific molecular features. It is characterized by the activation of the SHH signaling pathway [35], which is involved in the regulation of cell proliferation and differentiation during embryonic development [36]. The main players of the SHH pathway include the HH ligands Sonic (SHH), the Patched trans-membrane receptors (PTCH1 and 2), the G protein-coupled-like receptor Smoothened

(SMO), and the glioma-associated oncogene (GLI) transcriptional factors (GLI1, GLI2 and GLI3). SHH released from the Purkinje cells acts on overlying GCP cells in the external granule cell layer (EGL), leading to proliferation. The pathway is crucial for normal development of the cerebellum, because it governs the proliferation of GCPs cells, the most abundant neuronal population in the brain [37]. GCPs are a population of cells derived from the rhombic lip that populates the surface of the cerebellum and forms the EGL [38]. In mice, after one week of proliferation, GCPs start to differentiate and migrate towards the internal granule cell layer (IGL) and the EGL disappears completely at the third postnatal week (Figure 3a,b) [39].

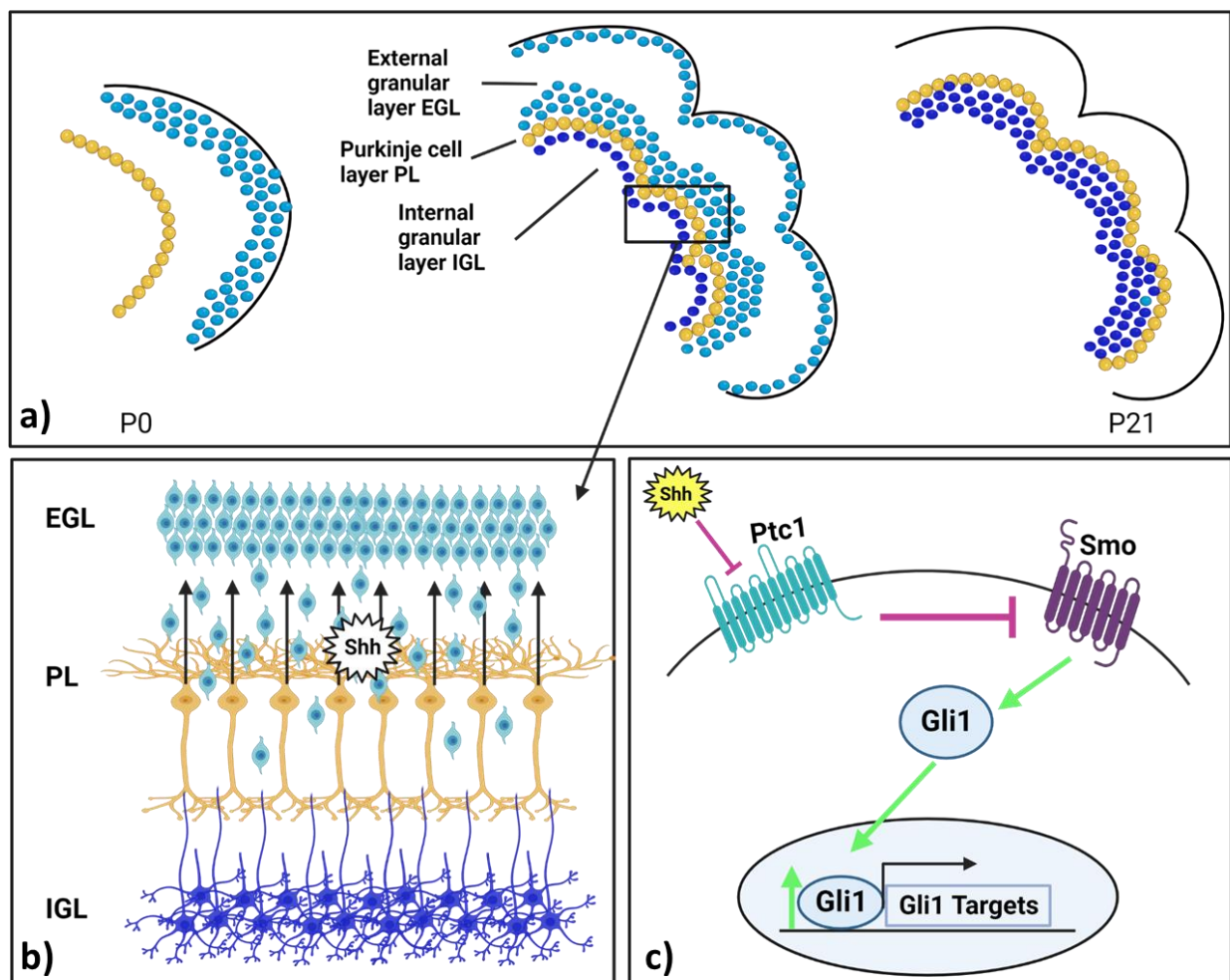


Figure 3. The SHH pathway activities during normal cerebellum development in mouse. SHH released from the Purkinje cells acts on overlying GCP cells in the EGL, leading to proliferation. After this period of SHH-dependent proliferation, granule neurons exit the cell cycle, begin to differentiate, and migrate inwards, past the Purkinje cell layer to reside in the IGL in the mature cerebellum. P0, post-natal day 0; P21, post-natal day 21; Ptc1, Patched1 protein; Smo, Smoothed protein; Gli1, Glioma-associated oncogene 1; PL, Purkinje cells layer.

GCPs cells are believed to be the cells of origin for MB; it has been suggested that MB arises as an aberration of normal developmental of GCPs due to genetic alterations/mutations in the components of SHH pathway [40]. As a result of the genetic alteration, the GCPs cells fail to exit the cell cycle at the appropriate time and remain in the EGL, eventually expanding to form a tumor. The first indication that mutations in the SHH pathway contribute to MB was the finding that Gorlin syndrome patients, who inherit germ line mutations in the *Ptc1* gene, are predisposed to MB [41,42]. One common genetic alteration in MB is the loss of function mutations in the *Ptc1* gene [43], which is responsible for the negative regulation of the SHH signaling pathway. *Ptc1* gene encodes a Ptc1 trans-membrane protein that functions as a receptor for SHH ligand (Figure 3c). In the absence of SHH, Ptc1 maintains the SHH pathway in the “off state” by acting on SMO protein [44]. Upon SHH binding to Ptc1, this repression is lifted and a signal is transduced through SMO to the nucleus increasing expression of target genes including *GLI1* and *Ptc1* itself [45] (Figure 4).

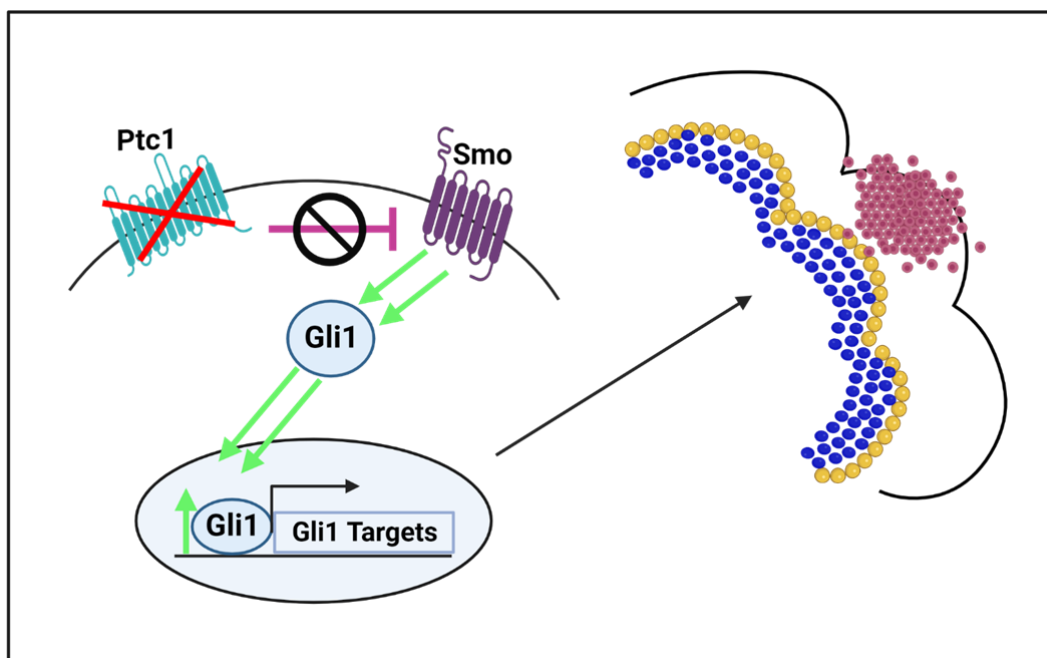


Figure 4. Somatic mutations of *Ptc1* gene, a component of SHH pathway, may contribute to sporadic medulloblastoma.

Another common genetic alteration in MB is related to the uncontrolled amplification of the SMO gene [46], which encodes the SMO protein that transduces the SHH signal to the downstream signaling components. In addition to these genetic alterations, other genetic changes can also contribute to the development of MB. Genome-wide analysis has revealed that SHH-MB have a high frequency of copy number alterations, with gains in chromosomes 7 and 17 and losses in chromosomes 1, 9, and 10 [47]. These genomic alterations lead to the activation of oncogenes, such as MYC and TP53 [48,49], and the inactivation of tumor suppressor gene, such as PTEN [50,51]. The molecular and genetic characterization of SHH-MB has important implications for the development of new targeted therapies.

1.1.3 SHH-MB mouse model

Mouse models have played a critical role in advancing our understanding of brain tumor biology and developing new treatments for these devastating diseases. Brain tumors are among the most complex and heterogeneous cancers, and mouse models offer a powerful tool for studying their molecular and cellular characteristics *in vivo*. Genetically engineered mouse models that recapitulate MB alterations found in human brain have been generated (Table 2).

Table 2. Medulloblastoma genetically engineered mouse models [52].

Genotype	MB profile	MB incidence	Tumor latency (week)	Ref.
Ptc ^{+/-} / Ptc ^{neo67/+}	SHH/desmoplastic	14%	5-25	[43][53]
Ptc ^{+/-} Ink4c ^{-/-} or ^{+/-}	SHH/desmoplastic	30%	12-36	[54]
Ptc ^{+/-} Kip1 ^{-/-} or ^{+/-}	SHH/desmoplastic	60-70%	16-18	[55]
Ptc ^{+/-} Hic1 ^{-/-}	SHH/desmoplastic	40%	by 25	[56]
Math1-Cre/Ptc ^{C/C}	SHH/desmoplastic	100%	8-12	[57]
Gfap-Cre/Ptc ^{C/C}	SHH/desmoplastic	100%	3-4	[57]
Sufu ^{+/-} /P53 ^{-/-}	SHH/desmoplastic	58%	by 28	[58]
Hemizygous ND2-SmoA	SHH/desmoplastic	48%	25	[59]
Homozygous ND2-SmoA	SHH/desmoplastic	94%	4-8	[60]

Gfap-Cre/Rb ^{loxP/loxP} /Tp53 ^{-/-or loxP/loxP}	SHH/desmoplastic	>84%	12	[61]
Nestin-Cre/Xrcc4 ^{loxP/loxP} /p53 ^{-/-}	SHH/desmoplastic	87%	12-14	[62]
Nestin-Cre/Xrcc2 ^{loxP/loxP} /p53 ^{-/-}	SHH/desmoplastic	>90%	14-16	[63]
Nestin-Cre/Lig4 ^{loxP/loxP} /p53 ^{-/-}	SHH/desmoplastic	>90%	14-16	[63]
Nestin-Cre/Brca2 ^{loxP/loxP} /p53 ^{-/-}	SHH/desmoplastic	>90%	14-16	[63]
GTML	Classic or LCA	75%	by 29	[64]
Blbp-Cre/Ctnnb1 ^{+/-lox(ex3)} /Tp53 ^{flx/flx}	WNT-subgroup	15%	41	[24]

In this PhD thesis, we used the *Ptc1*^{+/-} knockout mouse model developed by Hahn and colleagues, maintained on CD1 background. This MB mouse model has been created using Cre-loxP technology. The *Ptc1* gene was inactivated by replacing the exons 6 and 7 on chromosome 13 with a neomycin resistance (NEO) cassette [53] (Figure 5).

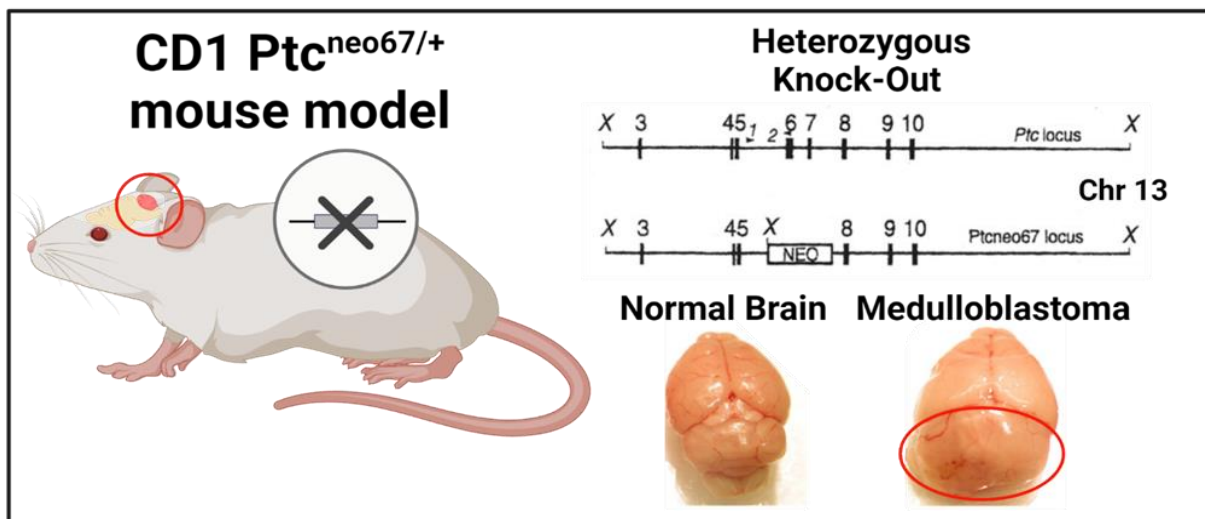


Figure 5. Schematic illustration of SHH-MB mouse model present at ENEA Research Center.

This mouse model has been extensively studied and characterized in the Laboratory of Biomedical Technologies (TEB) of the Department of Sustainability of Territorial Production Systems (SSPT) of the ENEA Casaccia Research center, where SHH-MB has been studying for over twenty years. Studies of exposure from low to intermediate doses of ionizing radiation to identify biological factors influencing tumor response conducted on the *Ptc1*^{+/-} mouse model, have shown that it is a radiosensitive model and that the degree of susceptibility is closely related to the age of the mice during exposure [65–69]. Indeed, *Ptc1* mutated mice spontaneously develop MB with an incidence of 8%, but if

subjected to irradiation with doses of 3 Gy in postnatal age (P2) the onset of the tumor increases up to 80%. This MB model, in addition of being useful for studying and understanding the risks of exposure to low doses of radiation, is useful for studying the molecular mechanisms of MB tumorigenesis and evaluating the efficacy of new therapies.

1.1.4 Conventional SHH-MB treatment

The treatment of SHH-MB remains a significant challenge for pediatric oncologists due to the difficulty in achieving complete remission, the risk of long-term toxicity, and the high likelihood of relapse. The current management strategies for this disease involves a combination of treatments, including maximal surgical resection followed by cranio-spinal irradiation (CSI) and adjuvant chemotherapy [70]. Surgery is the primary modality used to treat SHH-MB. The goal of surgery is to remove as much of the tumor as possible without causing significant neurological damage. The surgical approach for SHH-MB patients typically involves craniotomy followed by subtotal resection or biopsy [71]. Craniotomy is associated with a relatively low risk of morbidity and mortality, and has been shown to improve overall survival compared to biopsy alone [72]. However, craniotomy has several limitations, including the risk of infection, bleeding, and the need for a long period of rehabilitation [71,73]. In addition, subtotal resection or biopsy may not be possible in all cases, particularly when the tumor is located in an area of the brain that is difficult to access [71].

Radiation therapy is another important component of the treatment plan for SHH-MB. This treatment uses high-energy beams, such as X-rays, to destroy the cancer cells and shrink the tumor [74]. Radiation therapy is usually administered after surgery to reduce the risk of the cancer recurring. The most common radiation therapy approach for SHH-MB patients is CSI [75], which involves the delivery of radiation to the entire spinal cord and brain. CSI has been shown to significantly improve overall survival and reduce the

risk of relapse compared to surgery alone [76]. However, CSI is associated with a significant risk of long-term toxicity, including intellectual and neurocognitive impairment, endocrine dysfunction, and cardiovascular diseases [77,78].

Chemotherapy is also a common component of the treatment plan for SHH-MB. This treatment uses drugs to kill cancer cells and stop them from dividing and growing. Chemotherapy is usually administered in combination with radiation therapy to further improve the outcome for patients [79]. The most common chemotherapy regimen used to treat SHH-MB is high-dose methotrexate [80], a folate antagonist that is toxic to rapidly dividing cells. High-dose methotrexate has been shown to improve overall survival and reduce the risk of relapse, particularly when used in combination with radiation therapy [81]. However, methotrexate is associated with a significant risk of neurotoxicity [82], kidney damage, and liver damage [83].

1.2 New therapeutic approaches for the treatment of SHH-MB

In recent years, more and more researchers, with the aim of improving outcomes for patients with SHH-MB, have focused on developing new therapeutic approaches based on the precise targeting of the SHH pathway, which plays a crucial role in the development of SHH-MB.

1.2.1 SHH inhibitors

Targeted therapy is one of the most promising new approaches for SHH-MB treating [84]. This type of treatment is designed to target specific proteins or signaling pathways that are involved in the growth and survival of cancer cells. In the case of SHH-MB, several proteins have been identified as potential targets, including the SHH signaling pathway and the GLI family of transcription factors [85–87]. Targeted SHH inhibitors are small molecules that specifically target the SHH signaling pathway to inhibit its activity and ultimately reduce the growth and proliferation of tumor cells [88]. One of the most well-studied targeted therapy inhibitors for the treatment of SHH-MB is vismodegib [89,90]. Vismodegib is a selective SHH pathway inhibitor that blocks SHH

signaling by binding to SMO protein and inhibiting activation of downstream SHH target genes [91] (Figure 6).

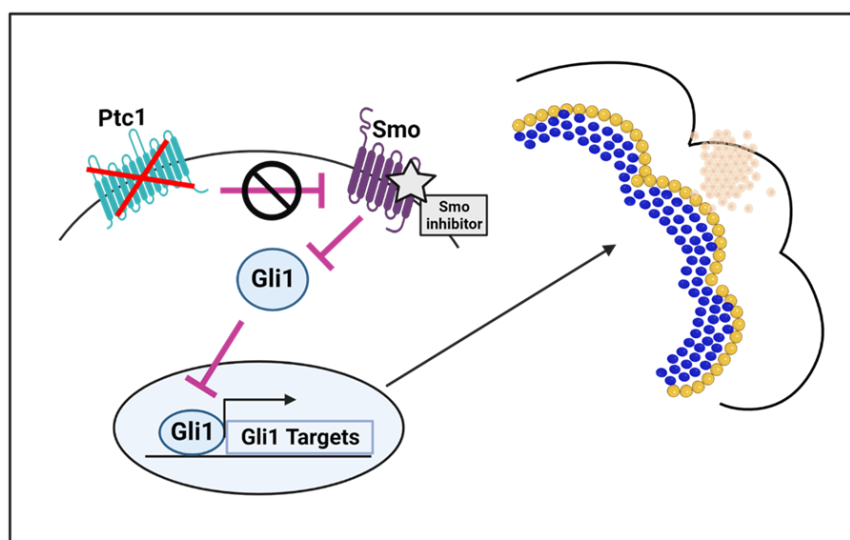


Figure 6. Schematic representation of inhibition of the SHH pathway mediated by a molecule targeting the SMO protein.

Clinical trials have shown that vismodegib is effective in reducing the size of SHH-MB tumors in some patients [92,93]. Another promising targeted therapy inhibitor for the treatment of SHH-MB is sonidegib [94], structurally similar to vismodegib, but with a slightly different mechanism of action. In fact sonidegib directly inhibits the activity of the GLI transcription factors, which are the downstream effectors of the SHH signaling pathway [95,96]. In preclinical studies, sonidegib has been shown to be effective in reducing cancer cells growth *in vitro* and *in vivo* [95,97]. In addition to vismodegib and sonidegib, there are several other targeted therapy inhibitors that have been investigated for the treatment of SHH-MB [22]. One of these is lomustine, a drug molecule that has been shown to inhibit the activity of the SHH signaling pathway in MB cancer cells [98]. However, the real efficacy of lomustine is still unclear, and more research is needed to determine its effectiveness in patients.

1.2.2 Gene therapy

Gene therapy is another innovative therapeutic approach that has shown promise for the treatment of MB [99,100]. This therapy involves the delivery of therapeutic genes to cancer cells in order to alter their behavior. One approach is to use siRNA molecules to block the expression of genes responsible of the SHH pathway alteration. Another approach involves delivering a functional copy of a tumor suppressor gene directly into the tumor cells to restore normal function and inhibit tumor growth. One recent study described the use of a viral vector system to deliver a functional SMO gene, which is a critical component of the SHH signaling pathway, into glioblastoma cells [101]. The study showed that this approach was effective in reducing the expression of the downstream SHH target genes and inhibiting tumor cell proliferation *in vitro*. In addition, some researchers are investigating the use of CRISPR-Cas9 technology to modulate endogenous Myc expression in mouse model of G3 MB [102]. In fact, this technology allows for precise editing of the genome, including the correction of genetic mutations that contribute to tumor onset, for the development of novel treatment strategies or murine models. Although gene therapy offers great potential for treating several types of cancers, especially those driven by the overactive SHH pathway, its use for MB is still in its early stages of development.

1.2.3 Immunotherapy

Immunotherapy is a rapidly-growing field of cancer research and has shown great promise for the treatment of MB [103–105]. This type of therapy involves boosting the body's natural immune response to cancer, either by activating the immune system directly or by blocking signals that prevent it from recognizing and attacking cancer cells. One type of immunotherapy that has shown promises in the treatment of SHH-MB is the checkpoint inhibitor therapy [106,107]. Checkpoint inhibitors are drugs that target specific proteins on the surface of immune cells that act as "brakes" on the immune sys-

tem, preventing it from attacking cancer cells. By blocking these brakes, checkpoint inhibitors unleash the full power of the immune system against the cancer, allowing it to recognize and destroy the cancer cells more effectively. Several checkpoint inhibitors, as anti-PD-1 and anti-CTLA-4, have been developed and tested in clinical trials for the treatment of SHH-MB (NCT02359565: Pembrolizumab and NCT03173950: Nivolumab, respectively) [106,108]. Another type of immunotherapy that has shown promise for the treatment of SHH-MB is adoptive cell therapy [104]. Adoptive cell therapy is a type of treatment that involves removing immune cells from a patient, modifying them to make them more effective against cancer, and then re-infusing them back into the patient [109]. One type of adoptive cell therapy that has been used in the treatment of Group 3 MB is CAR T-cell therapy [110–112], in which T cells are engineered to express a chimeric antigen receptor (CAR) that targets a specific protein on the surface of cancer cells [110]. These CAR T cells can then home the cancer cells and destroy them, providing a powerful immune response against the cancer. In addition to checkpoint inhibitor therapy and adoptive cell therapy, other types of immunotherapy are being explored for the treatment of SHH-MB, including vaccine therapy [113] and oncolytic virus therapy [114,115].

Despite the encouraging results of the new innovative strategies for the treatment of MB, the combined approach of surgery, radiotherapy and chemotherapy remains the most used strategy. There is therefore an evident need to develop new therapeutic approaches for the treatment of MB patients, especially those with high-risk or recurrent/relapsing disease [116,117]. Patients with high-risk disease, including those younger than aged 3–6 years or those with metastatic disease, large cell or anaplastic histology, or poorly resected tumors, [116–118] have lower survival rates than patients with standard-risk disease; 5-years survival rates are 55%–76% and 70%–85%, respectively [116,119]. In addition, most survivors suffer from long-term toxicities associated with the current standard-of-care treatment [117,120,121]. In particular, CSI-induced neurocognitive toxicities, which are inversely related to patient age, can be severe [117–119,121]. For this reason, CSI is not recommended for patients younger than aged 3–6 years [117–119]. In these

patients, postsurgical chemotherapy is suggested and usually includes high-dose chemotherapy with stem-cell rescue [117]. However, using chemotherapy in children has always been a challenge. For example, although SHH pathway is a promising strategy for MB treatment, it is involved in development of CNS and its direct inhibition can result in severe adverse effect on children growth [122]. In this context, nanotechnologies can play a crucial role in simultaneously improving the efficacy and safety of chemotherapy treatments through the development of new drug delivery systems.

1.3 Nanotechnology in nanomedicine

Nanotechnology is an emerging branch of science that has brought significant advancements in various fields, including medicine. Nanomedicine, which refers to the use of nanotechnology in healthcare, has the potential to revolutionize the field of oncology [123]. Among different types of nanotechnology-based approaches, nanoparticle (NP)-based technology has shown immense potential in cancer treatment. In fact, by the development of NP-based targeted delivery systems, therapeutic agents can be selectively delivered to the tumor site [124,125] (Figure 7).

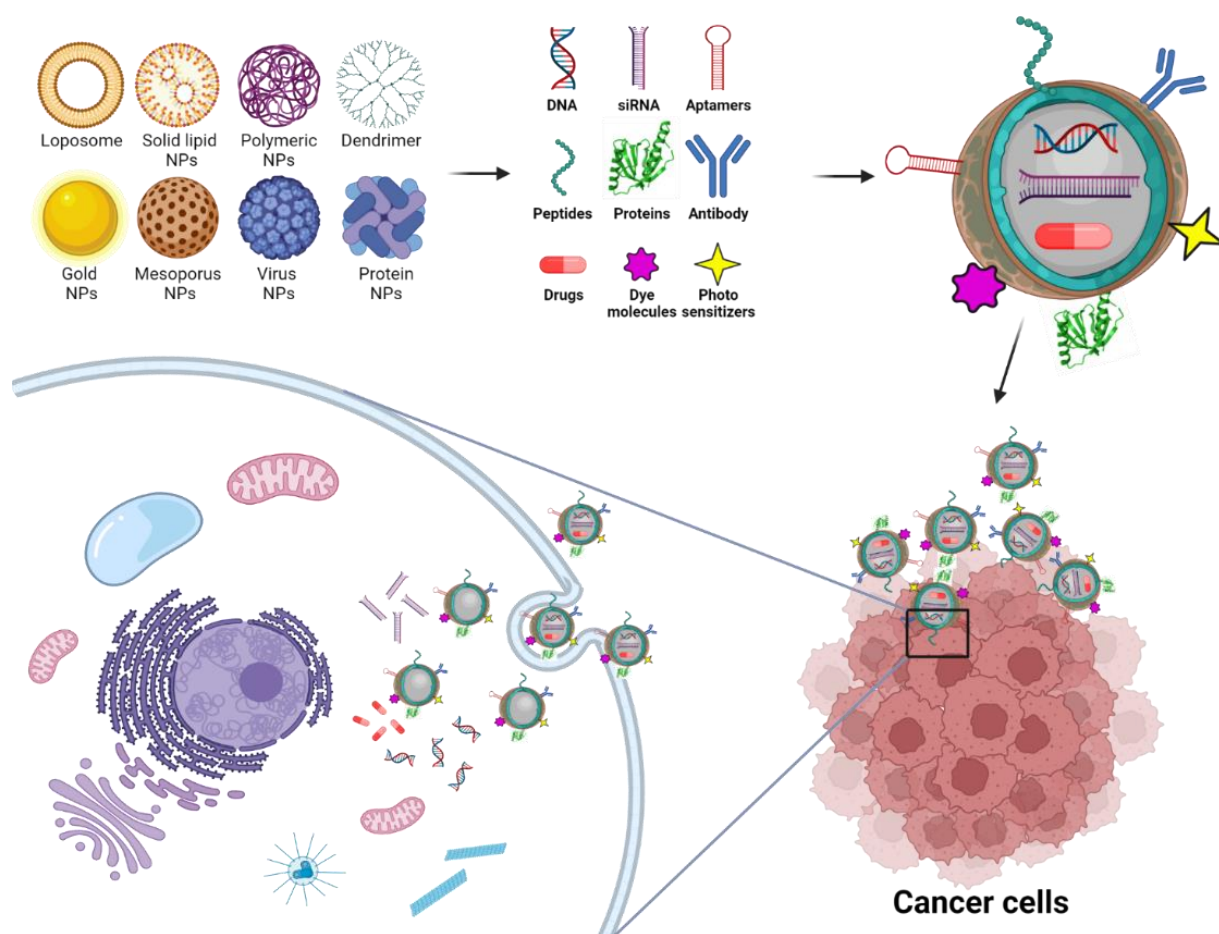


Figure 7. Illustration of different approaches to design a NP-based smart delivery systems. Various NPs, therapeutic molecules, targeted sites, and the different types of internal or external modifications with which a NP can be engineered are represented.

NPs are sub-micron particles with a size ranging from 1 to 100 nanometers that have shown great potential as drug delivery systems [126]. Due to their small size, NPs can easily penetrate through the tumor tissues and accumulate at the site of the tumor, increasing the efficacy of chemotherapy [127]. NPs can be engineered to possess specific physicochemical properties, such as size and surface charge/chemistry, which enable them to interact with biological systems in a controlled manner, an ideal characteristic for targeted drug delivery platforms [128,129]. NPs can be designed to carry a wide range of drugs such as small molecules, proteins, peptides, nucleic acids or other therapeutic agents to specific targets in the body [130] (Figure 7). Traditional cancer treatment, chemotherapy, can have significant side effects because the drugs are not specific to the tumor cells and can also kill healthy cells. However, NPs surface can be functionalized

with ligands or antibodies that bind to specific cell surface receptors, allowing for targeted drug delivery to cancer cells and protecting active principles from degradation and clearance by the immune system, leading to increased drug circulation time and improved bioavailability [131,132]. Depending by their nature, origin and source, NPs allow other promising applications:

- i) as radiosensitizers: to enhance efficacy of radiation therapy. When exposed to radiation, NPs can generate reactive oxygen species, which can damage the DNA of cancer cells, leading to their death [133];
- ii) as imaging tool: to improve the sensitivity of *in vivo* imaging. By labeling NPs with imaging agents, such as fluorescent dyes or magnetic resonance imaging (MRI) contrast agents, researchers can visualize the tumor tissues with high resolution and accuracy and detect early-stage tumors, allowing for early diagnosis and treatment [134];
- iii) as nanovaccines: to stimulate the immune system to recognize and attack cancer cells. By the exposure of tumor antigens on nanoparticles surface, they can be efficiently delivered to antigen-presenting cells, leading to the activation of immune cells [135]. Moreover, nanoparticles can also act as adjuvants, enhancing the immune response and promoting long-term immunity [131];
- iv) as delivery systems for nucleic acids, such as siRNA and miRNA: to silence specific genes involved in cancer progression [136]. By delivering nucleic acids to tumor cells, NPs can selectively inhibit the expression of genes that promote tumor growth, leading to cancer cell death;
- v) in addition to drug delivery, imaging, and gene therapy, NP-based nanotechnology can be used also for photothermal therapy, since they can absorb light and generate heat, leading to the death of cancer cells [137].

This broad field of applications are closely associated with the prerogative of achieving tumor targeting. In oncology tumor targeting is essential, as it allows for the selective delivery of therapeutic agents to the tumor site, while minimizing toxicity to normal tissues [138]. There are several strategies for tumor targeting, including passive and active targeting [139] (Figure 8).

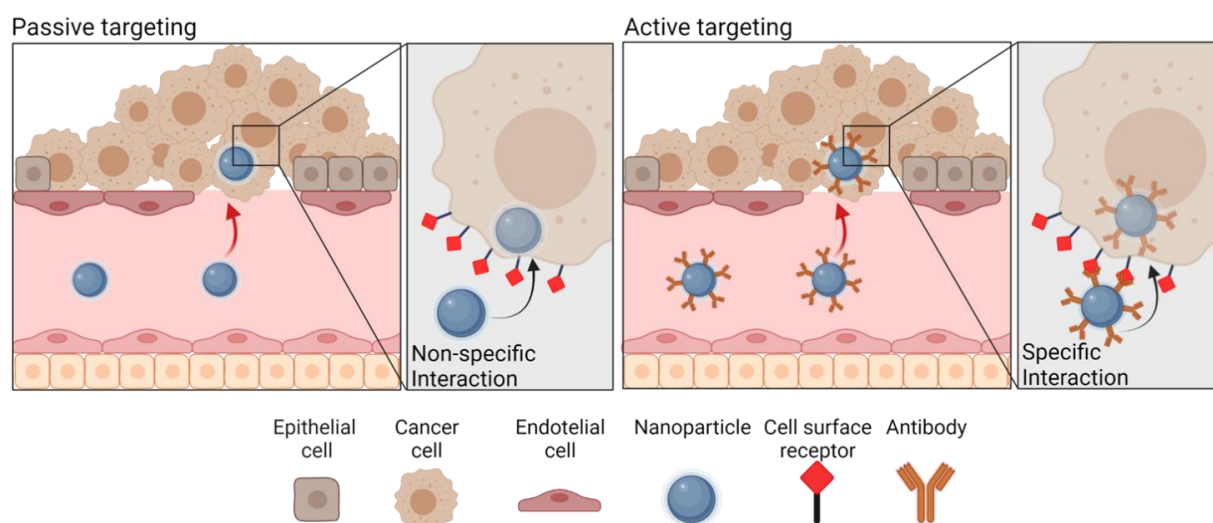


Figure 8. Schematic overview representing transport mechanisms of systemically administered NPs in biomedical applications. In passive targeting nonspecific interaction with tumor cells may occur upon paracellular NP transport. In contrast to passive targeting, ligand-coated NPs may interact specifically with tumor cells, potentially leading to increased NP retention and improved cell uptake.

Passive targeting relies on the enhanced permeability and retention (EPR) effect [140], which occurs due to the leaky vasculature and poor lymphatic drainage of solid tumors [141]. In this case, NPs can exploit this effect by accumulating in the tumor *via* passive diffusion, leading to higher drug concentrations in the tumor compared to normal tissues [142]. However, passive targeting has limitations, particularly in heterogeneous tumors with varying EPR characteristics [143]. Active targeting instead, can overcome this limitations and increase the specificity and efficacy of NP delivery to the tumor site [144] (Figure 8). Active targeting involves the use of specific ligands or antibodies that can selectively bind to receptors or antigens on the surface of cancer cells, leading to enhanced uptake and retention of the NPs in tumor tissue [139,145]. For example, NPs that target specific receptors or antigens on the surface of cancer cells, such as HER2 [146], EGFR [147], and VEGF [148], have shown promising results in preclinical studies, and some of these agents are currently being evaluated in clinical trials.

1.3.1 Different type of NPs

Over the past decades, the progress of nanotechnology has opened new avenues for medical science, especially in the field of drug delivery. Traditional drug carriers have

been developed based on liposomes/lipids [149], polymers of synthetic and natural origin [150], and inorganic nanoparticles [151] (Figure 9).

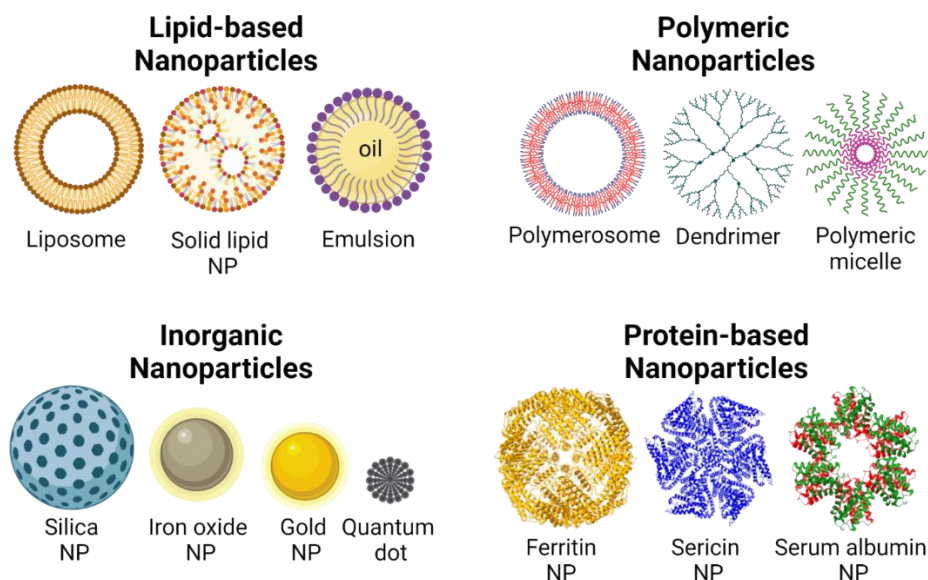


Figure 9. Some of most common classes of NPs used in nanomedicine for targeted therapy against tumors or as *in vivo* imaging systems.

1.3.2 Lipid- based NPs

Lipid-based NPs include various subset structures but are most typically spherical platforms comprising at least one lipid layer surrounding one internal aqueous compartment. As a delivery system, lipid- based NPs, such as such liposomes, lipid NPs and oil emulsion are the most common used as they offer many advantages including formulation simplicity, self-assembly, biocompatibility, high bioavailability, ability to carry large payloads and a range of physicochemical properties that can be controlled to modulate their biological characteristics [149,152]. For these reasons, lipid- based NPs are the most common class of FDA-approved nanomedicines [153,154].

1.3.3 Polymeric NPs

Polymeric NPs can be synthesized from natural or synthetic materials, as well as monomers or preformed polymers allowing for a wide variety of possible structures and characteristics [155]. The most common forms of polymeric NPs are polymersomes, micelles

and dendrimers. Polymersomes are artificial vesicles, with membranes made using amphiphilic block copolymers. They are comparable to liposomes but are reported to have improved stability and cargo-retention efficiency [156]. Polymeric micelles are also typically responsive block copolymers that self-assemble to form nanospheres with a hydrophilic core and a hydrophobic coating; this serves to protect aqueous drug cargo and improve circulation times [157]. Dendrimers are hyperbranched polymers with complex three-dimensional architectures for which mass, size, shape and surface chemistry can be highly controlled. Active functional groups present on the exterior of dendrimers enable conjugation of biomolecules or contrast agents to the surface while drugs can be loaded in the interior. Dendrimers can hold many types of cargo, but are most commonly investigated for the delivery of nucleic acids and small molecules [158,159].

1.3.4 Inorganic NPs

Inorganic materials such as gold, iron and silica have been used to synthesize nanostructures for various drug delivery and imaging applications. These inorganic NPs are precisely formulated and can be engineered to have a wide variety of sizes, structures, and geometries. Gold NPs (AuNPs), which are the most well studied, are used in various forms such as nanospheres, nanorods, nanostars, nanoshells and nanocages [160]. Magnetic iron oxide NPs, composed of magnetite (Fe_3O_4) or maghemite (Fe_2O_3), possess superparamagnetic properties at certain sizes and have shown success as contrast agents, drug delivery vehicles and thermal-based therapeutics [161]. Other common inorganic NPs include calcium phosphate and mesoporous silica NPs, which have both been used successfully for gene and drug delivery [162,163]. Quantum dots, typically made of semiconducting materials such as silicon, are unique NPs used primarily in *in vitro* imaging applications, but they show promises for *in vivo* diagnostics [164,165].

1.3.5 Protein-based NPs

Biopolymer-based NPs, such as protein NPs, are actively used as pharmaceutical and functional tools owing to their low toxicity and biodegradability. As exhibited throughout many studies that have been conducted on this subject [166], proteins exhibit unique functions and properties in biological materials and manufacturing fields, and can be used as base materials for production of nanoparticles. Protein NPs have been prepared from various sources including water-soluble proteins (ferritin, bovine and human serum albumin, silk sericin) and partially soluble or insoluble proteins (silk fibroin, zein, and gliadin) [167].

Minimal toxicity, biocompatibility and biodegradability are pivotal features to achieve NPs clinical impact. However, some synthetic carriers are limited by toxicity and/or low delivery efficiency, so alternative systems need to be developed [168]. Virus nanoparticles (VNPs) and their noninfectious counterparts, virus-like particles (VLPs), have emerged as a promising delivery system for therapeutic agents such as drugs, genes, and proteins. Viruses are naturally occurring nanoscale structures composed of proteins and nucleic acids that have evolved to efficiently transport their genetic material into host cells. Interestingly, the production strategies of these natural NPs are based on the use of biological systems such as bacteria, yeasts or mammalian cells, and also to completely green strategies based on the use of plants as biofactories, called plant molecular farming (PFM) [169].

1.4 Plant Molecular Farming (PMF)

Plant molecular farming is a modern and rapidly expanding field of biotechnology that involves the production of high-value proteins, metabolites, and other compounds using genetically engineered plants as a bioreactor (Figure 10).

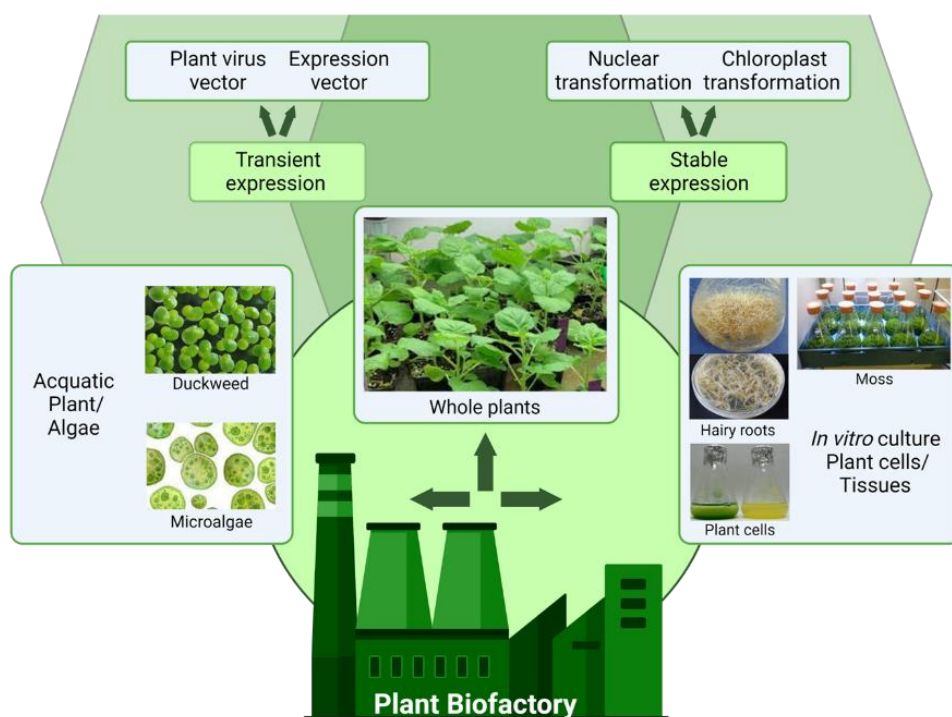


Figure 10. Plant platforms for the expression of recombinant proteins

The concept of using plants as bioreactors for the production of valuable compounds dates back to the early 1990s, when researchers first demonstrated the expression of functional immunoglobulin in transgenic tobacco plants [170]. Being a higher eukaryotic organism, plants allow expression of heterologous proteins with structural and functional characteristics similar to those of native proteins, including post-translational modifications [171], therefore they are a suitable host system for the production of human pharmaceuticals. Moreover, compared to traditional biomanufacturing methods that rely on microbial or animal cell cultures, the production of recombinant proteins in plants can be scaled up easily and inexpensively by using conventional agricultural methods, and without risk of mammalian pathogens contaminations [172]. Another advantage of PMF is the potential for producing complex and biologically active proteins that are difficult or impossible to produce using traditional biomanufacturing strategies [173].

Plant-based platforms are classified according to the transformation method (stable or transient) and the plant tissue used to target the expression. The basic principle of stable

transformation consists in inserting a foreign gene encoding the protein of interest into the genome (nuclear or plastid) of plant cells that are subsequently used to regenerate an entire plant through a callus phase [174]. The gene of interest is usually placed under the control of a strong and specific promoter that ensures its expression in the desired tissues [175]. By this approach, all the cells of the regenerated organism are genetically transformed, including germ cells that can transmit the heterologous information to the progeny [176]. Protein expression and accumulation can be targeted in leaves, seeds or any other storage organ [177,178]. The yields of the recombinant product that are obtained through stable transformation are generally not too high because the gene is inserted randomly into the plant genome (position effect) and also requires the screening of a large number of plants [179]. All the process (transformation, regeneration and growth) is generally long and time consuming also because several generations are needed to obtain plants that are homozygous for the transgene [173]. Other challenges are the regulatory framework for the production and use of transgenic plants. The use of transgenic plants for the production of pharmaceuticals and other products is subject to strict regulations and safety assessments by regulatory agencies, such as the FDA and USDA in the United States, and the European Medicines Agency in Europe [180]. Another challenge is the risk of contamination of the environment and food chain by genetically modified plants [181]. Although the risk of gene flow from transgenic plants to wild relatives or non-target organisms is low, it cannot be completely eliminated [182]. To face these challenges, transient protein expression strategies can bring significant advantages. In fact, it can considerably reduce costs to current cell-based manufacturing systems, while avoiding the complex procedures needed in stable plant transformation and risk assessment. Transient systems have been demonstrated to be environmentally friendly in both indoor and outdoor test [183].

Two approaches can be used for transient expression: standard binary plant expression vectors and virus-based expression vectors [184–187]. Binary expression vectors are introduced into intact plants using an *Agrobacterium tumefaciens*-mediated transfer-DNA

delivery system (Agro-infiltration, i.e. vacuum infiltration of aerial parts of the plant to introduce *Agrobacterium* cells containing expression vectors into the plant cells) [188,189]. The co-expression of silencing suppressor proteins, derived from plant viruses such as TBSV p19, has been shown to be a key factor for yields optimization [190–192]. Agro-infiltration have been used to produce a wide range of biopharmaceutical proteins [188,193,194].

Virus-based expression vectors offer advantages over standard plant expression systems by exploiting the cytoplasmic replication cycle of the virus [186,195,196]. These systems allow rapid and efficient expression level, obtaining considerable amount of the recombinant protein in as little as 4-8 weeks, while leaving no heritable changes in the progeny (no foreign gene is transmitted in the pollen) [195]. Virus-based expression vectors generally fall into two categories: "full virus" or "minimal virus". Full-virus vectors containing the entire genome of potexviruses (including potato virus X; PVX), tobamoviruses (including tobacco mosaic virus; TMV), comoviruses (including cowpea mosaic virus, CPMV), and many others have been developed [186,195]. The protein of interest or the viral particles accumulate in large quantities in the tissues of the infected plants and through purification protocols can be retrieved and collected. Usually, for recombinant protein expression, the sequence encoding the protein of interest is placed under the activity of subgenomic promoters, a typical feature of some kind of viruses [197]. Moreover, through genetic engineering strategies involving the gene encoding for the coat protein the full virus vectors can be modified to produce chimeric virus particles exposing on their surface a heterologous sequence. In contrast, minimal-virus mainly exploit the highly efficient viral replication system, and some of the "accessory" viral proteins involved in the infection process are eliminated. This approach makes it possible to increase the size of the genetic sequence inserted in viral genome [198]. Since these minimal vector viruses are unable to move from cell to cell into the inoculated plants, agroinfiltration procedures are used to initiate the infection process [196,199,200]. Compared to full-virus vectors, production times are considerably reduced as it is not necessary to wait for the infection to spread systemically [196,200].

Plant virus can be used not only as expression vectors, but also *per se*, as nano-objects for several nanotechnological application, biomedicine in particular. Therefore, PMF is a suitable approach for the production of protein-based delivery systems VLPs, plant VLPs and VNPs (pVLPs and pVNPs, respectively) *in primis*.

1.4.1 Virus Nanoparticles as delivery systems

The better understanding of the structure and function of viruses has revealed a collection of natural molecular assemblies and containers with a variety of shapes, sizes, stabilities, dynamic properties, and chemical reactivities [201]. Viruses are increasingly being used in materials science, engineering, and nanotechnology as tools and building blocks for electronics, chemistry, and biomedical science [202]. Although evolved in nature to invade and infect host cells for the efficient delivery of genomic cargo, recent developments in nanotechnology have revolutionized viruses as safe delivery vehicles. In fact, the VLPs are self-assembled structures that mimic the structural and functional properties of viruses, but the lack of genetic material makes them safe for biomedical applications [202] (Figure 11).



Figure 11. Schematic representation of the self-assembly of an icosahedral virus-like particles.

The structural proteins of the virus form the building blocks of the VLPs and are produced in a recombinant expression system. In this way it is possible to directly purify VLPs already assembled *in vivo* or the single structural units subsequently assembled *in vitro* through a variety of methods, including chemical crosslinking, heat shock, or self-assembly through electrostatic interactions [203,204].

One example of the use of VLPs in drug delivery is the development of VLPs derived from the hepatitis B virus (HBV). *Escherichia coli* produced HBc (Hepatitis B core) VLPs were used to deliver the anticancer drug doxorubicin (DOX) to HeLa and HT29 cells [205] and glioblastoma cells [206]. DOX was encapsulated within the VLPs, modified to display a tumor-targeting peptide on their surface. These VLPs were shown to be highly effective in delivering the drug to the cancer cells, resulting in increased cytotoxicity compared to free DOX. VLPs have also been investigated as potential imaging agents for diagnostic purposes.

For example, Qbeta bacteriophage VLPs have been used to enhance magnetic resonance imaging (MRI) [207,208]. They have high payload capacity and can carry a large amount of imaging agent, such as gadolinium (Gd), within their interior. Another study demonstrated the potential of M13 VLPs as an imaging agent for the detection of breast cancer [209]. Specifically, AF488 fluorophore has been chemically attached to the phage capsid which displayed antibody fragments to EGFR and HER2 proteins. In addition to mammalian and bacterial viruses, also plant viruses are promising delivery systems for therapeutics, such as drugs, genes, proteins, and imaging agents.

1.4.2 Plant Virus Nanoparticles

Since plant viruses were discovered in the late nineteenth century, their impact has changed from harmful pathogens to useful molecular machines with applications in plant molecular farming and bio-nanotechnology. Although public perception of a plant virus is often associated with a harm, viruses can easily be reorganized into environmentally friendly and safe structures [210–212]. The expansion of the overall knowledge

and understanding of viral genomes, architecture, and biophysical properties has allowed the use of plant viruses as vectors for recombinant protein expression and the production pVNPs. pVNPs are particularly valuable because they are not only biocompatible and biodegradable, but also considered non-infectious and nonhazardous in humans and other mammals [213–215]. pVNPs are well-characterized, monodisperse structures (many solved at atomic resolution) that can be produced in large quantities using plants. The production and purification of pVNPs from plants are rapid, cost effective and can easily be scaled up depending on demand [216]. Plant viruses have very simple shapes (rod-shaped, spherical, quasi-spherical, or filamentous), consisting of multiple copies of one or a few coat protein (CP) subunits, forming a protein capsid around the viral genome. Some of the many biological applications of pVNPs include vaccine production and innovative delivery mechanisms for therapeutic compounds, diagnostic reagents, enzymes, pesticides, immunogenic peptides, and many others [217]. Approximately 900 plant viruses, identified mainly in crops, are known [218]. Several of them, such as the TMV, PVX, CPMV, cowpea chlorotic mottle virus (CCMV), brome mosaic virus (BMV), cucumber mosaic virus (CMV), papaya mosaic virus (PapMV), tomato bushy stunt virus (TBSV) are utilized in biotechnology and plant viral-based nanotechnology [219,220]. With advances in biochemistry and molecular biology, continuous progresses have been made in the development of viral nanocarriers for drug delivery and theranostic applications. The most advanced pVLPs and pVNPs platforms for drug delivery applications are those based on TMV, CPMV, and PVX (Figure 12).

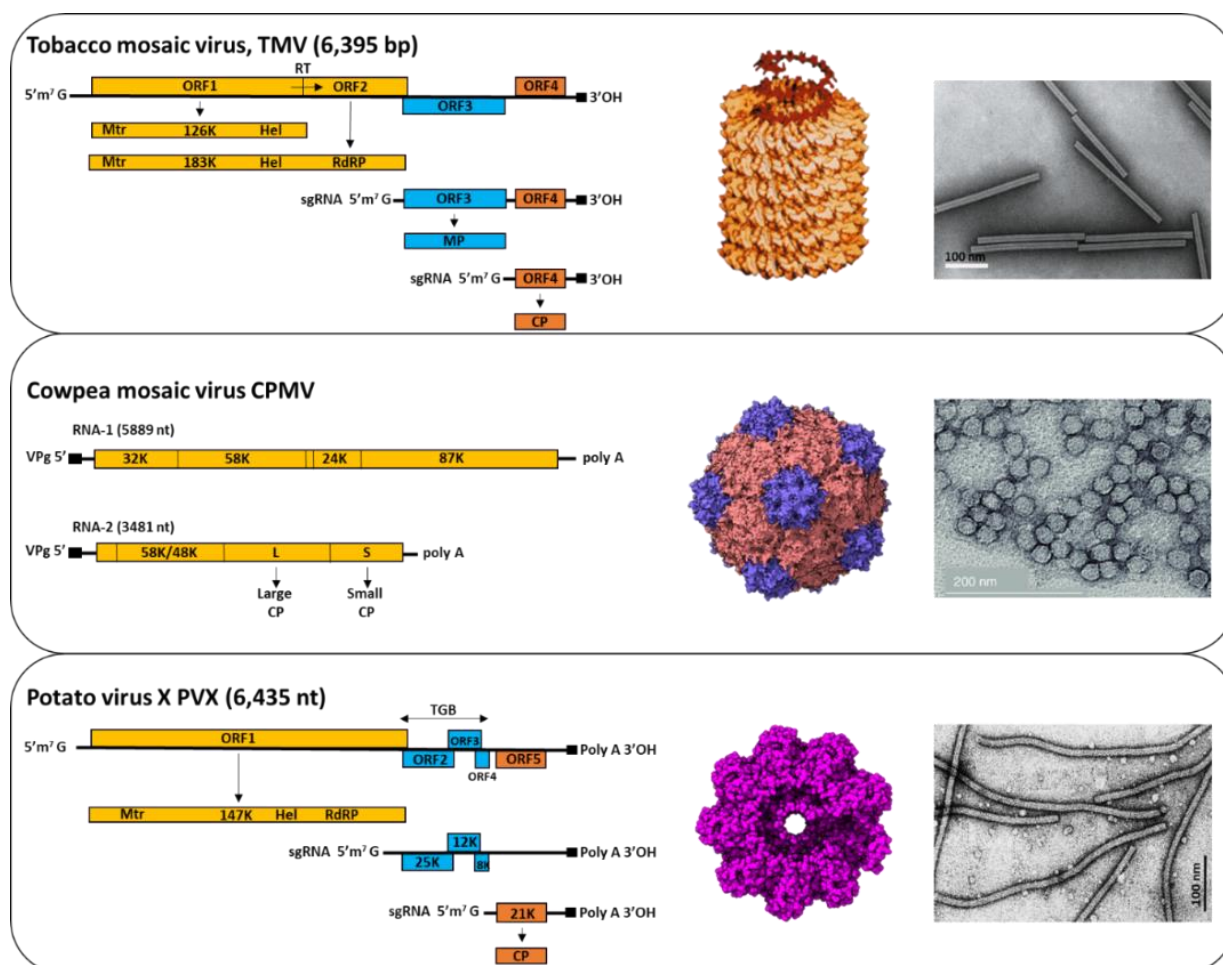


Figure 12. Schematic illustration of the main plant viruses used in nanotechnology. The figure shows the characteristics about the genome, the 3D structure and the (TEM) images of the TMV (scale bar 100 nm), CPMV (scale bar 200 nm) and PVX (scale bar 100 nm) viruses. ORF, “open reading frame”; RT, read-through translation mechanism Mtr, Methyltransferase domain; Hel, helicase domain; RdRp, RNA dependent RNA polymerase; MP, movement protein; VPg, viral protein genome-linked.

Tobacco mosaic virus is a well-known plant virus that has been extensively studied due to its unique rod-shaped structure and high stability. Ever since its initial characterization in the 19th century, TMV has played a prominent role in the development of modern virology and molecular biology. More recently, this knowledge has been applied to the development of novel reagents and structures for applications in biomedicine and bio-nanotechnology. TMV rod shaped particles have a length of approximately 300 nm and a diameter of 18 nm. The virus consists of a single-stranded RNA genome that is enclosed within a helical protein coat. The capsid is composed of approximately 2130 identical CP subunits [221]. At neutral pH and in the absence of RNA, the CPs assemble

into a 18 nm double disk, a 20S aggregate or nano-ring containing two layers of 17 CP molecules which can serve as a nanoscale scaffold [222]. TMV has been demonstrated to efficiently deliver Cisplatin [223] and Phenanthriplatin [224,225]. The drugs were loaded into the TMV NP cavity using charge-driven interactions or by synthesizing stable covalent adducts. Such a TMV-based drug delivery system was proven to enable superior targeted cytotoxicity as well as increased ease of uptake in HepG2 and MCF-7 cancer cell lines [226,227]. Another anti-cancer drug, mitoxanthrone (MTO), which is a topoisomerase II inhibitor, has been encapsulated into TMV [228]. TMV NPs exhibited tumor-reduction in mouse models, while precluding severe cardiac outcomes that sometimes accompany direct delivery of MTO. Yet another anti-neoplastic and antimitotic drug, valine-citrulline monomethyl auristatin E (vcMMAE), was bound to the exterior of TMV NPs which targeted non-Hodgkin's lymphoma [229].

Potato virus X is a member of the family Alphaflexiviridae, genus Potexvirus, an important plant pathogen for the Solanaceae family, and specifically infects potato, tomato and tobacco [230,231]. It has a 6.4-kb positive-stranded RNA genome [232] and multiple copies of CP are assemble around the genomic RNA to form the capsid. The PVX particle is 515×14.5 nm in dimension and comprised of 1,270 subunits of CP [233]. The C-terminus of each CP subunit is located internally and the N-terminus projected externally to the assembled particle, which provides a suitable site for modification [234–237]. Unlike other reported viruses, the assembly of PVX CP subunits into filamentous pVLP, *in vivo* or *in vitro*, is not possible in the absence of genomic RNA. This reflects the unique connection between virus RNA and CP [238]. The PVX-DOX combination was shown to be highly effective in stimulating cytokine/chemokine levels while prolonging the survival of mice in melanoma models compared to that obtained through the administration of either PVX or DOX alone [239]. An efficient and new drug delivery system has been reported for Non-Hodgkin's B cell lymphomas (NHL) based on PVX binding affinity towards malignant B cells. PVX loaded with monomethyl auristatin (MMAE) and administered to tissues harboring malignant B cells lead to inhibition of NHL growth in a mouse model [240]. PVX nanoparticles are increasingly used

for immunotherapy of tumor microenvironments. The monoclonal antibodies of Herceptin or Trastuzumab can be loaded onto PVX nanofilaments, which successfully induced apoptosis in breast cancer cell lines [241].

Cowpea mosaic virus belongs to the Comovirus genus, is an icosahedral shaped virus with a diameter of approximately 27 nm. It is composed of RNA-1 and RNA-2 of 6 and 3.5 kb, respectively, packed in 60 copies each of large and small CP [213]. CPMV is one of the most developed VNPs for biomedical and nanotechnology applications due to its ability to target specific tissues and act as an efficient drug delivery system. It is also reported to be well-adapted for the attachment of a variety of molecules to the CP. Five reactive lysine residues of CPMV CP provide sites to chemically conjugate to various compounds such as fluorescent dyes [242]. CPMV is also used to produce VLPs which can be loaded with heterologous material, increasing the number of possible applications for CPMV-based particles [243]. The CPMV-DOX conjugate was developed using eighty molecules of DOX covalently bound to carboxylates at the external surface of the CPMV NP. This drug delivery vehicle was found to be more cytotoxic than free DOX when used in low concentration, however, CPMV-DOX cytotoxicity is time-delayed at higher concentrations [211]. CPMV NPs have been reported as an *in situ* vaccine to stimulate an anti-tumor response and overcome local immunosuppression [244]. The strategy for immunotherapy resulting antitumor efficacy is promising and involved the formation of aggregates of CPMV and polyamidoamine, generating 4 dendrimers (CPMV-G4). Administration of CPMV-G4 effectively reduced ovarian cancer [245]. CPMV NPs thus provide a therapeutic application for tumor targeting and therapy [246].

1.5 Plant virus modification

Plant virus nanotechnology is an interdisciplinary field located at the interface of virology, chemistry, materials science, and medicine and wide variety of protocols and strategies have been developed and implemented to modify pVNPs for biomedical applications.

1.5.1 Genetic engineering

The knowledge of the complete genomic sequence of many plant viruses and the remarkable developments in the field of molecular biology have made the genetic manipulation of pVNPs feasible at entire viral genome level. In fact, it is possible to intervene in order to fuse sequences coding for entire proteins, or their small portions (peptides), to viral CPs [247]. The N- or C-terminal regions of viral CP or any other exposed domains, are exploited to fuse the heterologous sequence of interest [248]. The choice of the N-ter or C-ter position is closely related to the type of virus used, its shape, the size of the fragment and the decision to use external or internal surface of the virion [249,250]. The viral vectors obtained through this type of approach have led to the development of two expression systems. The first is defined "epitope-presentation" (Figure 13a) and viruses engineered by this strategy are therefore made up of chimeric CPs which, once assembled, expose the exogenous sequence on their surface [251–254]. This approach offers considerable potential as regards the preparation of vaccines. For example, helical plant viruses, such as, bamboo mosaic virus (BaMV), cardamom mosaic virus (CdMV), johnsongrass mosaic virus (JGMV), papaya mosaic virus (PapMV), papaya ringspot virus (PRSV), plum pox potyvirus (PPV), PVX, potato virus Y (PVY), tobacco etch virus (TEV), TMV, and zucchini yellow mosaic virus (ZYMV) have been genetically engineered to display immunogenic epitopes on their surfaces to provide effective vaccination against several disease pathogens and for immunotherapy [255]. However, not all viruses have the flexibility to tolerate this type of change without losing their functionality. For this purpose, it is important that heterologous sequence be specific characteristics in terms of length, pI, charge and amino acid composition in order to obtain infectious recombinant pVNPs [249,250]. The second strategy is the so-called Overcoat® system (Figure 13b), used when it is necessary to genetically fuse whole proteins or large protein fragments and the use of a peptide linker (for example composed by Gly-Pro or Gly-Ser repetitions) is not sufficient to prevent problems of steric hindrance, CP folding and virus assembly [256,257]. In this case it is possible to introduce, between the CP and the exogenous fragment, a leaky stop codon if exposed at the C-terminus, or the 2A

peptide deriving from the foot and mouth disease virus (Foot and Mouth Disease Virus, FMDV) [258], if exposed at the N-terminus. The presence of peptide 2A induces premature ribosome leakage during translation [259], allowing alternating expression of normal (wild-type) and modified viral CPs, and thus generating only partially chimeric virions.

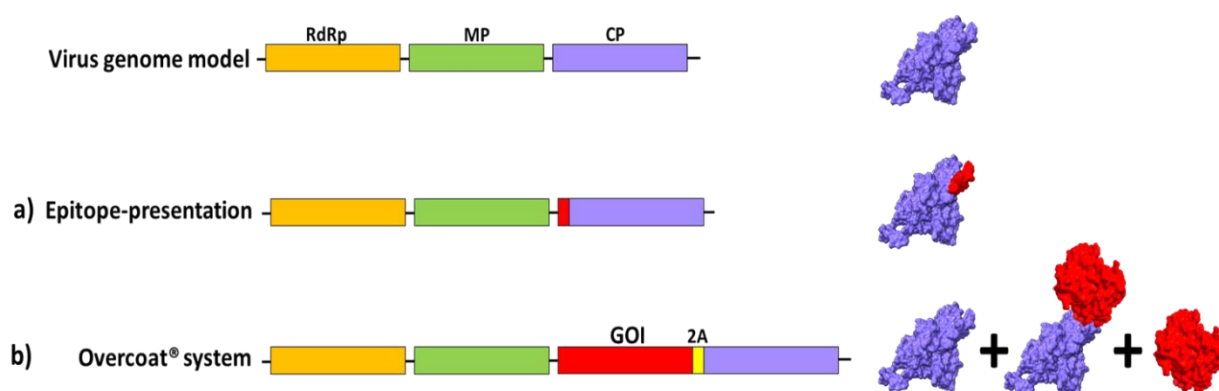


Figure 13. Scheme of the different genetic engineering strategies adopted to modify the genome of plant viruses. (a) fusion of the peptide of interest to the viral CP, "epitope presentation"; (b) fusion of the GOI to the viral CP via the 2A peptide "Overcoat®". RdRp, RNA dependent RNA polymerase; MP, viral movement protein; CP, capsid protein; GOI, gene of interest/peptide ("gene of interest"); 2A, 2A peptide from FMDV.

1.5.2 Chemical conjugation

An alternative to genetic engineering is chemical conjugation. The presence of reactive amino acid residues on the capsid can be exploited to form a covalent bond with a wide range of molecules. The functional groups mostly used for chemical conjugation reactions are amines (Lys), carboxylates (Asp, Glu), thiols (Cys) and aromatic groups (Tyr, Trp) [260–262] (Figure 14). Through chemical reactions of N-Hydroxysuccinimide (NHS), carbodiimides, maleimides and click chemistry the functional groups on the capsid surface can be modified [260,262]. For example, the carboxylic acid groups on CPMV have been activated by the carbodiimide method using 1-ethyl-3-(3 dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and NHS to conjugate DOX, becoming a more effective cytotoxic agent against HeLa cells than free DOX [211]. Another example of the

carbodiimide coupling method is the conjugation of folic acid to hibiscus chlorotic ring-spot virus (HCRSV) to selectively increase DOX uptake and cytotoxicity in OVCAR-3 ovarian cancer cells [263]. When the functional groups are inert, alternative methods such as click chemistry can be used to set up the chemical conjugation reaction [261,262]. A combination of NHS, EDC and diazo coupling reactions in association with click chemistry was used to modify N-terminus, glutamic acid/aspartic acid residues and tyrosine in tobacco mild green mosaic virus (TMGMV) [264]. Functional groups such as azide, alkyne, aldehyde, hydrazide or alkoxyamine are of great interest for click chemistry reactions [265,266]. The Cu(I)-catalyzed functional group reaction, termed as Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC), and that catalyzed by diazonium salts are widely used for highly efficient bioconjugation. [267]. The use of these methods to modulate the surface properties of TMV has been successfully used to construct new materials [267–269]. For example, TMV-based hydrogels based on dual diazonium reagents could be used to encapsulate small molecules and promote their sustained release. Using the CuAAC technique, the exterior or interior of the rod-shaped TMV can conjugate paramagnetic Gd to form TMV-based MRI contrast agents that can increase imaging performance [270].

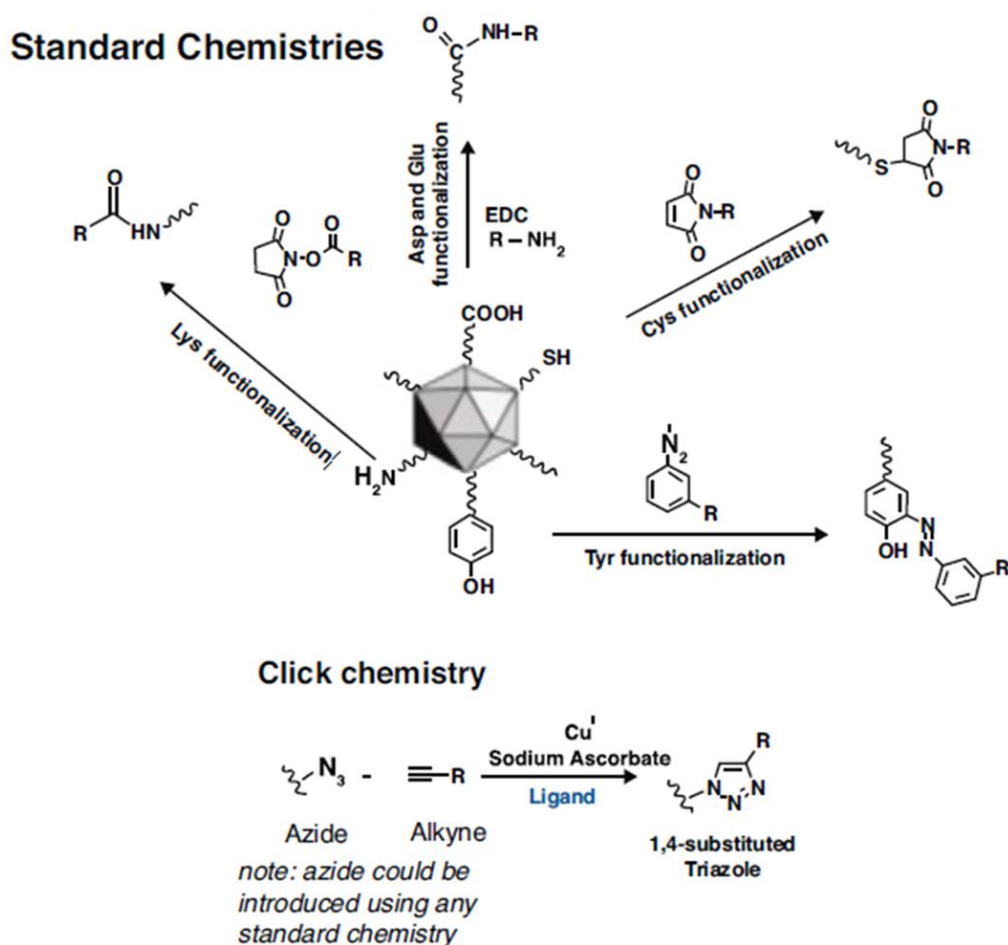


Figure 14. Some examples of the chemical conjugation strategies with which pVNPs/VLPs can be functionalized. Bioconjugation of lysine using N-hydroxysuccinimide ester (NHS); functionalization of cysteine with maleimide; Carbodiimide (EDC) mediated direct conjugation of Asp/Glu carboxylates groups; tyrosine-diazonium coupling and copper-catalyzed azide alkyne cycloaddition [271].

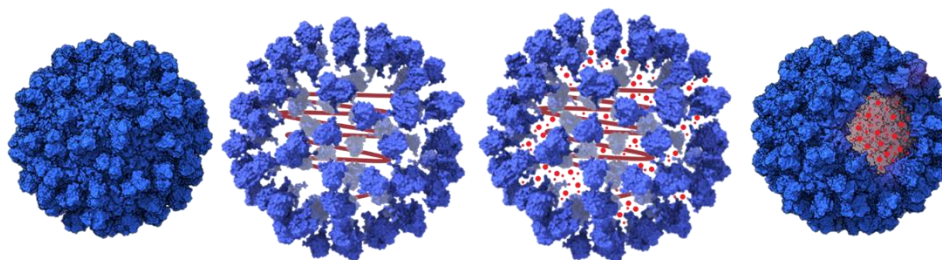
1.5.3 Plant VNPs and VLPs loading

Viruses with their particular shape and size have all the requisites to be considered natural "nano-cages" and to be used to entrap small molecules. Several studies have explained the ways in which the formation of the viral capsid takes place, demonstrating that, in addition to the presence of RNA, other factors such as pH and the presence of divalent ions play a fundamental role in the virion assembly process [272,273]. The techniques mostly used to introduce the substances of interest into the viral cavity are infusion and caging (Figure 15). By increasing or reducing the pH and the concentration of Ca^{2+} and Mg^{2+} ions it is therefore possible to induce conformational changes in the quaternary structure of the virus and thus regulate its assembly. The method required

to obtain a reversible swelling of TBSV was described by Krüse et al. [274] and relies on chelation and then addition of divalent cations to cause opening and then the closing of pores on the virus capsid. This method was used to load TBSV virions with ethidium bromide [275] and a similar strategy was used by Loo et al. [276] as well as by Lockney et al. [260] to load the chemotherapy drug doxorubicin into necrotic mosaic virus particles of red clover necrotic mosaic virus (RCNMV, Tombusviridae). The same technique was used to load doxorubicin into distantly related CMV particles [212]. This strategy can be used not only in the nanomedicine but also in the agronomy field, Cao et al. [277] exploit the high soil mobility of RCNMV to load the nematicide Abamectin inside the viral particles. Abamectin-loaded RCNMV have been observed to have the same bioavailability as the free form, but with better soil mobility, making it more effective at protecting crop roots from nematode infection. In some cases, the infusion does not even require swelling of the viral particles to allow entry of the cargo; the nucleic acid content of the virions can be used as an electrostatic sponge to attract and hold a positively charged cargo. This is the strategy that was used by Yildiz et al. [246] as well as by Wen et al. [278] to load CPMV particles with imaging agents and therapeutic molecules. The caging strategy is based on the assembly of viral particles around the cargo, either *in vivo* or after disassembling the virions *in vitro*. The conditions required to disassemble the virus particles have been described by Adolph [279] and are based on changes in the pH and ionic strength of the solution in which the particles are incubated. The reversibility of the process allows the disassembly-reassembly mechanism to be used to remove the native nucleic acid from the particles and replace it with the cargo of interest [280]. For example, CCMV particles were used as gene therapy candidates; the native viral RNA was removed after disassembly of the virions, and the capsid dimers were subsequently reassembled around the mammalian Sindbis virus heterologous RNA [281]. The authors demonstrated, as a proof-of-concept, that heterologous RNA was released and expressed in mammalian cells after transfection with the chimeric virus particles. Comellas and colleagues disassembled CCMV virions and reassembled them around an individual horseradish peroxidase molecules to investigate single-enzyme processes by

adding the substrate, demonstrating that this virus capsid is permeable for both the substrate and the product [282]. Sanchez also used a similar method for the encapsulation of bacterial cytochrome p450 within CCMV VLPs. These VLPs were enzymatically functional and could transform tamoxifen and resveratrol prodrugs into active cytotoxic forms, an important result for any implications in targeted drug delivery [283].

Infusion



Caging

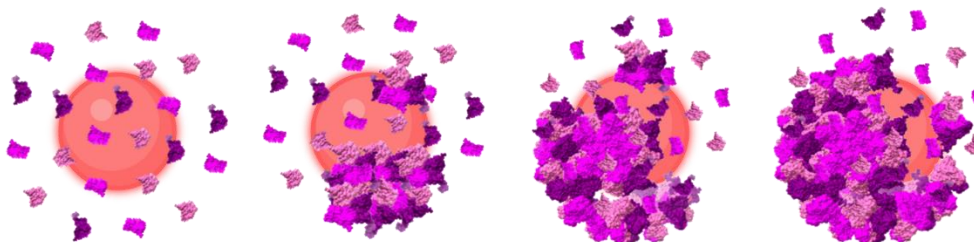


Figure 15. Small molecule trapping strategies in pVLPs. The infusion strategy involves the reversible swelling of viral particles which allows entry of the cargo, viral RNA is not lost and may aid loading efficiency. Caging is the most used strategy with VLPs, after the disassembly phase, in which RNA is lost, the viral components are reassembled to embrace the cargo.

1.6 Tomato Bushy Stunt Virus (TBSV)

Tomato bushy stunt virus belonging to the Tombusvirus genus and the Tombusviridae family [284] is an icosahedral phytopathogenic virus (with a T=3 symmetry). The virus capsid is formed by 180 copies of the same CP (subunits) with a diameter of about 30-35 nm (Figure 16a). The inner cavity contains a monopartite genome made of 4800 nucleotides long single strand of RNA with positive polarity (+) devoid of both the methylguanosine cap (m⁷GpppG) at the 5' and the poly(A) tail at the 3' end. The genome includes 5 independent ORFs, which encode for 5 different proteins: p33 and p92 constitute the

viral replicase complex (RdRp) [285], p22 the movement protein, p19 the post-transcriptional gene silencing (PTGS) inhibition protein, and p41 the CP (Figure 16b). p33 and p92 are directly translated from the viral genomic RNA with p92 produced by translational read-through of the p33 amber stop codon (UAG) [286]. During replication, two subgenomic RNAs (sgRNAs) are produced through a mechanism known as "premature termination" of negative polarity RNA synthesis. The resulting complementary RNA (-) then acts as a template for the transcription of sgRNA (+) [287]. The viral CP is expressed by sgRNA1, while p22 and p19 by sgRNA2, through different start codons by the leaky scanning process involving the 40S ribosomal subunit [288]. The tertiary structure of the viral CP has three specific domains: starting from the N-ter region, the positively charged residues 1-65, together with the β ring region (residues 67-85) and residues 86-101 constitute the R domain (RNA binding), which resides inside the capsid and serves to bind the viral RNA; in the central part (residues 102-268) there is the S domain (Shell), consisting of a pair of anti-parallel β -sheets which form the envelope of the viral capsid; finally, in the C-ter part two overlapping β sheets form the P (Projection) domain which protrudes on the outer surface of the capsid [289,290] (Figure 16c).

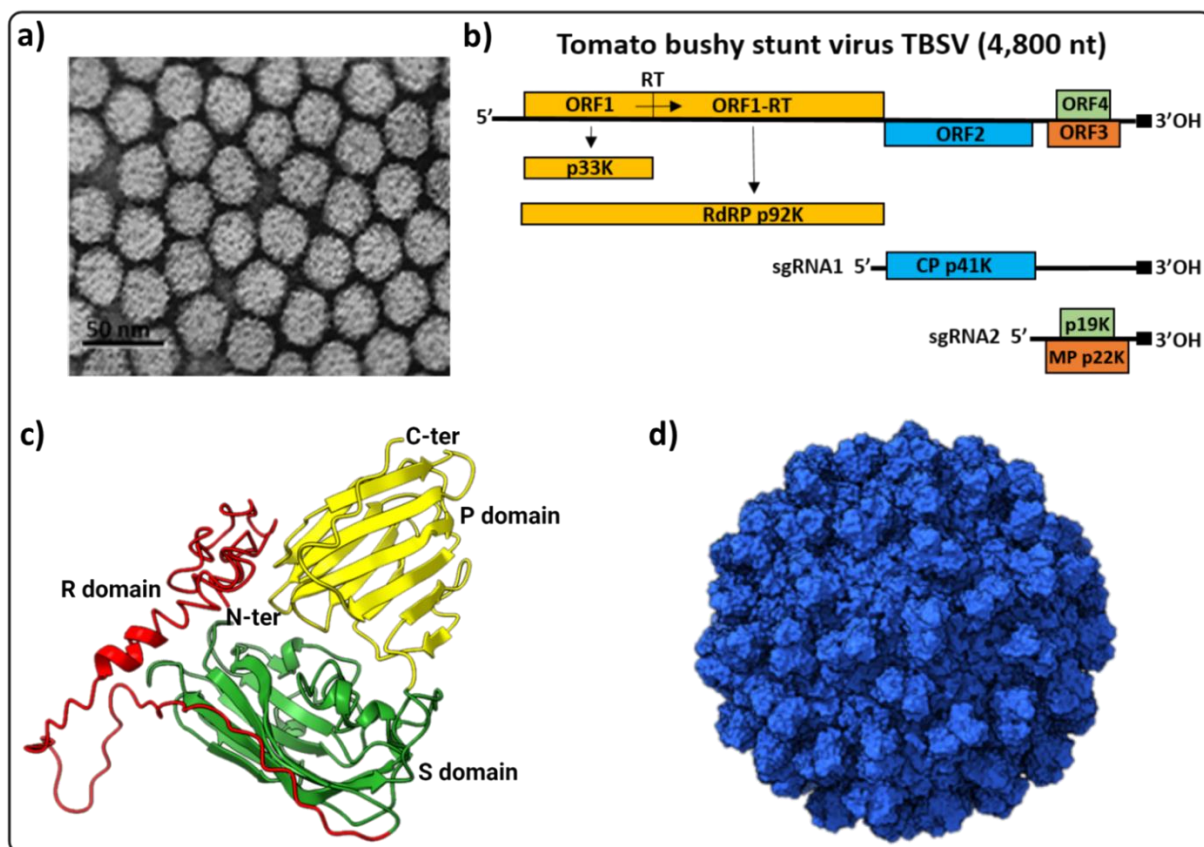


Figure 16. Tomato bushy stunt virus TBSV. (a) Transmission electron microscope images of the virions, scale bar = 50 nm; (b) Schematic representation of the viral genome; ORF: "open reading frame"; RT: read-through translation mechanism; p33K, p92K: proteins of 33 and 92 kDa, respectively, which constitute the viral replicase complex (RdRp); p41K, capsid protein (CP); p22K, viral movement protein; p19K, silencing suppressor; sgRNA, subgenomic RNA; (c,d) exemplary models of the tertiary structure of the CP with highlighted N-terminus/C-terminus domains and the icosahedral surface of the virion.

Although the use of TBSV in the biomedical field has not yet been extensively studied, encouraging results suggest that TBSV is very suitable for this purpose. First, its icosahedral shape makes it particularly suitable for encapsulating molecules of pharmacological interest. Furthermore, the C-ter domain of the viral CP is outwardly projected and can be exploited for the exposure of target antigens or peptides. In a first study a 13 amino acid peptide derived from the V3 loop of human immunodeficiency virus (HIV-1) glycoprotein 120 (gp120) was attached as a C-terminal gene fusion to the CP of TBSV. The HIV epitope was detected on the surface of the virus capsid by a V3-specific monoclonal antibody and human sera from HIV-1 positive patients, demonstrating the potential of using TBSV chimeric particles for diagnostic purposes. The chimeric virus also

induced a specific immune response to the HIV foreign epitope when administered to mice [291]. Following the same principle an epitope of 16 amino acids from the ricin A-chain was exposed on TBSV VLPs demonstrating that TBSV-RTA16 particles are capable of eliciting a strong immune response against the ricin epitope when injected into BALB/c [292]. Grasso and colleagues were able to fuse 50 amino acids (i.e. general tags) to the TBSV CP without affecting either the assembly or the infectivity of the virus, demonstrating its ability in supporting long epitopes fusion for-further applications; they subsequently introduced ethidium bromide molecules, through infusion strategy, into the chimeric particles produced and assembled in plants, assessing its feasibility in loading cargos [275]. In another work it was demonstrated that DBA/1 mice immunized with TBSV particles displaying immunodominant peptides (pFADK2 or pLIP1, specific for rheumatoid arthritis) can achieve complete remission of arthritis signs even if pVNPs are released after disease onset [293]. TBSV particles also possess intrinsic biosafety, in fact *in vitro* toxicity studies and *in vivo* biodistribution studies demonstrate that the virus is neither toxic nor teratogenic [215,294]. Through *in vitro* haemolysis tests the authors demonstrated that TBSV, even at high concentrations, has no effect on the integrity of erythrocytes. Furthermore, they used a chicken embryo assay to evaluate the effect of TBSV NPs on embryo development/survival compared with that induced by retinoic acid and carbon NPs, assessing that TBSV is neither toxic nor teratogenic [294]. In the biodistribution study [215], mice intravenously injected by tail vein with different doses of TBSV NPs did not show any symptoms of systemic toxicity (weight loss, organs histology). Moreover, this work analyzed in depth interaction of TBSV NPs with tissue/organs, demonstrating that TBSV particles still remain in the bloodstream up to the seventh day after inoculation, and in the liver, it localizes in hepatic macrophages, or Kupffer cells. Finally, in the spleen, the TBSV was found to be present exclusively in the outermost part of the organ, or red pulp. These findings paved the way for the studies performed in this thesis and further clinical applications.

Overall, data highlight the great versatility of the TBSV virus, a real "nano-object", safe and with a triple aptitude, for the exposure of antigens or peptides for targeting, for the

transport of drugs and, even if not yet fully demonstrated, as an *in vivo* imaging system (Figure 17).

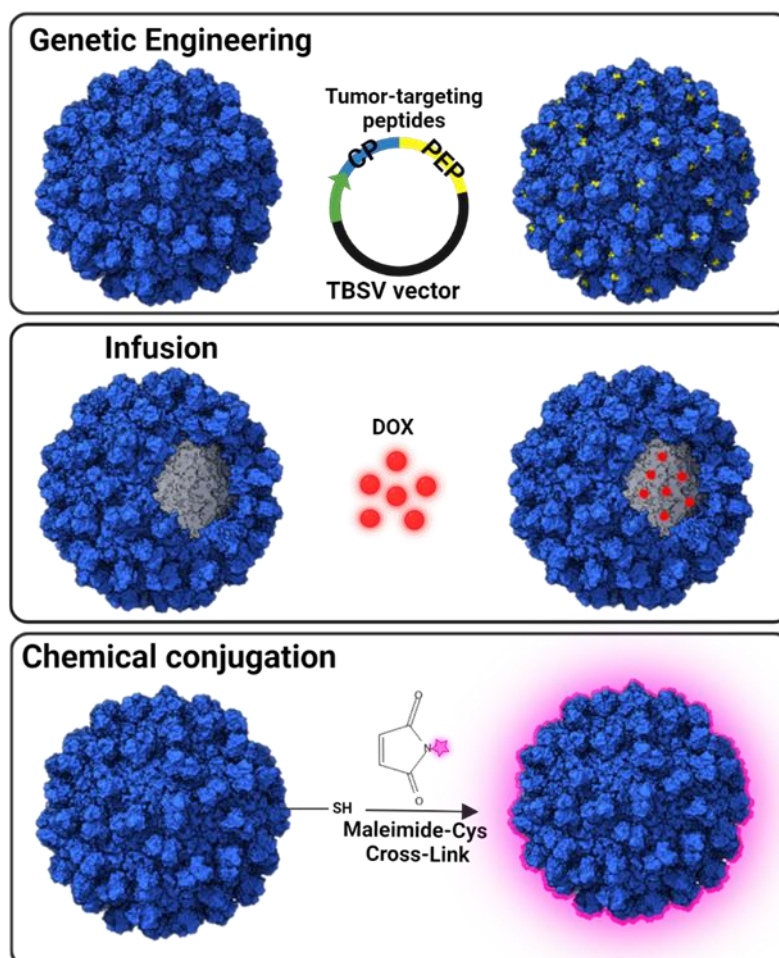


Figure 17. Schematic representation of the different strategies used in this thesis to modify the TBSV NPs. Genetic engineering for the fusion of tumor targeting peptides (PEP) on the viral surface; infusion to introduce the chemotherapeutic agent DOX into the viral particles; chemical conjugation to bind fluorescent molecules to the virus surface through the reaction between maleimide and thiol groups exposed on the viral surface.

References are listed in Appendix I

Figures have been Created with BioRender.com

2. AIMS AND SCOPE

SHH-MB is a highly aggressive brain cancer that predominantly affects children. The conventional treatments include surgery, radiation therapy, and chemotherapy. The use of radiation therapy can lead to long-term complications such as cognitive impairment and growth problems, while chemotherapy has a number of toxic side effects that can be particularly harmful to children. Therefore, there is an urgent need for developing new therapeutic strategies that can enhance the efficacy of treatment while minimizing the undesirable side effects. NP-based drug delivery systems represent a promising approach to achieve this goal. In last years, nanotechnology has revolutionized the field of medicine by providing innovative platforms for developing targeted drug delivery systems. Nanoparticles offer numerous advantages such as nanometric size, selective targeting, and high drug-loading capacity, which make them attractive candidates for drug delivery. In particular, pVNPs have emerged as a promising strategy for developing NP-based drug delivery systems. pVNPs offer several advantages over other types of NPs, such as their biocompatibility, easy production in plant and the possibility to be engineered using different strategies.

This PhD thesis has been developed as part of the NANOCROSS project (Plant virus nanoparticles for blood-brain barrier crossing and medulloblastoma targeting) funded by the Italian Foundation for Cancer Research (AIRC). With a nanotechnological approach, the project aims to develop a targeted drug delivery platform, based on the use of pVNPs, for the treatment of SHH-MB. The candidate virus we have chosen to develop this innovative targeted drug delivery platform is the TBSV. Thanks to its nanometric size, spherical shape and unique physical and chemical characteristics, TBSV is extremely versatile as it can support different types of genetic, chemical and physical modifications by which the particle can be engineered into a specific tumor targeting and drug delivery system. After selecting a series of tumor targeting peptides, these were genetically fused to the viral surface, in order to provide TBSV NPs with tumor specific

targeting ability. For the large-scale production of TBSV NPs we exploited *Nicotiana benthamiana* plant as a biofactory, following the concept of plant molecular farming. After the extraction and purification procedures, TBSV NPs were further engineered by introducing a well-characterized chemotherapeutic agent, doxorubicin, into the virus. Chimeric particles loaded with doxorubicin were then tested on primary murine MB cells, *ex vivo*, and subsequently *in vivo* on a MB mouse model.

Data obtained in this Thesis allowed the production of three peer-reviewed manuscripts, described in the following sections, one for each paper, with a brief introduction provided at the beginning of each chapter.

3. TOMATO BUSHY STUNT VIRUS NANOPARTICLES AS A PLATFORM FOR DRUG DELIVERY TO SHH-DEPENDENT MEDULLOBLASTOMA

Adapted from: Int J Mol Sci. 2021 Sep 29;22(19):10523. doi: 10.3390/ijms221910523.

Chiara Lico, Barbara Tanno, Luca Marchetti, Flavia Novelli, Paola Giardullo, Caterina Arcangeli, Simonetta Pazzaglia, Maurizio S Podda, Luca Santi, Roberta Bernini, Selene Baschieri, Mariateresa Mancuso.

3.1 INTRODUCTION

In this chapter, the use of TBSV as a novel candidate for targeted drug delivery to the murine Shh-MB brain tumor was evaluated. Usually, TBSV particles lack the innate ability to target tumor specifically but can acquire it if the viral surface is functionalized with peptides or ligands that selectively target tumor cells. To this end, the starting point of this work was to select a series of peptides, known in the literature to bind receptors overexpressed in tumor cells, in order to use them to generate cTBSV NPs with the modified surface that we subsequently produced in *Nicotiana benthamiana* plants. To evaluate the targeting efficiency and cellular uptake of modified TBSV NPs, we set up *in vitro* experiments with primary MB cells, our main target. Furthermore, having available protocols for the culture, maintenance and induction of cell differentiation, the *in vitro* evaluation was also conducted with GCPs (cells which in case of aberrant activation of the Shh pathway give rise to MB) and with the same cells artificially induced to differentiate into neuronal cells. Subsequently, we used docking and molecular dynamics simulations to evaluate the binding interaction between the selected peptides and their receptors, both in their free form and when fused to the viral particle. To complete the development of the TBSV-based targeting and delivery platform, we exploited the chemical-physical characteristics of the viral capsid as a "nano-cage" to trap molecules DOX (a known chemotherapeutic agent widely used to treat tumors) and subsequently tested the *in vitro* delivery efficacy of loaded TBSV NPs with MB cells. The last phase of this experimental part concerns the *in vivo* evaluation of the targeting specificity of the TBSV-modified NPs. In this regard, TBSV NPs functionalized with peptides, but not loaded

with DOX, were administered intravenously by tail vein in symptomatic *Ptch1^{+/-}* MB knockout mice. Twenty-four hours after inoculation, target tissues were harvested and analyzed by Real Time (RT)-qPCR to quantify the amount of particles that have reached the tumor.

3.2 MATERIALS AND METHODS

3.2.1 TBSV genetic engineering, production in plants, purification and characterization

In order to construct the cTBSV NPs, the sequences encoding tumor targeting peptides were inserted in the TBSV-vector at the 3'-end of the p41 gene, encoding the C-terminus of the CP [1]. The vector was digested *Apal/PacI* (New England Biolabs, Ipswich, MA, USA) and ligated with DNA fragments with compatible protruding ends obtained by the *in vitro* annealing of synthetic oligonucleotides couples encoding the peptides (Table 1). The oligonucleotides were designed using preferential *N. benthamiana* codon usage. To obtain infectious RNA transcripts, all the vectors (pTBSV, pTBSV-RGD, pTBSV-RiGiD, pTBSV-ApoE, pTBSV-CooP, and pTBSV-tLyp1) were linearized by *XmaI* digestion and *in vitro* transcribed using the MEGAscript T7 High Yield Transcription kit (Thermo Fischer Scientific, Rodano, MI). Each RNA was used to inoculate six to eight weeks old *N. benthamiana* plants, grown under controlled conditions (24 °C, 16 hrs light/8 h dark) in a containment greenhouse, by abrading the adaxial side of 2 leaves/plant with carborundum (silicon carbide; VWR International, Radnor, PA). Two cycles of reinfections were performed using saps from infected leaves prepared by homogenizing the systemic tissue of an infected plant in 1xPBS. After each cycle of infection, the onset of symptoms was monitored, the RNA was extracted from symptomatic systemic leaves with the RNeasy plant mini kit (QIAGEN, Hilden, Germany) following manufacturer's instructions, and the RT-PCR was performed using the QuantiTect Reverse Transcription Kit (QIAGEN), with specific primers up- and downstream the TBSV CP gene 3' end (Table 1). Finally, the obtained PCR fragment was analysed by sequencing (BMR Genomics, Padova, Italy). TBSV particles were purified following a previously developed protocol [1]. Briefly, infected leaves were collected and ground to a fine powder. After plant material homogenization with 3 ml/g of 50 mM sodium acetate, pH 5.3, supplemented with 1 % ascorbic acid and a cocktail of protease inhibitors (Sigma), the

extract was clarified by low-speed centrifugation (8,000g for 15 min at 4°C). After adjusting the pH to 5.3, the supernatant was ultra-centrifuged for 1 h at 90,000g at 4 °C. The obtained pellet was finally resuspended in 50 mM sodium acetate, pH 5.3.

Table 1. List of the oligonucleotides used for peptides cloning, molecular screening and PCR/RT-qPCR analysis.

Name	5' → 3' Sequence
5'tLyp1	CGGAGGTTGTGGAATAAGAGAACTAGGTGAGAGCTCTTAAT
3'tLyp1	TAAGAGCTCTCACCTAGTTCTTATTTCCACAACCTCCGGGCC
5'CooP	CGGAGGTTGTGGTCTTTCTGGATTGGGAGTTGCTTGAGAGCTCTTAAT
3'CooP	TAAGAGCTCTCAAGCAACTCCCAATCCAGAAAGACCACAACCTCCGGGCC
5'RiGiD	CGGAGGTGGGCCCGGAGGAGGTGCTAGAGGAGA- TAAGGGTCTGCTAGGGGTGACAAATGAGAGCTCTTAAT
3'RiGiD	TAAGAGCTCTCATTGTACCCCTAGCACCAGGACCCTTATCTCCTCTAGCAC- CTCCTCCGGGCCACCTCCGGGCC
5'ApoE	CGGAGGTCTTAGAAAGTTGAGGAAAAGATTGCTTCTCAGGAAGTTAAGGAAAC- GACTTCTGTGAGAGCTCTTAAT
3'ApoE	TAAGAGCTCTCACAGAAGTCGTTTCCTTAACITCCTGAGAAGCAATCTTTTCCTCAACTTTCTAAG ACCTCCGGGCC
5'RGD	CGGAGGTAGAGGAGATTGATTAAT
3'RGD	TAATCAATCTCCTCTACCTCCGGGCC
TBSV-For	TGACATTTCTGCAACTGG
TBSV-Rev	AAGATCCAAGGACTCTGTGC
qTBSV-For	CGGTTGCAGTCACCAGACAA
qTBSV-Rev	CCCAGACGTCCTGCCAGTA
qGADPH-For	CATGGCCTTCCGTGTTCTTA
qGADPH-Rev	GCGGCACGTCAGATCCA
qβ-ACTIN-For	CACAGCTTCTTTGCAGCTCCTT
qβ-ACTIN-Rev	CGTCATCCATGGCGAAGTG
qRlp13a-For	CTGCTCTCAAGGTTGTTCGGCT
qRlp13a-Rev	CCTTCCGTTTCTCCTCCAGAGT
qRlp32-For	TGTGCAACAAATCTTACTGTGCT
qRlp32-Rev	TGCACACAAGCCATCTACTCA
qTBP-For	GAAGAACAATCCAGACTAGCAGCA
qTBP-Rev	CCTTATAGGGGAACCTCACATCACAG

3.2.2 Mouse model

A colony of mice lacking one *Ptch1* allele (*Ptch1*^{+/-}), derived by gene targeting of 129/Sv ES cells and maintained on the CD1 strain background, is continuously maintained in the ENEA Casaccia (Rome, Italy) animal facility by crossing *Ptch1*^{+/-} heterozygous males with CD1-wild-type females and viceversa, and genotyped as previously described [2]. Animals are housed under conventional conditions with food and water available ad libitum and a 12-hours light-dark cycle, thus observed daily upon unequivocal appearance of full-blown symptomatic MB (severe weight loss, paralysis, ruffling of fur, inactivity).

3.2.3 Primary cell cultures and TBSV NPs internalization

After tumor dissection, dissociated cells were maintained in complete medium Ultra-CULTURE™ cell culturing medium (Lonza BioWhittaker Inc.) supplemented with 2 mM L-glutamine (Lonza BioWhittaker Inc.), 1% Penicillin-Streptomycin (Life Technologies), SAG dihydrochloride (Sigma Aldrich) and 20% fetal bovine serum (Euroclone). Granule Cell Precursors were purified from *Ptch1*^{+/-} mouse cerebella at postnatal day 2 (P2) as previously described [3] and maintained in culture in DMEM/F12 (Gibco) supplemented with 0.6% glucose (Sigma), 25 µg/ml insulin (Sigma), 60 µg/ml N-acetyl-l-cystein (Sigma), 2 µg/ml heparin (Sigma), 20 ng/ml EGF (PeproTech, Inc.), 20 ng/ml bFGF (PeproTech, Inc.), 1X penicillin-streptomycin and B27 supplement without vitamin A (Gibco). To induce differentiation, GCPs were seeded in DMEM/F12 with N2 supplement (Gibco) and 2 µg/ml heparin, 0.6% glucose, 60 µg/ml N-acetyl-l-cysteine, containing 1% Calf Serum (Euroclone) and PDGF 10 ng/ml (PeproTech, Inc.) for 7 days. MB cells were seeded at the density of 50000 cells/cm². Twenty-four hours later, they were incubated with the NPs at the concentration of 6.6 ng/µl for 3 hours. GCPs and differentiated GCPs were seeded at the density of 20000-50000 cells/cm², respectively. Seven days later, both cell types were incubated with the NPs for 3 hours. To analyse the uptake of the functionalized TBSV by different cell lines, RNA isolation and RT-qPCR were performed as below described.

3.2.4 RNA Isolation and Real Time qPCR analysis

RNA isolation from cells was performed with the RNeasy Mini Kit (QIAGEN). cDNA synthesis was performed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), and RT-qPCR was carried out by StepOnePlus™ Real-Time PCR System (Applied Biosystems), using Power SYBR® Green PCR Master Mix (Applied Biosystems). Relative gene expression was quantified using Glyceraldehyde-3-phosphate (*Gadph*) as house-keeping gene. Oligonucleotide primers used for RT-qPCR are listed in Table 1. The $\Delta\Delta C_t$ quantitative method was used to normalize the expression of the reference gene and to calculate the relative expression level of the TBSV CP target gene.

3.2.5 Immunofluorescence analysis

To detect FABP3 and NRP-1, MBs, GCPs and differentiated GCPs cells were seeded on Lab-Tek Flask previously treated with Poly-D-lysine (Sigma-Aldrich) and allowed to adhere for 16 hours. Cells were fixed with 4% paraformaldehyde for 15 minutes at RT, permeabilized with 1xPBS, 0.1% triton X-100 for 2 minutes on ice and incubated in 1xPBS 5% bovine serum albumin (BSA). Cells were incubated overnight with anti-NRP-1 (monoclonal 1:300; Abcam) and anti-FABP3 (H-FABP; polyclonal 1:100; Abcam), and subsequently with the secondary antibody Alexa Fluor 488 (1:1000; Invitrogen), and DAPI (1:1000; Thermo Fisher). Images were acquired with an Eclipse 80i Fluorescence Microscope (Nikon), equipped with the Imaging Software NIS-Elements BR Version 3.2.

3.2.6 NRP-1 and H-FABBP molecular docking and molecular dynamics simulations

The structures of human NRP-1 (PDB id: 5IYY) [4] and FABP3/H-FABP (PDB id: 5CE) [5] were retrieved from RCSB Protein Data Bank and used as starting coordinates for the docking calculations and simulations. The structures of tLyp1 (CGNKRTR) and Coop (CGLSGLGVA) peptides were taken from our previous work [6]. Modeller [7] was used for generating the structural models of the asymmetric unit of the cTBSV in which at the C-terminus of each CP the tLyp1 or the Coop peptides were inserted with a GGPGG

linker. ClusPro protein-protein docking [8–10] and HADDOCK 2.2 [11] were used to obtain the receptor-peptide and receptor-ligands complexes, respectively. As positive controls, tuftsin (TKPR, a peptide known to bind NRP-1) and oleic acid ($\text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$, $\text{C}_{18}\text{H}_{34}\text{O}_2$, the natural ligand of FABP3/H-FABP) were docked with their respective targets. In both docking methods standard protocols were used and an ensemble of ten poses was generated for each docking run. All the MD simulations were performed using Gromacs2019 [12] and the trajectories were visualized and analysed with VMD [13] and with tools included in Gromacs.

3.2.7 Chimeric TBSV molecular docking and molecular dynamics simulations

The structural models of the asymmetric unit of the cTBSV in which at the C-terminal of each CP the tLyp1/CooP peptide was inserted by means of a GGPGG linker have been generated by homology modeling. The template consisted of the atomic coordinates of TBSV (PDB id: 2TBV (A, B, C), [14,15]. The calcium ions, which are known to play an important role in the stability of the viral particle, were also included during the construction of the models. The cTBSV models were then generated by expanding the asymmetric unit using the 60 transformation matrices provided by VIPERdb database tools [26; <http://viperdbscripps.edu>]. All structural models used or homology builded or obtained by docking procedure and used as starting structure for the MD simulations are available at GitHub link: <https://github.com/mspodda/TBSV-nanoparticles-as-a-platform-for-drug-delivery-to-Shh-dependent-medulloblastoma-PDB-models>. The MD simulations were performed by using the all-atom AMBER 99sb-ildn force field [17] for the peptides and proteins. The force field parameters for oleic acid (OLA) ligand were generated using the LigPar server [18–20]. Most of the systems (tLyp1-NRP-1, tuftsin-NRP-1, CPtLyp1-NRP-1 and CPCooP-FABP3) were placed in a dodecahedric box, whereas the remaining systems (CooP-FABP3 and OLA-FABP3) were placed in a cubic box and filled with TIP3P water model [21]. All the systems were neutralized using NaCl at the physiological concentration of 150 mM. Periodic boundary conditions were applied to avoid edge effects. The systems were energy minimized by steepest descent

(SD) [22], heated and equilibrated for 400 ps. MD simulations were carried out in the NPT ensemble for 100 ns, each step of 2 fs. The v-rescale algorithm [23] was used to keep the temperature at 310 K ($\tau_t=0.1$ ps). The average pressure was kept at 1 bar ($\tau_p=2$ ps) by using the isotropic Parrinello-Rahman barostat [24]. The particle mesh Ewald (PME) method was used to compute the long-range electrostatics and Van der Waals interactions with a cut-off of 1 nm. An equal cut-off was used for short-range electrostatics and Van der Waals interactions. Rotation and translational motions of systems were removed, and all bonds were constrained with the LINCS algorithm [25]. Initial velocities were assigned according to Maxwell-Boltzmann distribution. Cluster analysis was performed with the standard gromos-clustering algorithm [26]. The method counts the number of neighbours for each structure using a root mean square displacement (rmsd) cutoff distance (<0.15 nm) and assigns the structure with the largest number of neighbours along with all its neighbours to a cluster. Once assigned to a cluster, the structures are discharged from the pool of structures. The procedure is repeated until the exhaustion of all the structures. Hydrogen bond (H-bond) analyses were performed using VMD. An H-bond was assumed to exist during the simulation if the donor to acceptor distance was shorter than 0.35 nm and the hydrogen-donor-acceptor distance was shorter than 30° . Absolute binding free energies were evaluated through the MM/PBSA (molecular mechanics/Poisson-Boltzmann solvent accessible surface area) method by using the g mmpbsa tool of GROMACS [27,28]. The average free energies of solvation ($\Delta G_{\text{binding}}$) between the residues of the active site of the receptors and the tLyp1 and CooP peptides (both in their free version or as fusion to the viral CP) were calculated for four different temporal windows: 0-25 ns, 25-50 ns 50-75 ns and 75-100 ns.

3.2.8 Transmission Electron Microscopy

NPs morphology was characterized by transmission electron microscopy (TEM). For TEM images, samples were fixed using 4% paraformaldehyde (PFA) in 1xPBS, then

placed on formvar-carbon coated copper grids, washed, and negatively stained with 2% uranyl acetate. Samples were observed at a JEOL 1200 EX II electron microscope, and images acquired by the Olympus SIS VELETA CCD camera equipped with the iTEM software. To calculate virions diameter, micrographs were analyzed using scientific image manipulation software ImageJ (National Institutes of Health, USA). TEM analysis was performed by the Electron Microscopy Lab, Centro Grandi Attrezzature, (CGA) University of Tuscia, Viterbo.

3.2.9 Dynamic light scattering and ζ -potential analyses

Particles size, ζ -potential and the polydispersity index of NPs were determined by Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) using a Zetasizer Nano ZS instrument (Malvern-Panalytical) and the Malvern Zetasizer software for data analysis. ζ -potential was calculated using the Mixed Mode Measurement Phase Analysis Light Scattering (M3-PALS) technique (Malvern-Panalytical). Twenty μL of a 2 $\mu\text{g}/\mu\text{L}$ viral nanoparticles solution was diluted in a Folded Capillary Zeta Cell (for ELS) and in a 12 mm Square Polystyrene Cuvettes (for DLS) to a final volume of 800 μL using a physiological solution (0.9% NaCl). Data were collected at 25°C for 120 s (for DLS) and 180 s (for ELS), in triplicate. The data reported represent the size by intensity, as diameter of the peaks. All the analyses were performed by IXTAL srl, Novara, Italy.

3.2.10 Production of DOX loaded TBSV NPs

Approximately 2 mg/ml of purified TBSV NPs were incubated in swelling buffer (0.1 M Trizma base, 50 mM EDTA, pH 8.0) for 1 hour at RT in agitation. A 5000 molar excess of DOX (0.324 μg DOX/ μg virus) was then added and incubated overnight at 4 °C on a bench-top tube rotator. Samples were then incubated in association buffer (0.2 M Na Acetate, 25 mM CaCl_2 , 25 mM MgCl_2 , pH 5.2) for 1 hour in agitation at RT to reassemble the virus cages. DOX excess was removed by overlaying the preparation onto a 20% sucrose cushion ultracentrifuged at 100000xg for 1 hour at 4 °C in a swing-out rotor. Pellets were resuspended overnight in association buffer and stored at 4 °C. To confirm

the association of DOX to TBSV, about 10 µg of the NPs were separated on a native 0.8% TBE agarose gel and visualized under UV and white light, before and after Coomassie Blue staining. Finally, TBSV-DOX NPs were analysed by a UV/VIS spectrophotometer (Shimadzu UV-2600) at 485 nm to determine the number of DOX molecules loaded/virion. A DOX calibration curve was generated and the Lambert-Beer law used to determine the concentration of DOX associated to NPs [29]. The drug encapsulation efficiency (EE) and loading capacity (LC) of DOX in NPs were also determined according to the formula:

EE(%) = Amount of DOX in the NPs/total amount of DOX added (×100%)

LC(%) = Amount of DOX in the NPs/NPs weight (×100%)

3.2.11 Cell viability assay

Cell viability was measured using the RealTime-Glo™ MT Cell Viability Assay (Promega, Milan, Italy). MB cells (5000 cells/well) were plated in a 96-well plate according to the manufacturer's instructions. DOX-cTBSV concentrations were adjusted to maintain constant DOX concentration (ranging from 5 µM to 25 µM) and luminescence was measured every 24 hours using the plate-reader GloMax (Promega) up to 72 hours.

3.2.12 Absolute quantification of chimeric TBSV in target tissues after intravenous injection

Mice with clear MB symptoms (4 females and 5 males) were injected i.v. by tail vein with 200 µg of purified TBSV-CooP, TBSV-tLyp1 or TBSV-WT. Twenty-four hours later the animals were euthanized, brains explanted and MB isolated from normally appearing brain tissue (NB). MB and NB were weighted, and cells counted after proteolytic dissociation with Accutase (Euroclone, Milan, Italy). Total RNA was extracted from approximately 30 mg of MB or NB homogenized with gentleMACS™ Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany).

Total RNA (1 µg) was reverse transcribed and used to quantify the number of viral RNA genome copies through RT-qPCR. Reactions were performed in triplicate from each biological replicate and normalization based on 5 housekeeping genes, i.e., *Gadph*, *β-Actin*, *TATA box binding protein (TBP)*, *ribosomal protein L13A* and *32 (RPL13a and RPL32)*. Oligonucleotides used are summarized in Table 1. The absolute quantification of the viral RNA in each sample was performed by means of Absolute Quantification Method using a standard curve based on TBSV RNA. Knowing the molecular mass of the TBSV genome, the obtained value was converted in number of copies of viral genome (i.e., number of particles) and then in number of viral particles/1x10⁶ cells.

3.2.13 Statistical Analysis

All quantitative data were presented as mean ± SD and statistical significance (p) was calculated by two-tailed Student's t-test. All analyses were carried out using GraphPad Software.

3.3 RESULTS

3.3.1 Construction, production and purification of WT and chimeric TBSV NPs

cTBSV NPs were constructed by engineering the viral genome to display on their surface tumor targeting peptides as fusion to the C-terminus of the CP. The peptide RGD, in single or double copy (RiGiD), found in the sequences of extracellular matrix proteins, was selected because able to bind to integrins, a family of transmembrane receptors overexpressed also on different types of tumor cells, and cTBSV carrying this peptide was used as positive control [30]. The tLyp peptide is a linear truncated version of the cyclic peptide Lyp1, identified by the *in vivo* selection of a phage display peptide library, that through a C-terminal C-end rule motif (CendR) binds to neuropilin (NRP), overexpressed in angiogenic tumors, and activates cell internalization [31]. The CooP peptide is a glioblastoma homing peptide identified, like Lyp-1, by the *in vivo* selection of a phage display peptide library, whose interacting partner has been identified primarily to be the mammary-derived growth inhibitor (MDGI/FABP3/H-FABP) [32]. Finally, the ApoE peptide is derived from Apolipoprotein E, one of the major protein involved in lipid metabolism, that mediates the interaction with low-density lipoprotein receptor-related proteins overexpressed in the brain and in tumors herein [33] (Table 2).

Table 2. List of the peptides fused to the TBSV CP

Peptide Name	Peptide Sequence
RGD	GGPGG-RGD
RiGiD	GGPGG-ARGDK- GPG -ARGDK-
CooP	GGPGG-CGLSGLGVA
tLyp1	GGPGG-CGNKRTR
ApoE	GGPGG- LRKLRKRLLLRKLRKRL

The linker sequence is highlighted in italic; the spacer sequence is in bold.

A GGPGG linker was inserted between the CP and the peptide to avoid possible steric hindrance during CP folding and/or CP-CP interaction instrumental for virus particle assembly (Figure 1A-D), and at the same time to obtain a higher degree of flexibility,

thus increasing the chance of proper interaction with cell surface receptors. A GPG spacer was also inserted between the two copies of RGD in the RiGiD chimera. All the RNA transcripts obtained from the different viral constructs (TBSV-WT, -RGD, -RiGiD, -ApoE, -CooP, -tLyp1) were used to infect *N. benthamiana* plants and demonstrated able to induce on leaves the onset of typical infection symptoms (chlorotic vein clearing) (Figure 1E and F). Ten to eleven d.p.i. plant tissues were harvested, RNA extracted, retrotranscribed and cDNA fragments sequenced, confirming the presence of the heterologous sequences. Total protein extracts, containing cTBSV, were also obtained and used to infect a second set of plants, allowing to verify the genetic stability of the chimeric viruses through subsequent infection cycles. On this basis, all the constructs were then used to produce the cTBSV on large scale. Particles were purified with an average yield of 1 mg/g of fresh leaves tissue weight. No substantial differences in recovery were observed among WT and cTBSV, indicating that the genetic modifications did not affect viral fitness and in planta behaviour. Each batch of purified virus particles analysed by Coomassie Blue staining after SDS-PAGE confirmed the presence in all samples of the viral CP (both as a monomer or as dimer/aggregate) and purity of each preparation (Figure 1G).

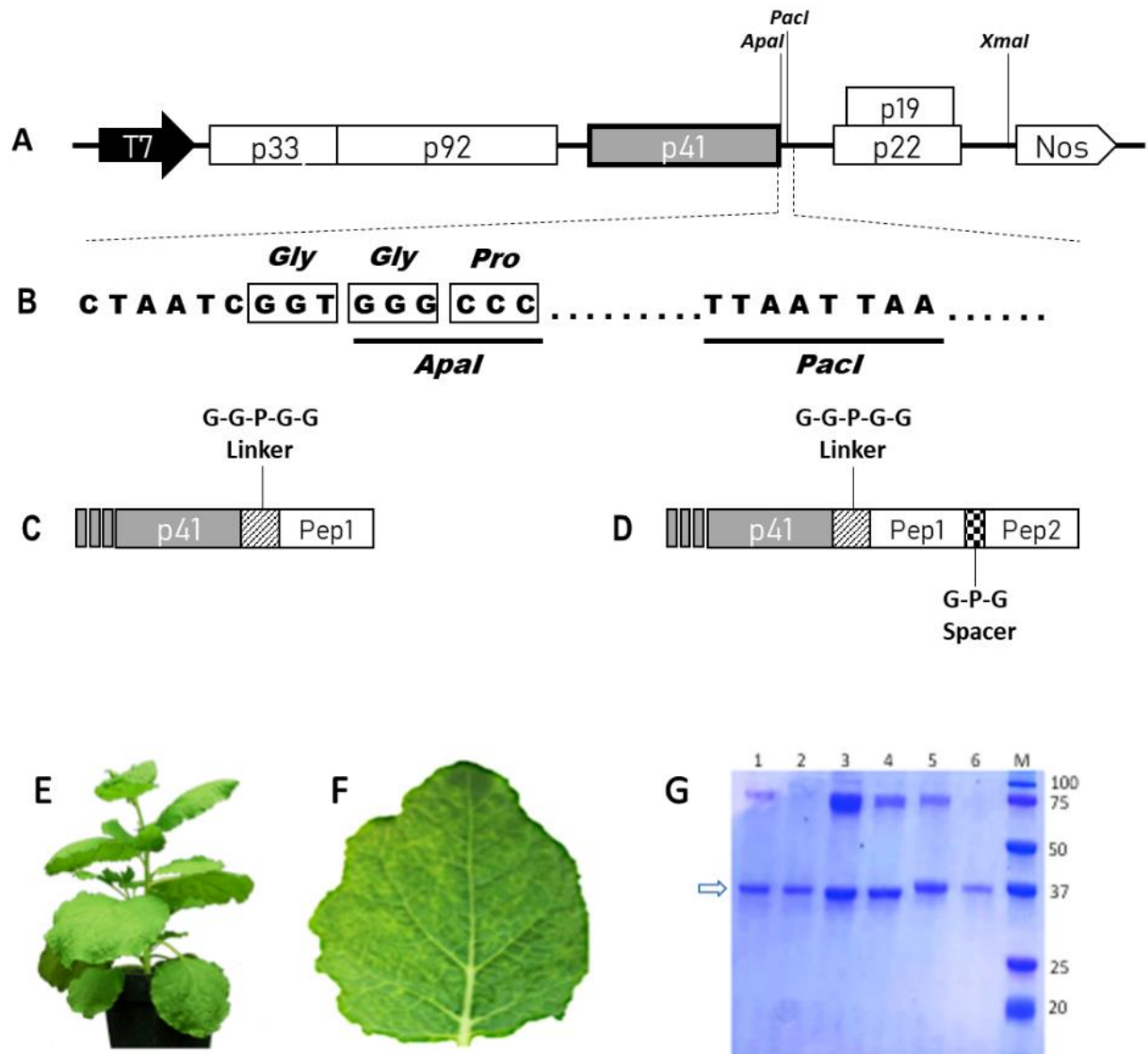


Figure 1. Schematic representation of TBSV cDNA constructs and TBSV NPs characterization. **(A)** Viral genome organization in the TBSV-vector plasmid. The 5 ORFs and the respective encoded proteins with their molecular masses (in kDa) are represented as boxes: p33 and p92 (RNA-dependent RNA polymerase), p41 (CP), p22 (movement protein), p19 (silencing inhibitor). T7, promoter sequence from T7 phage; Nos, terminator sequence from the *Agrobacterium tumefaciens* nopaline synthase gene. The *XmaI* restriction site used for vector linearization is also indicated. **(B)** Sequence detail of the CP 3' region in the TBSV-vector. The underlined sequences represent the restriction enzyme sites used to clone the heterologous sequences. **(C, D)** Schematic representation of single or double peptides coding sequences fused to the viral CP. As simplification, only the 3' region of the CP gene is illustrated. Striped box, GGPGG linker coding sequence; dotted box, GPG spacer coding sequence. **(E)** Adult *N. benthamiana* plant. **(F)** Typical TBSV symptoms (mainly chlorotic spots and vein clearing) induced at 7 d.p.i. **(G)** Coomassie Blue stained SDS-PAGE of TBSV NPs (5 µg/lane) purified from infected *N. benthamiana* plants. M: Precision Plus Protein Dual Xtra Prestained Protein Standard (Bio-Rad); 1: TBSV-WT; 2: TBSV-RGD; 3: TBSV-CooP; 4: TBSV-tLyp1; 5: TBSV-ApoE; 6: TBSV-RiGiD. The molecular masses of the marker bands are indicated. The arrow indicates the CP.

3.3.2 *In vitro* validation of chimeric TBSV uptake

Shh-MBs cells were isolated from *Ptch1*^{+/-} mice and maintained in culture in the appropriate medium. Subsequently, the cells were incubated for 3 hours with cTBSV, or TBSV-WT as control, to be then collected and used to extract RNA and verify the presence of the viral genome by RT-qPCR. All cTBSV were internalized by MB cells more efficiently than the WT virus. Data based on RT-qPCR analysis revealed that the uptake of TBSV-RGD, -RiGiD, -ApoE, -tLyp1 and -CooP was 69, 37, 40, 99 or 123 times the uptake of TBSV-WT, respectively (Figure 2A). Moreover, referring the internalization of the cTBSV NPs to TBSV-RGD (a well characterized tumor-targeting peptide) [30], it was observed that the uptake of TBSV-tLyp1 and -CooP was increased of 1.43 and 1.78 times, respectively, while the internalization efficiency of TBSV-RiGiD and -ApoE was reduced (0.54 and 0.59 times respectively). On this basis, the subsequent functional characterization studies were carried out only on TBSV-tLyp1 and TBSV-CooP.

GCPs are the highly proliferating and undifferentiated cells residing in the external granular layer of the cerebellum during its postnatal development, from which MB arises [34]. After isolation and purification from *Ptch1*^{+/-} mouse cerebella at P2, GCPs were incubated with TBSV-WT, -RGD, -tLyp1 or -CooP. The results of RT-qPCR analysis revealed that, compared to TBSV-WT, the internalization of TBSV-tLyp1 and -CooP was 8.2 and 4.7 fold higher respectively, while the uptake of TBSV-RGD was 0.46 fold lower (Figure 2B, left panel). These experiments were repeated on GCPs induced to differentiate, to mime the physiological process occurring during the cerebellum development (Figure 2B, right panel). Results showed that internalization of TBSV-tLyp1 and TBSV-CooP was significantly reduced (2.21- and 4.69-folds lower, respectively) compared to the uptake by undifferentiated GPCs, although TBSV-tLyp1 was still internalized more efficiently than the WT virus.

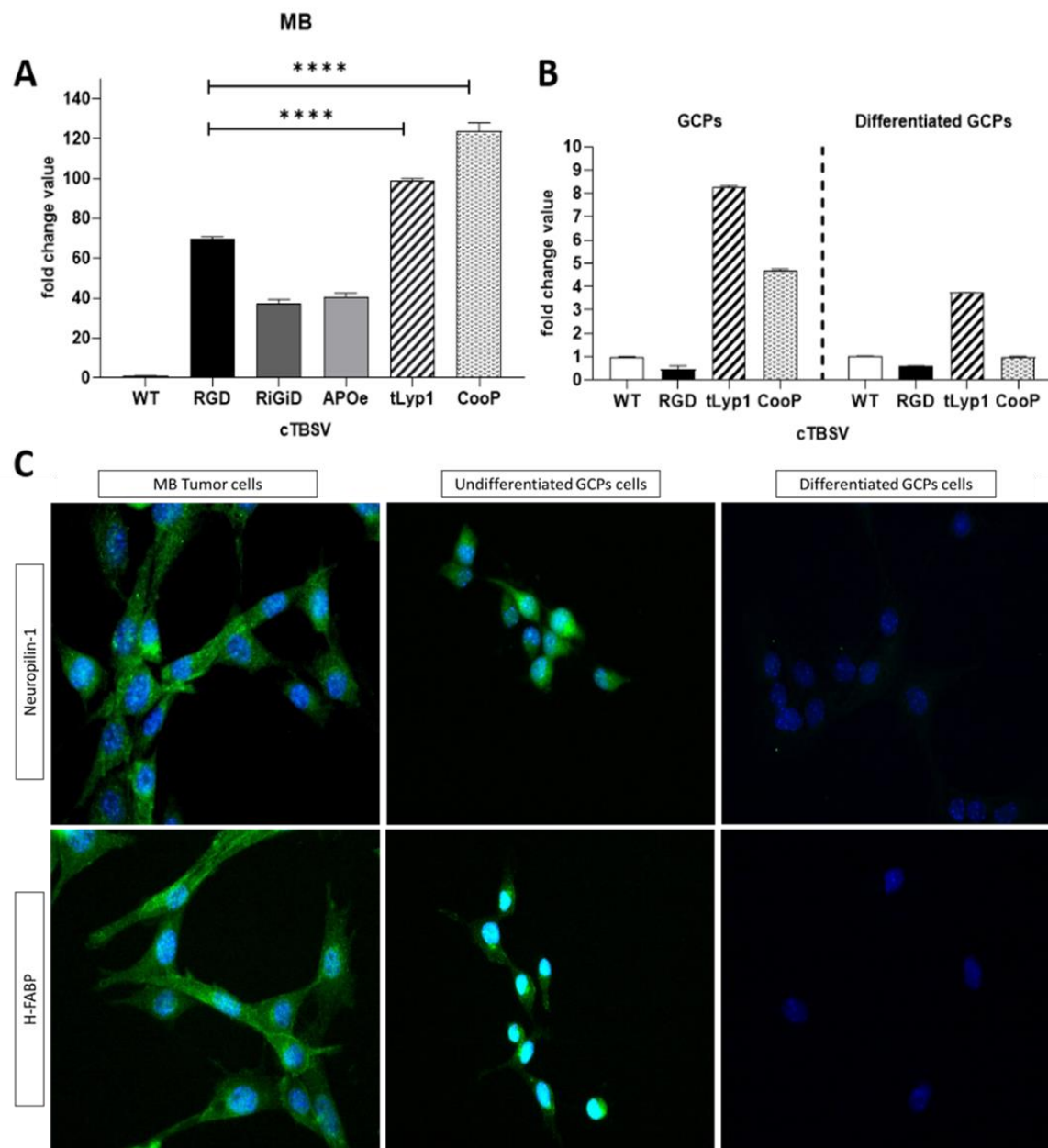


Figure 2. TBSV NPs uptake and receptors immunolocalization. Quantification through RT-qPCR of cTBSV internalized by (A) Shh-MB primary cells, (B) GCPs (left panel) and differentiated GCPs (right panel) using primers specific for a region in the CP gene common to all cTBSV NPs. Data are presented as mean $\pm$ SD of biological triplicates. Values obtained with TBSV-WT were taken as 1. (C) Localization by immunofluorescence of tLyp1 and CoopP peptides interacting partners NRP-1 and FABP3, respectively. NRP-1 (top line) and H-FABP (bottom line), in Shh-MB, GCPs and differentiated GCPs. Bars: 10 μ m. *** p <0.0001 (Student's t-test).

3.3.3 Immunolocalization of the interaction partners

It has been established that the interacting partners of CooP and tLyp1 peptides are the mammary-derived growth inhibitor (MDGI/H-FABP/FABP3) [35] and neuropilin 1 (NRP-1) [36], respectively. To evaluate if the specific uptake of the cTBSV NPs displaying these peptides may be mediated by these proteins, their expression and localization in Shh-MB cells, GCPs and differentiated GCPs were evaluated by immunofluorescence. As shown in Figure 2C, MB cells and GCPs showed high expression of both NRP-1 and H-FABP, that conversely are not detected in differentiated GCPs. Altogether these results suggested that the specificity of the uptake of TBSV-tLyp1 and TBSV-CooP by MB cells and GCPs may be receptor-mediated.

3.3.4 Docking and MD simulation of the tLyp1-NRP-1 and CooP-FABP3 complexes

The possible binding mode of the tLyp1 and CooP peptides (both in their free version or as fusion to the viral CP) with their respective interacting receptors was investigated by molecular docking and MD approaches. MD simulations were employed to enlighten the structural changes occurring in the active binding sites and to monitor the overall stability of the complexes predicted by docking calculations (Table 3).

Table 3 Molecular Docking Results.

ClusPro ^(a) or HADDOCK ^(b) complexes	Receptor	Ligand	ΔG (kcal/mol)	H-Bonds	
				receptor	ligand
tLyp1 – NRP1^(a)	NRP-1	tLyp1 peptide	-8.9	ASP 320	ARG 5
				ASP 320	THR 6
				TYR 353	LYS 4
CPtLyp1 – NRP1^(a)	NRP-1	tLyp1 peptide fused to CP of TBSV	-8.7	TYR 297	LYS 4
				PRO 317	ARG 7
				ASP 320	ARG 5
				ASP 320	ARG 7
				GLU 319	GLY 393 (L)
				TYR 322	ASN 385
				ASN 323	LEU 386
tufstin – NRP1^(a)	NRP-1	tufstin peptide (natural ligand)	-7.0	TYR 297	LYS 2
				TYR 297	PRO 3
				ASP 320	ARG 4

				LYS 351	ARG 4
CooP – FABP3^(a)	FABP3/ H-FABP	CooP peptide	-12.4	TYR 19	GLY 7
				ALA 75	LEU 6
				GLN 95	LEU 6
				ARG 126	ALA 9
CP CooP – FABP3^(a)	FABP3/ H-FABP	CooP peptide fused to CP of TBSV	-12.7	ASN 59	PRO390(L)
				ALA 75	LEU 6
OLA – FABP3^(b)	FABP3/ H-FABP	oleic acid (natural ligand)	-9.4	TYR 19	OLA 133
				TYR 128	OLA 133

Note: HADDOCK tool was used to obtain the OLA-FABP3 complex, since ClusPro is not suitable for docking chemicals, such as oleic acid (OLA), to proteins. Binding free energies (ΔG) and hydrogen bonds (H-Bonds) between receptors and ligands of the best ClusPro or HADDOCK derived docked complexes as calculated by Prodigy webserver.

As reference, the interaction mode of tuftsin with NRP-1 and of oleic acid with FABP3, natural binders of these receptors, were also simulated (Table 3, Figure 3 and 4). The clustering of the structural conformations adopted by the different complexes during the MD simulation showed that the natural ligands form complexes with their receptors for approximately 98% of their trajectory (panels A and B of Figure 3 and 4) and that such stability is mainly due to the formation of persistent H-bonds (panels C of Figure 3 and 4).

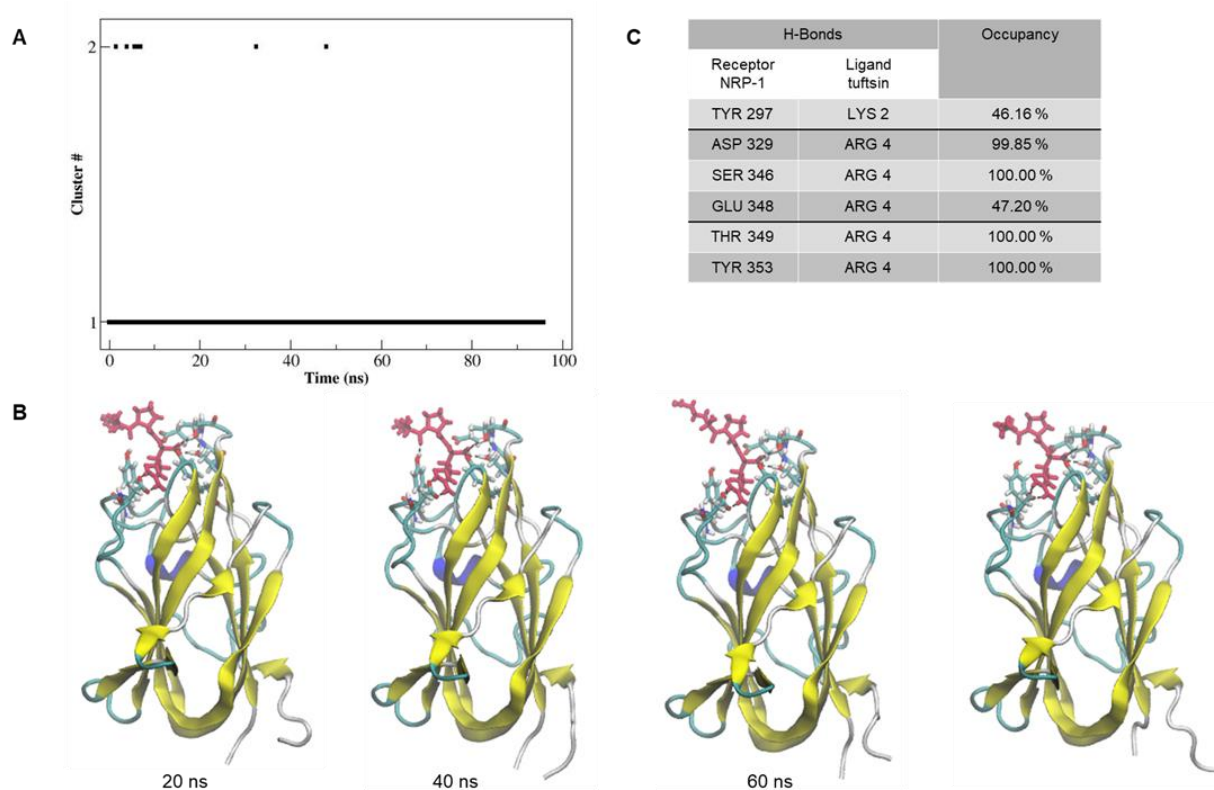


Figure 3. Dynamics and stability of the interaction of NRP-1 with tuftsin, the native ligand. (A) Number of clusters as a function of simulation time and their occupancy throughout the trajectory of the tuftsin-NRP1 complex. (B) Hydrogen bonds between peptide and receptor and their occupancy (%) along the simulation. (C) Simulation snapshots of the tuftsin-NRP1 complex, taken at selected times of the MD trajectory. The residues of the receptor involved in the interaction with tuftsin are represented by licorice model. Tuftsin is coloured in red and depicted in licorice model. The black dashed lines show the hydrogen bonds. The representative structure of the most populated cluster and its percentage of occupancy along the trajectory is also indicated. The interaction mode of tuftsin with NRP-1 was simulated as a reference. The clustering of the structural conformations adopted by the complex during the MD simulation showed that the natural ligand forms a stable complex with its receptors for approximately 99% of their trajectory (panels A and B) and that such stability is mainly due to the formation of persistent H-bonds (panels C).

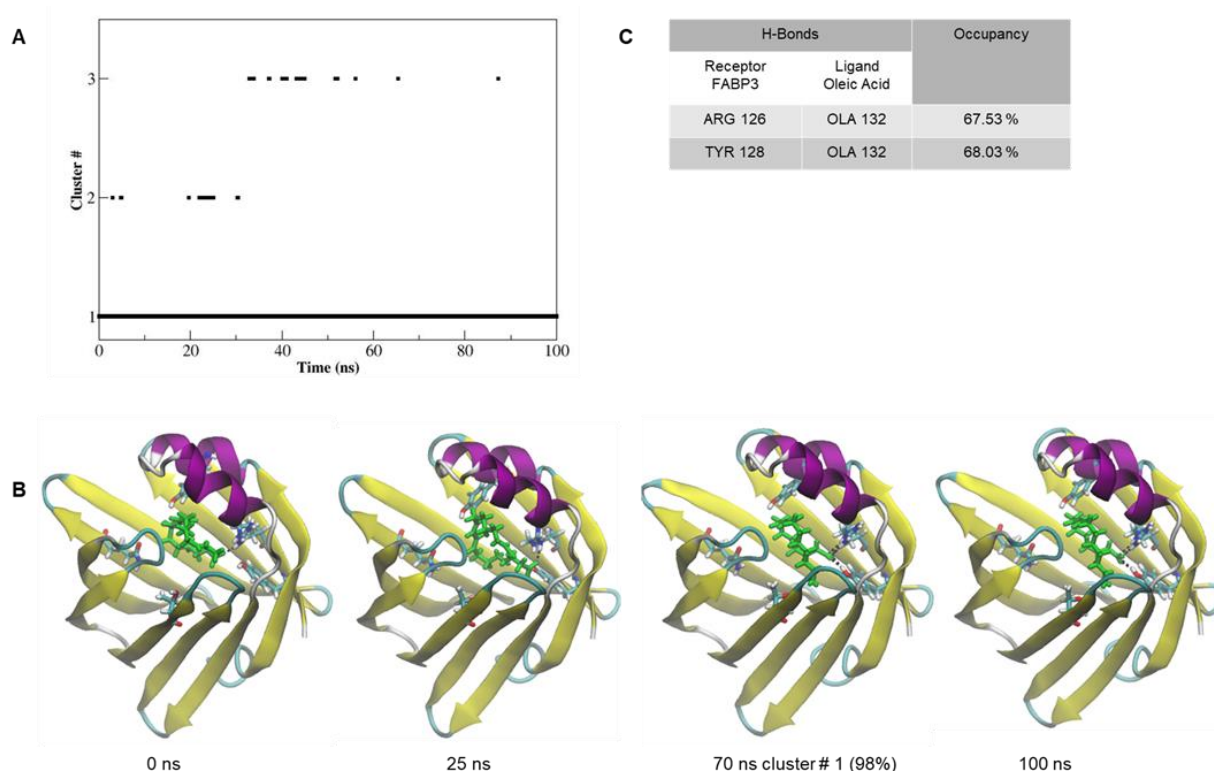


Figure 4. Dynamics and stability of the interaction of FABP3 with oleic acid, the native ligand. (A) Number of clusters as a function of simulation time and their occupancy throughout the trajectory of the OLA-FABP3 complex. (B) Hydrogen bonds between peptide and receptor and their occupancy (%) along the simulation. (C) Simulation snapshots of the OLA-FABP3 complex, taken at selected times of the MD trajectory. The residues of the receptor involved in the interaction with oleic acid are represented by licorice model. Tufstin is coloured in green and depicted in licorice model. The black dashed lines show the hydrogen bonds. The representative structure of the most populated cluster and its percentage of occupancy along the trajectory is also indicated. The interaction mode of OLA with FABP3 was simulated as a reference. The clustering of the structural conformations adopted by the complex during the MD simulation showed that the natural ligand forms a stable complex with its receptors for approximately 98% of their trajectory (panels A and B) and that such stability is mainly due to the formation of persistent H-bonds (panels C).

Concerning the tLyp1-NRP-1 complex, as shown in Figure 5A, approximately 67% of the trajectory was mainly described by two of the 21 identified structural clusters. After 72 ns of simulation, the MD trajectory indicated that several transitions among the clusters occurred, suggesting that the complex was unstable. Although the hydrogen-bonding pattern (Table 4) appeared rich in interactions between tLyp1 and NRP-1, only two H-bonds (Gly318-Arg7 and Asp320-Arg5) showed occupancy for more than 40% of the trajectory. After about 72 ns of the simulation, the tLyp1 peptide moved out from the receptor active site and the contact was maintained by the interaction of Arg7 with

Glu319 of NRP-1 (Figure 5B). Interestingly, when tLyp1 is fused to the CP (Figure 5C), the binding to NRP-1 appeared to be more stable, with several H-bonds (Table 4) contributing to stabilization. After about 60 ns of simulation, some residues (Gly375 and Lys397) of the distal part of NRP-1 appear to interact also with Asp191 of the viral CP giving a further contribution to the attraction towards the cTBSV. The time evolution of the binding free energy (Table 5) shows that after 75 ns of simulation the interaction of free tLyp1 with the receptor becomes unstable ($\Delta G = -43.229$ kJ/mol) whereas the conjugation of tLyp1 to the viral CP seems to enhance of two times the strength of the binding with NRP-1 ($\Delta G = -91.07$ kJ/mol).

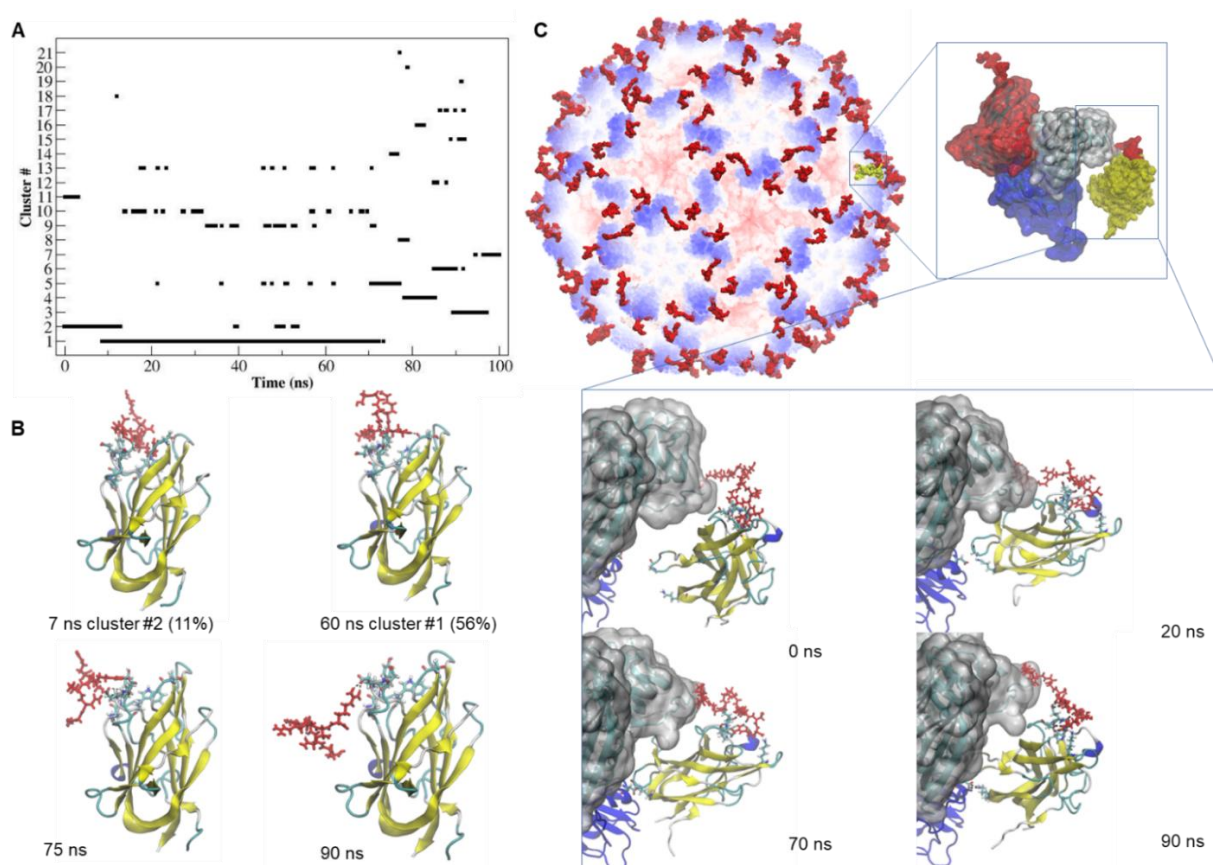


Figure 5. Dynamics and stability of the interaction of tLyp1-NRP-1 and TBSV CPtLyp-NRP-1. (A) Number of clusters as a function of simulation time and their occupancy throughout the trajectory of the tLyp1-NRP1 complex. (B) Simulation snapshots of the tLyp1-NRP1 complex, taken at selected times of the MD trajectory. The residues of NRP-1 involved in the interaction with tLyp1 are represented by licorice model. tLyp1 is coloured in red and depicted in licorice model. The representative structures of the most populated clusters and their percentage of occupancy along the trajectory are also indicated. Black dashed lines: hydrogen bonds. (C) Structural model of TBSV-tLyp1 in complex with NRP-1. Zoom views: simulation snapshot of the asymmetric unit of the cTBSV docked with NRP-1 receptor (shown in yellow and by Surface representation model) and simulation snapshots of the CPs of the asymmetric unit docked with

NRP-1 (shown by New Cartoon representation model) taken at selected times of the MD trajectory. The tLyp1 peptide is depicted in licorice model and coloured in red.

Table 4 H-Bonds Analysis.

tLyp1			tLyp1 fused to CP of TBSV		
H-Bonds		Occupancy	H-Bonds		Occupancy
<i>NRP-1</i>	<i>tLyp1</i>		<i>NRP-1</i>	<i>tLyp1</i>	
GLN 296	ARG 7	19.25 %	PRO 317	ARG 7	<u>70.94 %</u>
TYR 297	LYS 4	14.43 %	GLY 318	ARG 5	<u>60.16 %</u>
TYR 297	ARG 7	14.54 %	GLU 319	ARG 5	<u>59.22 %</u>
GLY 318	ARG 7	<u>41.70 %</u>	GLU 319	ASN 3	<u>51.88 %</u>
GLU 319	ARG 7	16.92 %	ASP 320	ARG 7	<u>93.93 %</u>
ASP 320	ASN 3	28.57 %	LYS 351	ARG 7	<u>59.55 %</u>
ASP 320	ARG 5	<u>43.96 %</u>	<i>Residues of CP of TBSV</i>		
ARG 323	ARG 7	11.56 %	GLY 375	ASP 191	30.98 %
THR 413	ARG 5	13.74 %	LYS 397	ASP 191	37.65 %

Note: H-Bonds between peptides and receptors and their occupancy (%) along the simulation. The H-Bonds with occupancy >40% along the trajectory are underlined. The binding to NRP-1 by their peptides fused to the TBSV CP seems to be stronger compared to the free peptides as assessed by the higher H-Bonds occupancy values.

Table 5 MM/PBSA Results.

Receptor	Ligand	Binding Free Energy (ΔG , kJ/mol)			
		0 – 25 ns	25 – 50 ns	50 – 75 ns	75 – 100 ns
NRP-1	tLyp1	-109.64 ± 101.47	-104.56 ± 93.00	-86.60 ± 71.98	-43.23 ± 78.39
	CP-tLyp1	-3.80 ± 83.15	-74.11 ± 85.42	-86.97 ± 91.77	-91.07 ± 74.47

Note: Binding Free Energy (ΔG) of the binding sites of receptors in complex with the free peptides and the peptides fused to the CP of TBSV, as predicted by MM/PBSA calculations.

The dynamics of the interaction of Coop with FABP3 (Figure 6) seemed to be very stable and comparable with that of oleic acid (Figure 4). In particular, the interaction of Ala9

of CooP with Arg126 in the active site of FABP3 was maintained all over the simulation time (Table 6).

The binding to FABP3 of the CooP peptide fused to the TBSV CP seems to be stronger compared to the free peptide despite few changes in the H-bonding pattern (Table 6). The values of the binding free energy (Table 7) clearly show that CooP peptide fused to the viral CP recognizes with almost 3-fold higher binding affinity the FABP3 receptor ($\Delta G = -377.07$ kJ/mol vs $\Delta G = -129.30$ kJ/mol).

These results, by supporting the idea that internalization is receptor-mediated, indicate also that the interaction of the peptides with their receptors, CooP in particular, is strengthened by fusion to the viral CP.

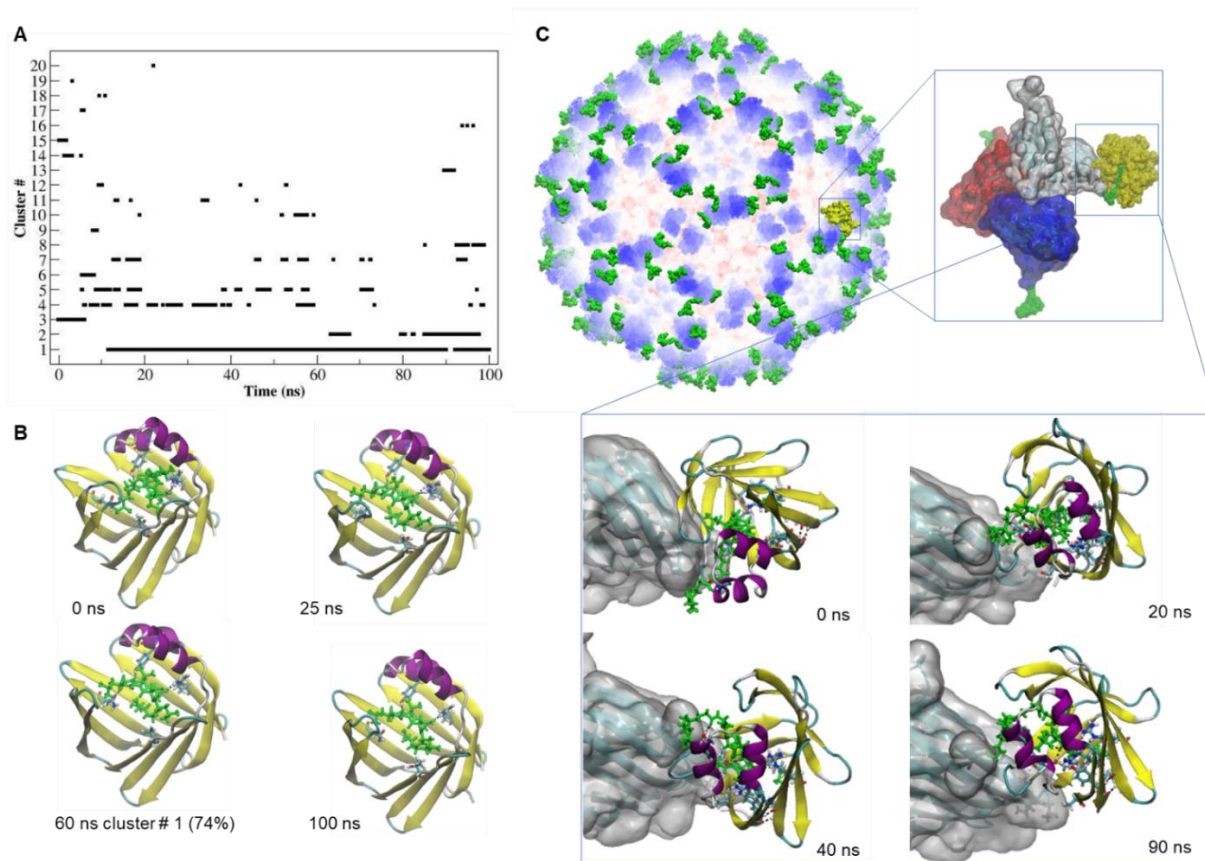


Figure 6. Dynamics and stability of the interaction of CooP-FABP3 and TBSV CPCooP-FABP3. (A) Number of clusters as a function of simulation time and their occupancy throughout the trajectory of the CooP-FABP3 complex. (B) Simulation snapshots of the CooP-FABP3 complex, taken at selected times of the MD trajectory. The residues of FABP3 involved in the interaction with CooP are represented by licorice model. CooP is coloured in green and depicted in licorice model. The representative structures of the most populated clusters and their percentage of occupancy along the trajectory are also indicated. Black dashed lines: hydrogen bonds. (C) Structural model of cTBSV in complex with FABP3. Zoom views: simulation snapshot of the asymmetric unit of the cTBSV docked with FABP3 receptor (shown in yellow and by

Surface representation model) and simulation snapshots of the CPs of the asymmetric unit docked with FABP3 (shown by New Cartoon representation model) taken at selected times of the MD trajectory. The CooP peptide is depicted in licorice model and coloured in green.

Table 6 H-Bonds Analysis.

CooP			CooP fused to CP of TBSV		
H-Bonds		Occupancy	H-Bonds		Occupancy
<i>FABP3</i>	<i>CooP</i>		<i>FABP3</i>	<i>CooP</i>	
TYR 19	GLY 7	<u>90.39 %</u>	THR 53	LEU 6	<u>46.85 %</u>
THR 60	GLY 2	10.49 %	ARG 106	GLY 7	<u>67.38 %</u>
GLN 95	GLY 7	<u>38.99 %</u>	ARG 126	LEU 3	<u>57.78 %</u>
ARG 126	ALA 9	<u>100.00 %</u>	TYR 128	SER 4	<u>49.94 %</u>
			<i>Residues of CP of TBSV</i>		
			LYS 52	ASP 341	<u>46.58 %</u>

Note: H-Bonds between peptides and receptors and their occupancy (%) along the simulation. The H-Bonds with occupancy >40% along the trajectory are underlined. The binding to FABP3 by their peptides fused to the TBSV CP seems to be stronger compared to the free peptides as assessed by the higher H-Bonds occupancy values.

Table 7 MM/PBSA Results.

Receptor	Ligand	Binding Free Energy (ΔG , kJ/mol)			
		0 – 25 ns	25 – 50 ns	50 – 75 ns	75 – 100 ns
FABP3	CooP	-118.27 $\pm$ 25.75	-118.20 $\pm$ 22.96	-128.41 $\pm$ 18.41	-129.30 $\pm$ 21.66
	CP-CooP	-502.9 $\pm$ 68.07	-457.36 $\pm$ 108.67	-391.34 $\pm$ 101.20	-377.07 $\pm$ 131.58

Note: Binding Free Energy (ΔG) of the binding sites of receptors in complex with the free peptides and the peptides fused to the CP of TBSV, as predicted by MM/PBSA calculations. The values clearly show that CooP peptide fused to the viral CP recognize with almost 3-fold higher binding affinity the FABP3 receptor ($\Delta G = -377.07$ kJ/mol vs $\Delta G = -129.30$ kJ/mol).

3.3.5 TBSV-tLyp1 and TBSV-CooP characterization and loading with DOX

The size homogeneity and monodispersity of TBSV-WT, -tLyp1 and -CooP preparations were verified by TEM and Dynamic Light Scattering (DLS), before proceeding with DOX-loading. Measurement of the NPs on TEM micrographs rendered a mean size of

32 nm (Table 8), consistent with the expected dimensions of TBSV. On the other hand, DLS analysis indicated that the mean diameter of the NPs was higher, suggesting the formation of agglomerates. This hypothesis is supported by the analysis of the polydispersity index that, even if below 0.7 as required by ISO standard ISO 22,412:2017 to define a polydisperse distribution of particles, ranged from 0.419 to 0.588 (Table 8). The presence of aggregates was detected in particular in the TBSV-CooP and TBSV-tLyp1 samples and this may be due to the fact that the ζ -potential values of these NPs is closer to neutrality, thus less prone to repulsion (Table 8).

Table 8. Characterization of TBSV nanoparticles in DLS/ ζ -potential evaluation and DOX loading.

NP	Size* (d.nm) by TEM	Size [§] (d.nm) by DLS	PDI	ζ -potential (mV)	DOX molecules/ particle [§]	Ng DOX/ μ g virus	[μ M]	EE [§] (%)	LC [§] (%)
TBSV-WT	32.7 $\pm$ 1.1	50.8 $\pm$ 18.4	0.429	-5.73	2009 $\pm$ 73	130.2 $\pm$ 4.7	207	38 $\pm$ 6	12 $\pm$ 2
TBSV-RGD	32.7 $\pm$ 0.9	48.5 $\pm$ 14.2	0.489	-5.95	1911 $\pm$ 394	136.4 $\pm$ 21.2	155	34 $\pm$ 1	11 $\pm$ 1
TBSV-CooP	32.7 $\pm$ 0.8	68.1 $\pm$ 17.7	0.588	-3.98	2174 $\pm$ 326	151.9 $\pm$ 15.9	379	47 $\pm$ 5	15 $\pm$ 2
TBSV-tLyp1	32.7 $\pm$ 0.9	66.5 $\pm$ 18.8	0.419	-3.61	2498 $\pm$ 749	174.1 $\pm$ 32.1	207	54 $\pm$ 10	17 $\pm$ 3

NP, nanoparticle; PDI: polydispersity index; EE, encapsulation efficiency; LC, loading capacity.

[§]The data obtained represent the mean value of 3 measurements $\pm$ SD.

*The data obtained represent the mean value of 50 measurements $\pm$ SD.

In order to “load” TBSV NPs with DOX, an *ad hoc* protocol was developed. Positively charged DOX was added in 5000 molar excess to the virus particles in swelling buffer in the presence of EDTA, in order to facilitate access into the viral cavity and interaction with negatively charged genomic RNA. Then, by restoring the initial pH conditions and proper Ca²⁺ and Mg²⁺ concentrations, the viral capsid was induced to re-establish the compact state, entrapping the drug into the shell. DOX in excess was removed from the samples performing a sucrose cushion passage. Electrophoresis analysis confirmed that

DOX migrates together with TBSV (Figure 7) and not towards the negative pole as it would be the case when it is free. All the TBSV NPs preparations showed a reproducible loading capacity in terms of ng of DOX/ μ g of TBSV: 100 for TBSV-WT, 113 for TBSV-RGD, 150 for TBSV-tLyp1 and 157 for TBSV-CooP, corresponding to 1543, 1736, 2314 and 2424 DOX molecules/virion, respectively. Results in Table 8 show that the encapsulation efficacy (EE) ranged from 31 to 48% and the loading capacity (LC) from 10 to 16%.

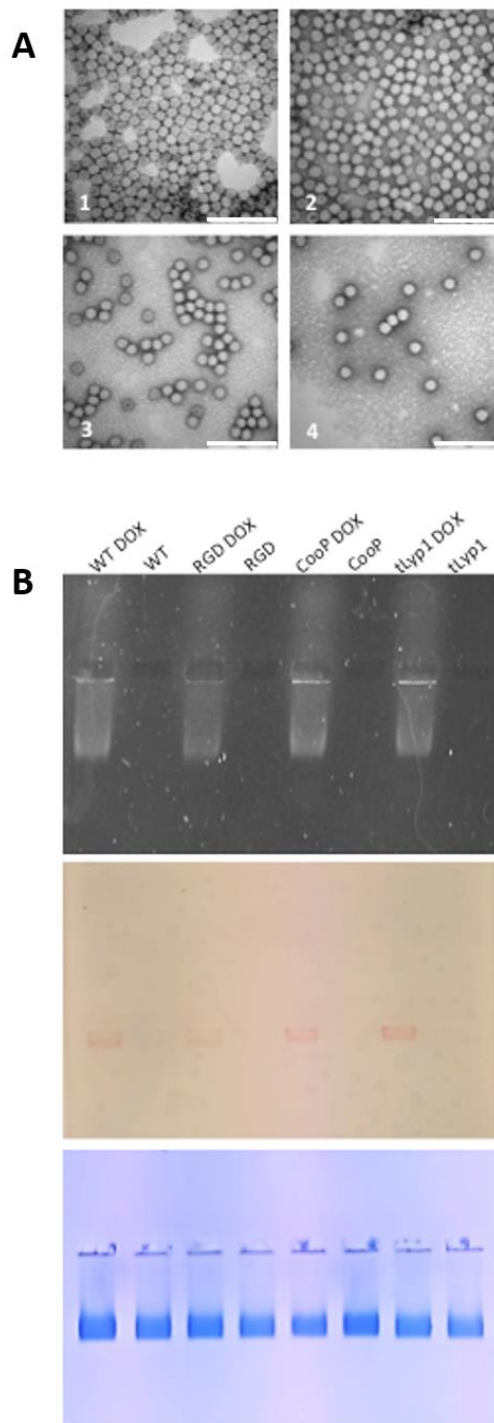


Figure 7. TBSV NPs transmission electron microscopy and DOX loaded TBSV NPs native agarose gel electrophoresis. (A) Visualization by negative staining and high-resolution transmission electron microscopy of purified TBSV NPs. 1: TBSV-WT NPs; 2: TBSV-RGD NPs; 3: TBSV-tLyp1 NPs; 4: TBSV-CooP NPs. Bar scale: 200 nm. (B) Native agarose gel visualized under UV light (upper panel), and whitelight before (middle panel) and after Coomassie Blue staining (lower panel). Four μ g of NPs were loaded in each well. TBSV NPs, loaded or not with DOX, are indicated for each well.

3.3.6 Chimeric TBSV-mediated delivery of DOX to Shh-MB cells

The ability of the cTBSV to deliver DOX was evaluated in MB cell cultures, measuring the cell viability after 72 hours of incubation with different concentrations of free-DOX or DOX-loaded NPs (TBSV-WT, -RGD, -tLyp1 or -CooP). The viability of the cells treated with the unloaded WT virus was set as 100%. As shown in Figure 8A, cell viability was significantly reduced by free-DOX in a dose-dependent manner, with maximum efficacy reached at a concentration of 25 μ M (90% of dead cells). DOX delivery through TBSV-WT and -RGD was able to significantly increase cell killing at lower DOX concentrations (around 85% of cell-death with TBSV-WT and 75% with TBSV-RGD at 10 μ M vs 50% with free-DOX). Moreover, the largest difference in cell-death rate among groups can be appreciated at the lowest DOX concentration (5 μ M), when delivered through TBSV-tLyp1 and TBSV-CooP producing over 90% of cell-death rate compared to 68% in TBSV-RGD, 46% in TBSV-WT and only 17% in free-DOX groups, indicative of the potential therapeutic gain of this targeted strategy of delivery.

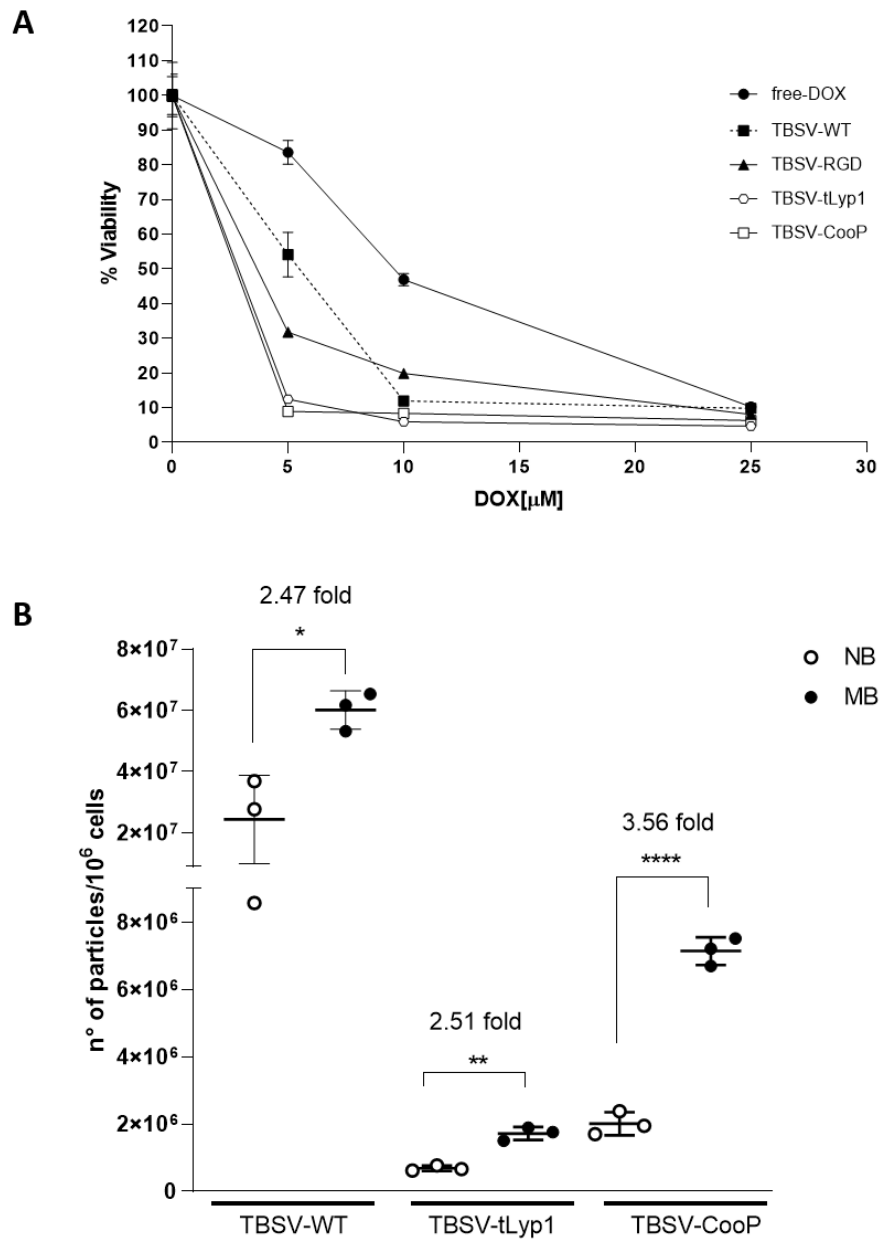


Figure 8. Validation of *in vitro* and *in vivo* TBSV targeting to MB. (A) Viability of Shh-MB cells after 72 hours of incubation with free-DOX or DOX-loaded NPs (TBSV-WT, -RGD, -tLyp1 or -CooP). The viability of the cells treated with the unloaded WT virus was set as 100%. Data are presented as mean $\pm$ SD of biological quintuplicates. (B) Absolute quantification of TBSV NPs in MB and NB determined by RT-qPCR after i.v. injection of 200 μ g TBSV-CooP, TBSV-tLyp1 or TBSV-WT in *Ptch1*^{+/-} mice with clear signs and symptoms of MB. Data are presented as mean $\pm$ SD of biological triplicates. *p=0.0171; **p=0.0010; ***p<0.0001 (Student's t-test). NB = normal brain; MB = Medulloblastoma.

3.3.7 ChimericTBSV NPs targeting to Shh-MB in vivo

To verify that TBSV-tLyp1 and TBSV-CooP were able to specifically target MB also *in vivo*, *Ptch1*^{+/-} mice developing the tumor were i.v. injected with TBSV-WT or chimeric

particles and 24 hours later the absolute quantity of TBSV NPs in MB and NB determined by RT-qPCR. The analysis (Figure 8B) indicated that TBSV-WT, as well as chimeric NPs, were able to reach the brain. Notably, even if the overall amount of TBSV-WT was higher compared to that of cTBSV NPs, TBSV-CooP showed the highest specificity in targeting MB cells (3.56-fold increase vs. NB, $P < 0.0001$) with respect to TBSV-WT and TBSV-tLyp1 (2.47- and 2.51-fold, respectively).

3.4 DISCUSSION

In the present study, NPs based on TBSV have been developed with the aim of targeting MB, a primary CNS tumor, which is one of the most frequent malignancies in childhood. Rapid advances in molecular genetics over the past two decades have provided consensus on the existence of four distinct molecular MB subgroups: Wingless (WNT), Sonic Hedgehog (SHH), group 3 and group 4 [37]. Accordingly, a new concept of therapeutic approach is now evolving, which could substitute or at least complement the standard treatment of MB, currently based on resection, chemotherapy and craniospinal irradiation. This approach is particularly relevant for SHH-MBs, the most common subgroup in infants (≤ 3 years old), in whom radiotherapy is precluded due to the severe neurological side effects. Targeted therapy is an example of alternative treatment and SHH pathway antagonists, primarily those inhibiting smoothened (SMO), are currently in clinical trials demonstrating improved progression-free survival [38]. However, such inhibitors might be ineffective when administered as monotherapy, due to the primary resistance observed in patients, or to the secondary resistance related to mutations occurring in the targeted receptor or in other involved proteins [39]. More efforts are thus necessary to develop new therapeutic options directed against SHH-MB and smart drug delivery systems represents the most promising. A wide range of different NP-based delivery systems have been investigated pre-clinically, which may lead to more NPs reaching the clinic. Glioblastoma (GBM) is the most explored tumor brain to test the efficacy of these type of devices, and 10 different nanomaterials are currently evaluated for possible clinical use [40]. Different NPs have been experimentally tested to investigate efficacy also against MB, including poly(lactic-co-glycolic acid) conjugated to polyethylene glycol (PLGA-PEG)-based NPs delivering the Shh pathway inhibitor HPI-1 [41,42], High Density Lipoprotein (HDL) NPs [43], biomimetic NPs decorated with a ligand targeting the stage-specific embryonic antigen 1 (SSEA-1) (anti-CD15) and loaded with the Shh inhibitor sonidegib (LDE225) [44], or polyoxazoline block copolymer micelles encapsulating the Shh pathway-inhibitor [45]. In recent years the interest is increasing towards the use

of biomaterials for nanomedical applications. Building blocks of biological origin are indeed ideal to construct biocompatible nanodevices for drug delivery. Viruses, and their non-infectious structural analogues (i.e., virus-like particles), are bio-based nanoparticles. The self-assembling properties of viral capsid proteins ensure the spontaneous formation of shells that are structurally uniform at the atomic level. In addition, targeting to specific cellular types may be in some cases intrinsic because related to viral tropism. In this context, plant viruses may be considered as a “natural collection” of NPs with conformations that range from spherical, to tubular or filamentous. Not being infectious for animal cells, they are replication incompetent thus intrinsically safe, and their half-life *in vivo* cannot be hindered by pre-existing immunity. These NPs may be rapidly produced on large scale at low costs using plants, in particular *N. benthamiana* that is permissive to the infection by a wide range of different plant viruses [46]. The intrinsic ability of plant viruses to target specific mammalian cells is not obvious; however, CPMV spontaneously binds to cervical and colon cancer cells through vimentin [47], a cell marker expressed also on GBM [48]. Nonetheless, specific targeting may be easily obtained decorating the viral CP with “homing” peptides by viral genome engineering or chemical interventions. By these approaches, a plethora of plant viruses, some of which, including TBSV, characterized for their biodistribution and formally demonstrated to be devoid of toxicity, have been investigated for very different applications in nanomedicine, ranging from vaccine formulations to tissue engineering, imaging, and diagnostic. Concerning drug delivery, the potentialities of plant viruses as possible vehicles have been tested mainly on melanoma, myeloma, colon, breast, prostate and ovarian cancer, both *in vitro* and *in vivo*, and only rarely on brain tumors [49]. In this scenario, the development of new plant virus-based delivery platforms appears to be important to increase the number of well characterized tools available for applications in biomedicine. Here we focused on TBSV NPs, displaying on the outer surface peptides selected from a larger panel whose structural, dynamical and physicochemical features were previously characterized in detail by exploiting the capabilities of MD

simulation [6]. All the peptides were correctly displayed and were able to induce a significant increase in the uptake of TBSV by primary Shh-MB cells. In particular, CooP and tLyp1 chimeras mediated a considerably higher internalization. To explain the advantage offered by these two peptides, the binding mode of tLyp1 and CooP (both in their free version or as fusion to the viral CP) with their interacting partners, NRP-1 and FABP3, respectively, were investigated by MD approaches. Interestingly, the dynamics of the interaction resulted to be more stable when peptides, CooP in particular, were fused to the CP, supporting the idea that internalization in MB cells is indeed receptor mediated. NRP-1, involved in cell survival and proliferation, seems to favor an undifferentiated phenotype in cancer cells [50] and its overexpression has been reported to be correlated with a poor prognosis in both GBM [51] and MB [52]. Furthermore, it has been demonstrated that 12-aminoacids within the NRP1 cytoplasmic domain are necessary to regulate Hedgehog signaling [53]. In agreement, we revealed NRP1 expression only in MB cells and in GCPs, both strictly depending on Shh deregulation. A similar trend characterized the immunostaining of FABP3, although, to the best of our knowledge, no correlation with Shh-dependence is reported in the literature. However, FABPs are expressed in the developing brain and their cellular distribution and genetic variation have been implicated in several brain diseases including human brain cancer. In particular, in a meta-analysis study of gene expression in murine and human MB, FABP3 has been reported to be downregulated [54]. The absence of expression of both receptors in differentiated cells well correlates with the significant reduction of cTBSV internalization, corroborating the high specificity for MB targeting offered by these NPs, with strong implications toward the safety of differentiated tissues. The functional test carried out *in vitro* with DOX-loaded cTBSV NPs highlighted that all NPs offered a significant advantage in terms of efficacy with respect to the delivery of low concentrations of free DOX. Five μm DOX delivered through TBSV-tLyp1 and TBSV-CooP were sufficient to induce a mortality rate of MB cells of 90%, a dose 5-fold lower than that necessary to induce the same mortality using the free drug. Importantly, at the same DOX-loaded concentration, these cTBSV particles offered a statistically significant reduction

of tumor cell viability compared to TBSV-RGD and TBSV-WT. Overall, these results are in agreement with data showing an increase in the specific uptake of both chimeras by MB cells and GCPs. Notably, when tested *in vivo*, CooP peptide increased the uptake of the plant virus NPs by MB cells compared to that of the unmodified NPs and to cTBSV displaying the peptide tLyP1 (3.5 fold compared to around 2.5 in the other cases). Beside the same experiment showed that the access to the brain of the cTBSV particles is overall less efficient if compared to that of the WT particles. This may be due at least to three main factors i.e., the slight tendency to aggregation of the chimeric particles when suspended in saline and/or the delivery route and/or deposition of a different protein corona. Moreover, it must be considered that the cellular composition of the tumor mass is less homogenous if compared to that of the cells in the *in vitro* cultures.

3.5 CONCLUSIONS

In summary, the presented data support the hypothesis that cTBSV NPs, and TBSV-CooP in particular, might be suitable bio-based vehicles, rapidly produced on large scale at low costs using plants, for the targeted delivery of chemotherapeutics to Shh-dependent MB, potentially overcoming problems related to low drug bioavailability and adverse off-target effects.

References are listed in Appendix II

Figures have been Created with BioRender.com

4. PEPTIDE-FUNCTIONALIZED AND DRUG-LOADED TOMATO BUSHY STUNT VIRUS NANOPARTICLES COUNTERACT TUMOR GROWTH IN A MOUSE MODEL OF SHH-DEPENDENT MEDULLOBLASTOMA

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4.1 INTRODUCTION

The third chapter is dedicated to the *in vivo* therapeutic efficacy of TBSV-CooP loaded with DOX which in previous experiments proved to be the most efficient, among other tested particles, in terms of MB tumor targeting. The onset of the tumor in *Ptch1*^{+/-} knock-out heterozygous mice occurs starting from 10-12 weeks of life, when the mice begin to show the evident symptoms of MB (arched and swollen head, matted hair, poor mobility of the lower limbs and general disorientation). In this phase, the survival rate of symptomatic mice is decidedly poor because the tumor is in an advanced stage of development, in this chapter defined full-blown MB. Unlike in the later stages of tumor development, in the early stages there is the possibility of slowing the progression of the tumor more successfully. In the early stages, although *Ptch1*^{+/-} mice did not show any tumor symptoms, from a histological point of view there is a high incidence of hyperplastic cerebellar lesions localized in the outermost regions of the cerebellum. These areas of hyperproliferation are defined as pre-neoplastic lesions and, in *Ptch1*^{+/-} mice, begin to appear between the fourth and sixth weeks of life with an incidence of 60-90%, respectively. In this regard, the first step was to design a preclinical study to verify whether multiple doses of TBSV-CooP loaded with DOX, administered between the sixth and eighth week of life (time frame in which 90% of the mice present the pre-neoplastic lesions at the "point of no return"), were able to inhibit PNLs progression towards MB. The second step was to verify whether a single dose of DOX-loaded TBSV-CooP

particles, administered to mice with full-blow MB, was enough to modulate pro-apoptotic/anti-proliferative molecular signaling.

4.2 MATERIALS AND METHODS

4.2.1 Mice treatment

Ptch1^{+/-} mice, of both sexes, were obtained at the ENEA Casaccia (Rome, Italy) animal facility by crossing *Ptch1*^{+/-} heterozygous males with CD1-wild-type females and viceversa, and selected after genotyping as previously described [1]. At postnatal day 2 (P2), pups were whole-body irradiated with a single 3 Gy-dose of X-rays (dose rate = 0.89 Gy/min). Irradiation was performed using a Gilardoni CHF 320G X-ray generator (Gilardoni S.p.A., Mandello del Lario, Italy; HVL=1.6 mmCu) operated at 250 kVp, 15 mA, with filters of 2.0 mm Al and 0.5 mm Cu. To test the inhibition of tumor progression, at six weeks of age a total of 52 *Ptch1*^{+/-} mice of both sexes were randomized in 3 groups and injected intravenously (i.v.) by tail vein 2 times per week for 2 weeks with 150 µl of: vehicle (n=17; NaAc pH 5.2), DOX-TBSV-CooP (n=18; DOX concentration 1 µg/g in NaAc), DOX-free (n=17; 5 µg/g in NaAc). During the treatment, mice were daily monitored to check the general health status and weighted immediately before each treatment; three days after the last injection, mice were weighted, sacrificed and organs (brain, spleen, kidneys, lung, liver) as well as blood collected. For molecular analyses, *Ptch1*^{+/-} mice (n=6 for each experimental group) with symptomatic MBs were i.v. treated with a single dose of vehicle, DOX-TBSV-CooP and DOX-free, with the same concentration aforementioned, and sacrificed 24 h after the treatment. Tumors were collected in two aliquots and immediately frozen in liquid nitrogen, then stored at -80°C for RNA and protein extraction. The experimental administration schedule is summarized in Figure 1. Throughout the experimental duration, animals were housed under conventional conditions with food and water available *ad libitum* and a 12-hours light-dark cycle.

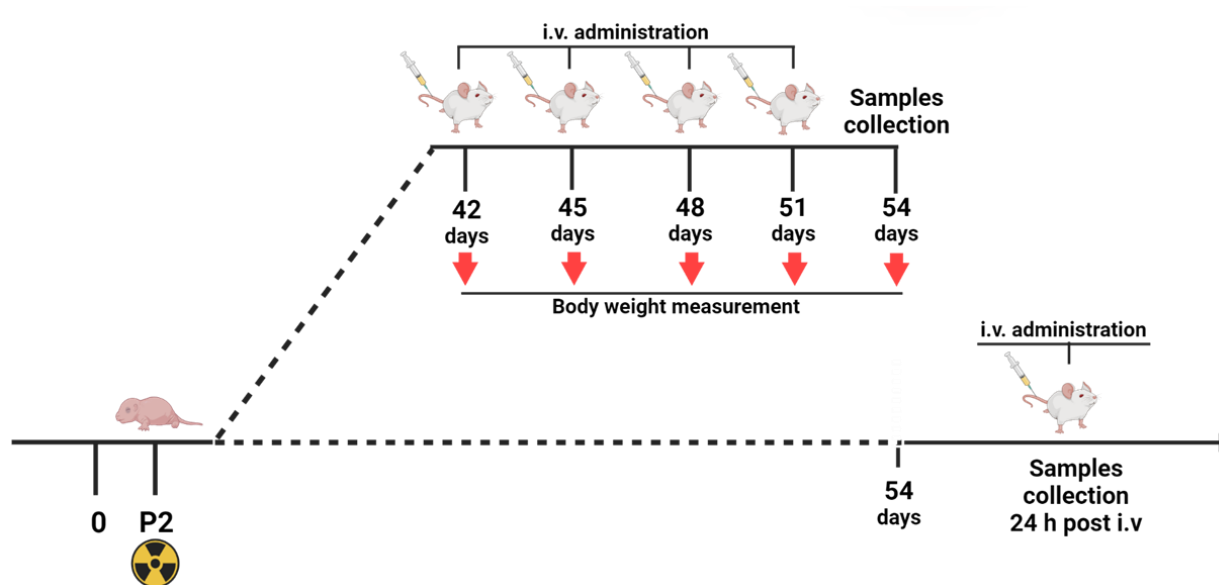


Figure 1. Experimental administration schedule.

4.2.2 Histology, morphometry and immunohistochemistry

Collected organs were formalin-fixed and paraffin-embedded (FFPE) for routine histology. To assess the percentage of pre-neoplastic lesions (PNLs), the entire cerebellum was examined recovering FFPE brain sections with intervals of 70 μm . At the recognition of abnormal cerebellar regions with morphology varying from focal subpial aggregates of cerebellar GCPs to overt nodules, microtumors - in general defined as PNLs - sections were mounted on slides and stained with standard haematoxylin and eosin (H&E) technique. PNLs cross sectional areas were carried out using imaging software NIS-Elements BR 4.00.05 (Nikon Instruments Europe B.V., Italy). For immunohistochemistry, PNL-positive FFPE brain sections (4 μm) were dewaxed for 20 min at 56°C and incubated in citrate buffer pH 6.0 for 20 min at 95°C. After peroxidases inhibition by 3% H_2O_2 for 10 min, sections were incubated with primary antibodies anti-Ki67 (Bethyl, Montgomery, TX, USA), anti-NeuN (Merck Millipore, Darmstadt, Germany) and anti-caspase 3-activated (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature in a moist chamber. After incubation with the secondary anti-rabbit antibody (Bethyl), the antigen-antibody reaction was revealed by DAB (Dako, Agilent Technologies, Santa Clara, CA, USA) and analyzed by HistoQuest (TissueGnostics, Vienna, Austria) software.

4.2.3 RNA extraction and Real Time qPCR

Tumor masses (~30 mg each) were homogenized with gentleMACS™ Octo Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and RNA extracted with the RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA synthesis was performed using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), and RT-qPCR was carried out by StepOnePlus™ Real-Time PCR System (Applied Biosystems), using Power SYBR® Green PCR Master Mix (Applied Biosystems). Relative gene expression was quantified using *Glyceraldehyde-3-phosphate (Gadph)* as housekeeping gene. Oligonucleotide primers used for RT-qPCR are listed in Table 1. The $\Delta\Delta C_t$ quantitative method was used to normalize the expression of the reference gene and to calculate the relative expression level of target genes.

Table 1. List of primers used for quantitative Real Time qPCR.

Gene	Forward primer	Reverse primer
<i>53BP1</i>	5'- TCACTGCCATGGAGGAGC -3'	5'- GGATGCCTGGTACTGTTTGG -3'
<i>Bax</i>	5'- ATCCAGGATCGAGCAGGGCG -3'	5'- ACTCGCTCAGCTTCTTGGTG -3'
<i>Cyclin D1</i>	5'- TCCGCAAGCATGCACAGA -3'	5'- AGGGTGGGTGGAAATGAACT -3'
<i>Gadph</i>	5'- CATGGCCTTCCGTGTTCTTA -3'	5'- GCGGCACGTCAGATCCA -3'

4.2.4 Western blot

Total proteins were extracted from ~15 mg of previously collected tumor tissues (n=6 for each experimental group) using T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Rodano, Italy) supplemented with proteinase and phosphatase inhibitors following manual procedure and subsequently quantified by Bradford assay. For each experimental group, equal amount of proteins was pooled together in a total of 50 µg. Then, the samples were mixed with SDS loading buffer and heated for 5 minutes at 100°C. Proteins were separated on 4-20% Tris-glycine TGX precast gel (BIO-RAD, Hercules, CA, USA) in reducing and denaturing conditions, before transferring onto PVDF membrane by using the Trans-Blot Turbo Transfer System (BIO-RAD). Membranes were blocked with 5% non-fat dry milk in PBS containing 0.5 % (v/v) Tween-20 (PBS-T) at

37°C for 2 h. After blocking, membranes were incubated over night at 4°C with primary antibodies, diluted in 3% non-fat dry milk in PBS-T, against H-FABP (Abcam, Cambridge, UK; 1:500), Bim (Cell Signaling Technology; 1:1000) and 53BP1 (Abcam; 1:1000). Housekeeping anti- β Tubulin (Abcam; 1:2500) was incubated at RT for 45 min. After washing three time with PBS-T, membranes were incubated at RT for 1 h with horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Abcam; 1:5000 for target genes and 1:15000 for housekeeping) diluted in 3% non-fat dry milk in PBS-T. The signal development was obtained on washed membranes by enhanced chemiluminescence (Amersham Biosciences, Amersham, UK) and the images acquired through iBright Imaging System (Thermo Fisher Scientific).

4.2.5 Immunofluorescence

Symptomatic *Ptch1*^{+/-} (n=3) and wild type (n=3) mice were perfused via the left ventricle using 20 ml of PBS. Brains were collected and fixed in cold 4% w/v paraformaldehyde in PBS at 4°C for 24 h, washed in PBS at RT for 1 h and cryoprotected in 30% w/v sucrose at 4°C overnight. Cryoprotected and fixed tissues were frozen in optimal cutting temperature compound (Killik-O.C.T. Compound; Bio-Optica, Milan, Italy), cryosectioned at 10 μ m and stored at -20°C. Air-dried tissue sections were rehydrated in PBS for 10 min, permeabilized in PBS + 0.2% v/v Triton at RT for 10 min thus incubated with anti-H-FABP antibody (Abcam, 1:200) at 4°C, overnight. After washing, incubation with secondary Alexa Fluor 564 goat anti-rabbit antibody (Abcam; 1:500) was performed at RT for 30 min, followed by washing and nuclear counterstaining with DAPI (1 μ g/mL in PBS) at RT for 8 min. Images were analyzed using Zeiss ZEN PRO software (Zeiss, Jena, Germany).

4.2.6 Enzyme-Linked Immunosorbent Assay (ELISA) for antibodies titration

Serum samples belonging to individual mice of DOX-loaded TBSV-CooP treated group were analyzed by ELISA to determine the concentrations of anti-TBSV CP antibodies. Briefly, ELISA Maxisorp plates (ThermoFisher Scientific) were coated overnight with

100 μ l 1 $\times$ Carbonate Buffer (0.2 M Na₂CO₃/NaHCO₃ at pH 9.4) containing 0.5 μ g of purified TBSV-WT. After washings three times with 1 $\times$ PBS supplemented with 0.1% Tween-20, and twice in PBS, plates were blocked in PBS-5% low-fat milk, 2 h at 37°C. Then, after washings as before, 100 μ l of individual mouse serum dilutions in 1 $\times$ PBS were added in triplicate to wells, and incubated overnight at 4°C. The presence of bound murine IgG antibodies was detected using an HRP-labelled sheep anti-mouse IgG polyclonal antibody (GE Healthcare Europe GmbH; 1:2500;), incubated for 1 h at 37°C and revealed, after washing, with 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonica cid) (ABTS; SeraCare, Milford, CT, USA) as substrate. The colorimetric reaction was measured by a microplate absorbance reader (Tecan, Mannedorf, Switzerland) at OD_{405nm}. Endpoint titres were defined as the reciprocal of the highest serum dilution giving an absorbance ≥ 0.1 OD above the blank (absorbance of the sera of sham treated mice). Geometric mean titres were determined for each group.

4.2.7 Statistical Analysis

Analyses were performed using GraphPad Prism v.7 for Windows (GraphPad Software, San Diego, CA). We used Student's t-test and Mann-Whitney test for comparison between groups. *P* values ≤ 0.05 were considered statistically significant.

4.3 RESULTS

4.3.1 DOX-loaded TBSV-CooP nanoparticles inhibit progression of MB PNLs

At 6 weeks of age almost the totality of neonatally-irradiated *Ptch1*^{+/-} mice are characterized by the presence of microscopically recognizable cerebellar abnormalities on their way to become full-blown tumors [1]. Thus, to evaluate the capability of the TBSV-CooP drug delivery strategy to efficiently reach the target, inducing a therapeutic effect, we designed a preclinical study focused on early phases of MB development. To this aim, we compared the effect induced by a dose of free DOX (5 mg/Kg) with a dose 5-fold lower (1 mg/Kg) loaded in TBSV-CooP NPs. Clinically, DOX is administered at doses of 1–10 mg/kg, intravenously. If 1–10% of the injected dose reaches the tumor site, the resulting intratumoral dose would equate to 0.01–1 mg/kg [2]; thus our dose is within a clinically relevant use range of DOX. From 6 to 8 weeks of age, mice were treated 4 times (2 times per week) with DOX-free, DOX-TBSV-CooP or vehicle (sham group) and sacrificed 3 days after the last treatment. According to histological and dimensional analyses, PNLs found in all FFPE mouse brains analyzed were classified as asymptomatic microtumors (area > 1×10⁶ μm²; Figure 2a) or small nodules (area < 1×10⁶ μm²; Figure 2b). Although the total incidence of positive mice with cerebellar abnormalities was 100% for each experimental group, a different size distribution of PNLs was observed among groups. As shown in Figure 2c, in vehicle-treated FFPE mouse brains, 41% (7/17) of microtumors and 59% (10/17) of small nodules were found, with a global proliferation index of 72% (ki67-positive/total cells; Figure 2d). The frequency of microtumors decreased to 33% (6/18) and 23.5% (4/17) in DOX-free and DOX-TBSV-CooP groups, respectively. The corresponding increased frequency of less progressed PNLs (67% and 76.5%) suggests a slowing down of tumor progression (Figure 2c and d) in both treated groups. This conclusion is corroborated by the statistically significant inhibition of the proliferation index in DOX-free (55.3%; *p* = 0.0097) and DOX-TBSV-CooP (58.7%; *p* = 0.0247) PNLs compared to that quantified in PNLs from vehicle-treated group.

Furthermore, immunostaining of small nodules with an antibody directed against NeuN, a marker of neuronal differentiation, highlights a statistically significant higher percentage of NeuN-positive cells in DOX-free (41.1 %; $p = 0.0241$) and in DOX-TBSV-CooP (35.9%; $p = 0.0117$) treated animals compared to the sham (22.5%) group, indicating a shift towards regression of small nodules to normal tissue and thus decreasing propensity to develop into MB (Figure 2e). Notably, in PNLs from treated groups, several figures associated to irreversible cell injury (apoptosis) were morphologically recognized (Figure 2g and h). They were also confirmed after immunostaining with caspase-3 antibody compared to sporadic apoptotic cells in vehicle-treated counterparts, where many cells in mitosis are present (Figure 2f-h, inserts). Quantification of apoptotic cells clearly demonstrated a statistically significant increase of apoptotic rate in MB from treated-groups (Figure 2i); notably, MB treated with DOX-TBSV-Coop showed the highest level of damaged cells ($p < 0.0001$ vs. vehicle; $p = 0.0040$ vs. DOX-free).

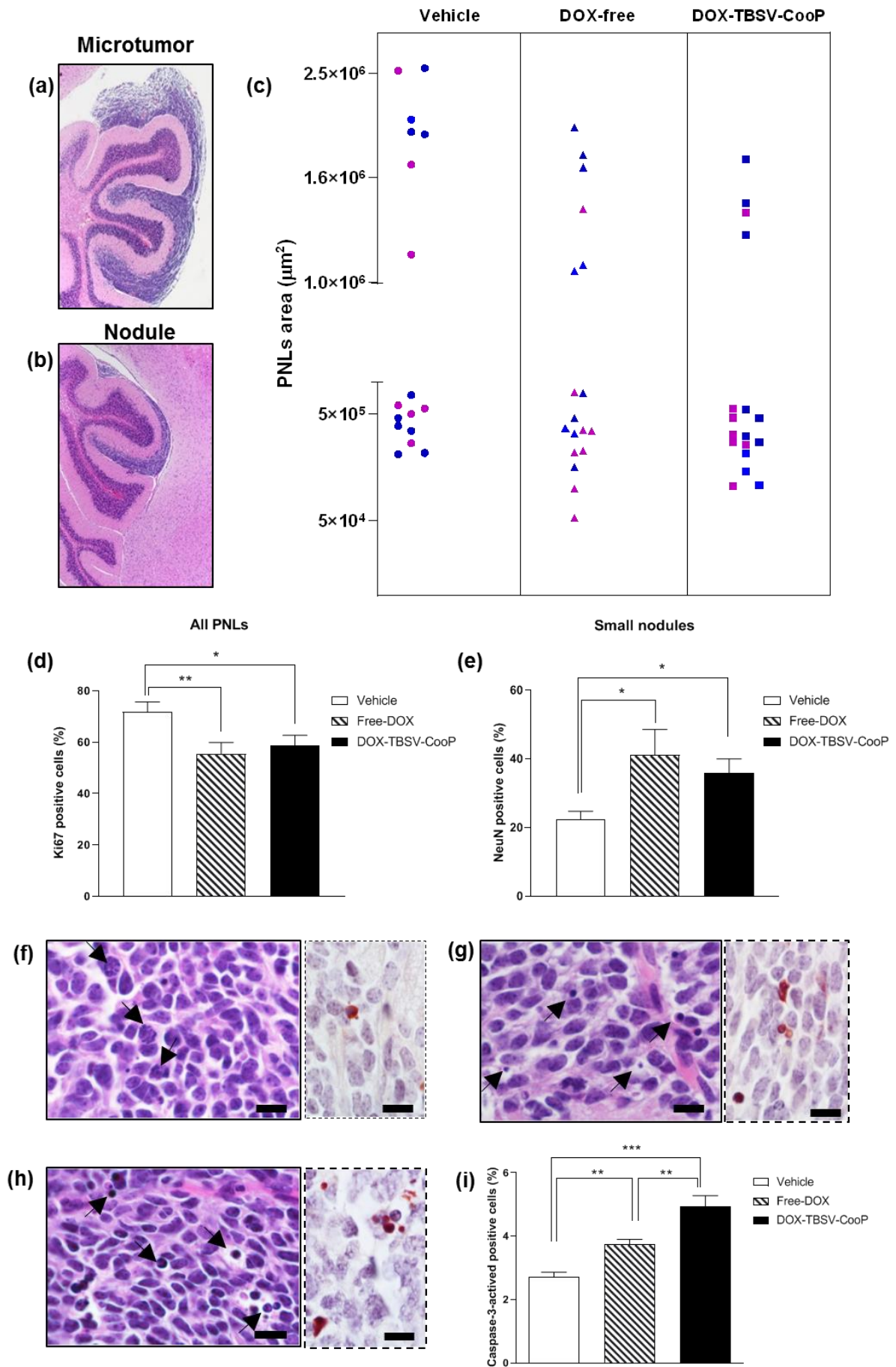


Figure 2. Histological and immunohistochemical analyses of MB PNLs. (a). Representative image of a PNL classified as microtumor (area > $1 \times 10^6 \mu\text{m}^2$). (b) Representative image of a PNL classified as small nodule (area < $1 \times 10^6 \mu\text{m}^2$). (c) Distribution of PNLs according to their cross-sectional area in sham (n=17), DOX-free (n=18) and DOX-TBSV-CooP (n=17) groups. Blue = male mice; magenta = female mice. (d) PNLs proliferation index calculated as percentage of Ki67-positive/total cells. Dataset represent the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$ (Student's t-test). (e) Rate of cell differentiation in small nodules calculated as percentage of NeuN-positive/total cells. Dataset represent the mean $\pm$ SEM. * $p < 0.05$ (Student's t-test). Representative images of PNLs sections from sham (f), DOX-free (g) and DOX-TBSV-CooP (h) groups. Arrowhead in (f) = Phases of mitosis. Arrowhead in (g) and (h) = apoptosis. Inserts in (f-h) = Immunostaining with an antibody against caspase-3-activated. Scale bars = 10 μm .

Despite microscopic examination of organs (spleen, kidneys, lung, liver) collected from mice of all the experimental groups did not show evident alterations of normal structure neither the presence of cell injury at the end of our experimental protocol (Figure 3), mice treated with DOX-free manifested clear symptoms of systemic toxicity as demonstrated by body weight loss after the third injection that was statistically significant compared to vehicle-treated group after the last treatment ($p = 0.0429$; Figure 4). On the contrary, no variation of body weight was observed in DOX-TBSV-CooP-treated mice all along the experimental time.

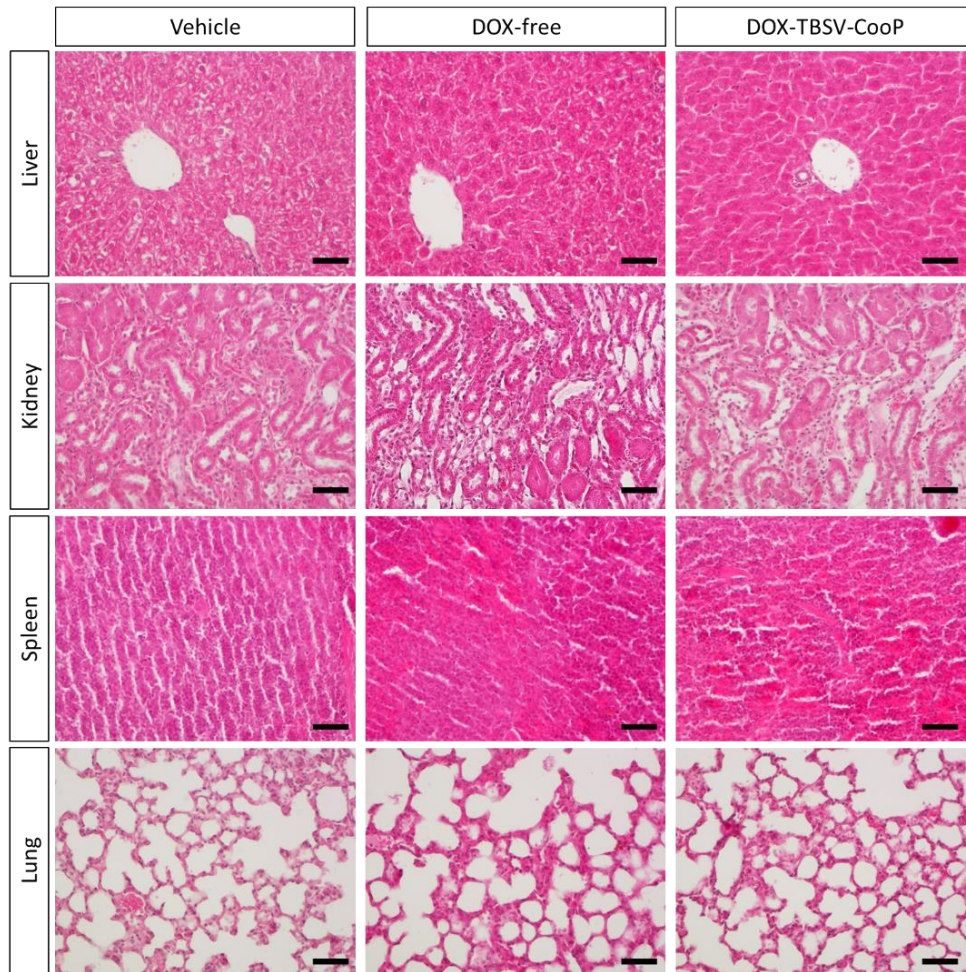


Figure 3. Microscopic examination of off-target organs. Representative histological images of haematoxylin and eosin (H&E) stained organs collected from vehicle-, DOX-free and DOX-TBSV-CooP treated mice. Bars = 50 mm

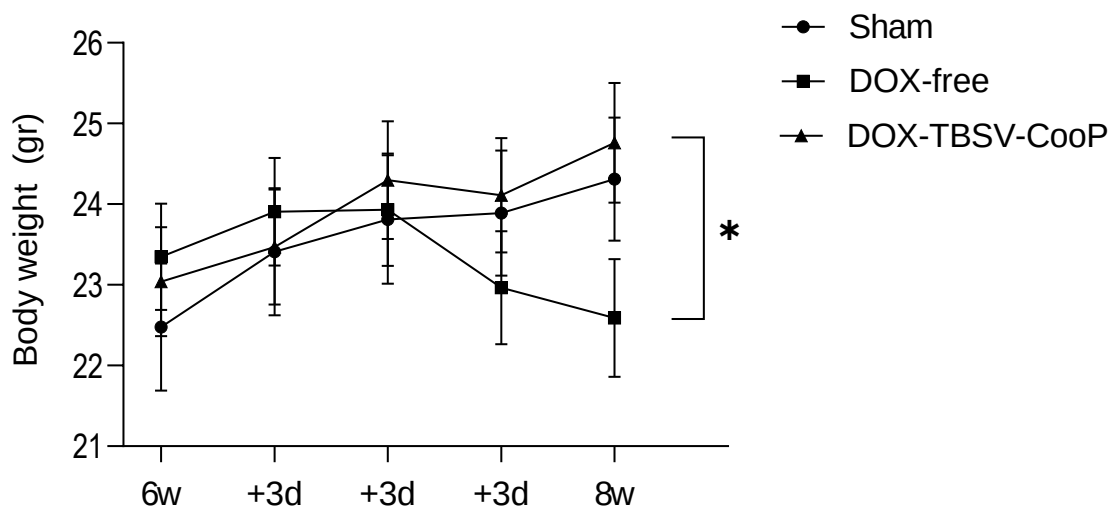


Figure 4. Evaluation of body weight registered immediately before each i.v. injection of NaAc (vehicle), DOX-free and DOX-TBSV-CooP and at the time of organs collection (8 weeks). Data represent the mean weight of each experimental group $\pm$ SD. * $p < 0.05$ (Student's t-test).

Overall, these results clearly demonstrate the advantage offered by the TBSV-based drug delivery system in contrasting MB PNLs towards their evolution in overt tumors, being able to inhibit tumor cell proliferation and to promote cell death as well as cell differentiation despite a drug dose 5 times lower than the freely administrated, with also absence of undesirable side effects.

4.3.2 Effects of DOX-loaded TBSV-CooP nanoparticles on full-blown tumors

Ptch1^{+/-} mice, irradiated at post-natal day 2 with a single dose of 3 Gy of x-rays, develop full-blown MBs starting from 10 weeks post-irradiation with maximum tumor incidence (60%) reached by 20 weeks [1]. Tumor development is accompanied by clearly recognizable symptoms (e.g. weight loss, skull deformation, impaired balance and paralysis) and, since the onset of these symptoms, mice survival is reduced to a few days. At this stage of MB development, tumor mass is well-recognizable in the posterior cranial fossa and it is easily detached from the remaining normal cerebellum (Figure 5a). To establish if our platform of drug delivery could offer a therapeutic benefit also at this late stage of MB development, symptomatic mice were i.v. injected by tail vein with vehicle (n=5), DOX-free (n=5) or DOX-TBSV-CooP (n=5) and 24 hrs later tumors collected for RNA and protein extractions. By RT-qPCR, mRNA expression levels of genes involved in DNA repair, apoptosis and cell proliferation were initially assessed. As shown in Figure 5b, a statistically significant higher expression of 53pb1 gene, a classic late marker of DNA damage response, was observed in both treated groups compared to sham. To note, in MBs from mice treated with DOX-free, 53pb1 mRNA expression was 1.26-fold higher with respect to controls ($p = 0.0480$); on the contrary, when DOX was delivered through the engineered virus, its capability to induce double strand breaks was more effective despite the five times lower concentration (2-fold increase vs. vehicle, $p = 0.0025$; 1.56-fold increase vs. DOX-free, $p = 0.0728$). In support of these data, the expression levels of the pro-apoptotic gene Bax, were also significantly up-regulated in tumors from DOX-TBSV-CooP compared to vehicle-treated mice ($p = 0.0480$; Figure 5c). Analyzing Cyclin D1, an important regulator of cell cycle progression, we did not find significant variation

in the expression levels among groups (Figure. 5d); however, a decreasing trend in both treated groups fits with the lower proliferation index of PNLs MB (Figure. 2d), suggesting that repeated administrations of the DOX-carrying nanoparticles would be able to inhibit tumor cell proliferation also during the late stage of MB development. As shown in Figure 5e, Western blot analyses of Bim, a direct activator of Bax [3] and Cyclin D1 are in agreement with the mRNA expression levels results. Bands intensities are reported in Table 2.

The interacting partner of the CooP peptide has been identified primarily to be the mammary-derived growth inhibitor (MDGI/FABP3/H-FABP) [4]. By molecular dynamic approach, we previously demonstrated that the binding mode of CooP with H-FABP was more stable when CooP was fused to the viral coat protein, suggesting the hypothesis that the internalization by MB cells could be receptor-mediated [5]. Expression analysis of H-FABP, obtained by western blot and immunofluorescence on three independent MBs, clearly showed an increase of this protein level in tumors compared to normal cerebellum (Figure 5f and g; bands intensities are reported in Table 2), highlighting that the higher induction of DNA damage and apoptosis obtained by DOX-TBSV-CooP compared to DOX-free treatment can be attributed, at least for Shh-dependent MB, to the presence of H-FABP and its internalization activity.

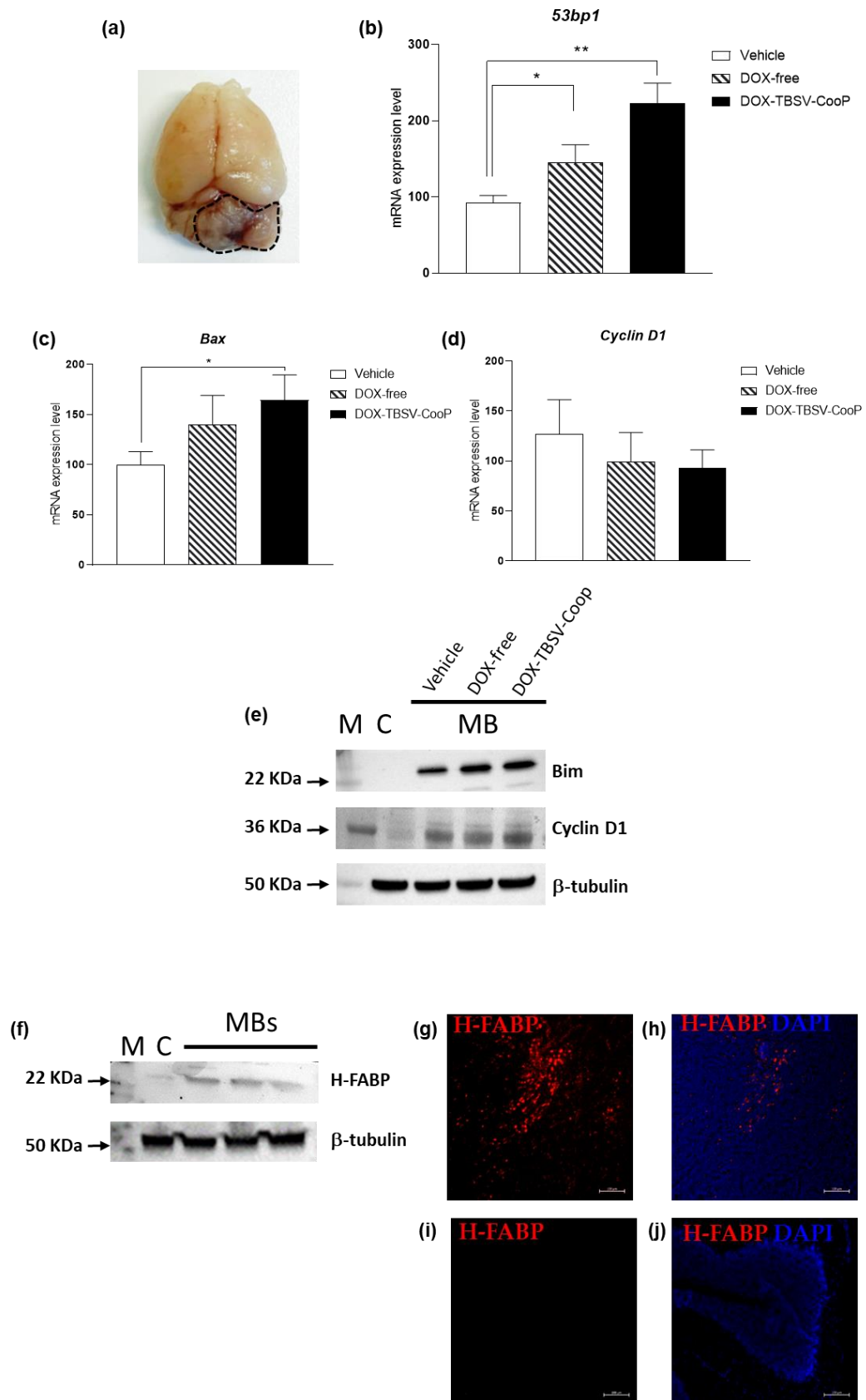


Figure 5. Effects induced by DOX-loaded TBSV-CooP nanoparticles on overt MB. (a) Representative image of an explanted brain from a symptomatic *Ptch1*^{+/-} mouse; dashed line = tumor mass. Evaluation of *53bp1* (b), *Bax* (c), and *Cyclin D1* (d) expression levels, determined by RT-qPCR analysis after a single i.v.

injection of vehicle, DOX-free and DOX-loaded TBSV-CooP in mice with clear MB symptoms. Each dataset represents the mean $\pm$ SEM of three independent biological replicates. * $p < 0.05$; ** $p < 0.01$ (Mann-Whitney test). (e) Western blot analysis of Bim and Cyclin D1 expression. (f) Western blot analysis of H-FABP expression. β -tubulin was used to normalize protein loading in (e) and (f). Cryosections of MB (g, h) and normal cerebellum (i, j) stained with anti-H-FABP antibody (red); cell nuclei stained with DAPI (blue). C = normal cerebellum. M = Precision plus protein Standards (BIO-RAD). Scale bars = 100 μ m.

Table 2. Densitometry of bands shown in Western Blots in Figure 5e and f.

	Cerebellum	Vehicle	DOX-free	DOX-TBSV-CooP
Bim	13	16432	24731	24628
Cyclin	9821	22140	22659	27553
Tubulin	27388	24394	24076	20956

	Cerebellum	MB1	MB2	MB3
H-FABP	2387	5487	5284	3284
Tubulin	18454	16976	16473	18764

Data have been obtained by iBright Imager Analysis Software, Thermo Fisher Scientific.

4.3.3 Antibody titration

The antibody response against the virion shell composed by a single type of CP has been evaluated. The dense experimental schedule of NPs administrations, concentrated in 2 weeks, did not allowed to sample the sera after each single dose, consequently the analysis has been performed by ELISA at the end of the experiment, at the day of the sacrifice, evaluating individual CP-specific IgG titers. No anti-TBSV antibodies have been detected in the sera of the vehicle-treated mice, while specific IgG were found in DOX-TBSV-CooP treated mice with an endpoint titer spanning from 1:35,841 to 1:66,805 (average 1:48,128), and no statistical differences between male and female.

4.4 DISCUSSION

In conventional cancer treatment, chemotherapy drugs are usually delivered systemically via the circulatory system but are dispersed in the whole body and often prematurely released before reaching the tumors. Consequently, the dose of the drug has to be increased considerably in order to reach the target organ in a quantity adequate to be effective, but have the disadvantage to induce side effects or cytotoxicity, causing damage to normal tissues or cells. Hence, the major goals of precision cancer medicine development are not limited to early diagnosis and phenotyping of the disease, but also to improvements in drug delivery efficacy. NPs may have great clinical potential in this field. Viral NPs, and plant virus NPs in particular, are intriguing from a nanomaterial science standpoint, given their self-assembling architecture and the easiness of production that can reach milligrams in a lab-scale frame. Their intrinsic quality-control mechanism ensures that all the particles are almost similar in size and shape, a feature, among several others, that is impossible to obtain in synthetic nanomaterials, achieving on the whole optimal cargo loading capacity, targeting efficiency, biocompatibility, ease of manufacturability, and cost-effectiveness. Finally, plant virus NPs can be custom modified by genetic engineering and are biodegradable, a clear advantage over lower modification efficiency typical of synthetic materials which rely on chemical synthesis and over their inherent persistence in the body for extended periods.

In this context, a TBSV-based drug system of targeted delivery for Shh-dependent MB treatment has been developed and characterized, moving from preliminary *in vitro* data to *in vivo* efficacy experiments. CooP-functionalized and DOX-loaded TBSV NPs have been demonstrated to be specifically uptaken by primary cultures of Shh-MB cells, allowing to reduce by 5 times the effective drug dose for inducing 90% decrease in tumor cells viability [5]. On the basis of *in silico* simulations, it was hypothesized that DOX-TBSV-CooP NPs internalization by MB cells could be mediated by MDGI/FABP3, the receptor expressed on glioblastoma cells recognized by CooP [6]. Here, with different experimental approaches, it was found that H-FABP, the best-characterized member of

the FABP family [7], is expressed also in Shh-MBs. A receptor-mediated target-specific mechanism of uptake well-explains why, although the dose of DOX delivered through TBSV NPs is significantly lower as compared to that delivered as free-drug, a similar therapeutic efficacy in counteracting MB growth is obtained.

The current chemotherapy approach adopted for MB treatment does not include DOX [8]. Nevertheless, DOX was selected as drug to be delivered through TBSV-NPs because it is fluorescent, thus allowing proper optimization of an *ad hoc* particles loading protocol, and in addition it has a poor intrinsic ability to cross the BBB and reach brain tumor cells. In agreement with the well-known capability of DOX to activate different regulatory mechanisms inducing either apoptosis or cell death [9], in both treated experimental groups at early and also late stages of MB development, we found a significant increase of apoptosis and activation of pro-apoptotic pathways. Notably, when DOX was delivered through the engineered TBSV-NPs, its capability to induce apoptosis was significantly higher than when freely administered at a 5-fold higher concentration. The higher pro-apoptotic effect induced by DOX-TBSV-CooP correlates with the significantly higher expression of the DNA damage response factor *53bp1*, found in the tumor already after a single injection of TBSV-NPs. Although a single dose of engineered TBSV-NPs was unable to significantly change *cyclin D1* expression levels, the reduced content in Ki67 resulting from the analysis of early stages of MB development suggest that TBSV-based DOX delivery platform inhibits tumor cell proliferation as effectively as a 5-fold higher dose of free-DOX. Another important advantage offered by the administration of the drug by means of proposed delivery system is the absence of systemic toxic effects. Although no cytotoxic damage to non-target organs was found in both treated groups, animals treated with multiple doses of free-DOX suffered from a significant loss of body weight. This effect may be related to the decrease in fat and skeletal muscle mass caused by DOX, due to its inhibitory effects on AMPk resulting in hyperglycemia and insulin resistance and culminating in atrophy, weight loss and anorexia [10]. Since TBSV is a “protein-based cage”, a potential disadvantage of its use as drug delivery system is that it is *per se* immunogenic. This is the reason why the antibody

response against the viral CP was evaluated. The titers of the antibodies after repeated administrations were higher in comparison to those measured after a single inoculum [5]. However, it is important to highlight that immunogenicity is a translational challenge to be addressed for many systems, including synthetic nanoparticles. In fact, the production of carrier-specific antibodies has been reported also for various inorganic and synthetic systems, as for example iron oxide NPs, liposomes, multi-walled carbon nanotubes [11][12][13], and obviously other plant viruses [14]. Moreover, the immune response is one of the natural clearance mechanisms and it does not pose per se any potential health risk or adverse effect. Generally, it is assumed that the immunogenic properties of protein-based carriers can be attenuated by stealth polymer coating, as polyethylene glycol (PEG) [15], able to decrease serum protein adsorption, thus clearance by the mononuclear phagocyte system. Nevertheless, more recently this paradigm is rapidly changing and antibody production against PEG in humans has also been reported [16][17][18]. Even if unintended immune responses may result in unfavourable accelerated clearance of delivery systems, apparently it does not affect dramatically the efficacy of the TBSV-based delivery strategy.

4.5 CONCLUSIONS

These results demonstrated the great versatility of TBSV-NPs as drug delivery system, which is grounded on a finely tunable plant based production and on different approaches for functionalization such as genetic engineering of the structural proteins and cargo loading. By confirming non-toxicity, it was verified that TBSV-NPs have great potential being able to specifically convey a chemotherapy drug to tumor cells in a murine model of MB, reducing tumor progression and allowing to decrease the drug dose. The TBSV-based delivery platform therefore encompasses all the characteristics of an elective candidate in theranostics application.

References are listed in Appendix III

Figures have been Created with BioRender.com

5. TARGETING OF TOMATO BUSHY STUNT VIRUS WITH A GENETICALLY FUSED C-END RULE PEPTIDE

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5.1 INTRODUCTION

This last chapter is dedicated to the part of the research activity carried out abroad at the Laboratory of Precision and Nanomedicine of the University of Tartu, in Estonia. Professor Tamber Teesalu and his group have been using *in vivo* phage display as a strategy for the discovery of new peptides for tumor targeting for years. This strategy allowed to identify tumor-penetrating peptides (TPPs), a class of peptides that usually contain a key sequence motif R/KXXR/K at the C-terminus, also referred to as the C-end rule (CendR), that drives the specific binding to neuropilin-1 (NRP-1), co-receptor for vascular endothelial growth factor involved in the occurrence and development of a variety of cancers. Among the various TPPs, the RPARPAR (RPAR) peptide is considered the prototype of this class of peptides and is often used as a positive control in NRP-1 receptor binding experiments. With the aim of exploiting the versatility of the TBSV viral particles with the targeting precision of the RPAR peptide, we set up proof of concept experiments to further validate the potential of the TBSV-based delivery platform with another tumor system, different from the one investigated so far. Also in this case we genetically fused the RPAR peptide on the surface of TBSV particles and after the in planta production we evaluated the interaction of TBSV-RPAR with NRP-1 following different strategies. Fundamental for this experimental part was to work with two different human cancer cell lines, the prostate cancer cell line PPC-1 (NRP-1 positive) and melanoma cell line M21 (NRP-1 negative) that we exploited *in vitro* as a "black or white" system to determine TBSV-RPAR/NRP-1 binding. Finally, in addition to genetic fusion

and loading of particles with DOX, we have adopted chemical conjugation to generate fluorescent particles with a cross-link reaction between the thiol groups exposed on the viral surface and the dye labeled-maleimide.

5.2 MATERIALS AND METHODS

5.2.1 Genetic engineering of TBSV and its production in plants

To generate TBSV-RPAR, first the DNA sequence encoding the RPARPAR peptide was inserted at the 3'-end of the p41 gene of the plasmid vector covering the full viral genome, pTBSV-WT as reported in [1]. Briefly, codon optimized in vitro annealed DNA oligos encoding the peptide with *ApaI*/*PacI* compatible ends (5'CGGAGGTAGAC-CAGCTAGGCCTGCAAGATGAGAGCTCTTAAT3', 5'TAAGAGCTCTCATCTT-GCAGGCCTAGCTGGTCTACCTCCGGGCC 3') were ligated into the properly double digested plasmid generating the pTBSV-RPAR. *XmaI* linearized pTBSV-WT and pTBSV-RPAR DNAs were in vitro transcribed using the MEGAscript T7 High Yield Transcription kit (Ambion Applied Biosystems) to generate infectious RNA molecules corresponding to viral genome. Virus RNA was used to inoculate 6-8 weeks old *Nicotiana benthamiana* plants, grown under controlled conditions (24° C, 16 h light/8 h dark) in a containment greenhouse, by abrading the adaxial side of 2 leaves/plant with carborundum (silicon carbide, VWR International). TBSV NPs were purified from infected plants according to previously developed protocol [1].

5.2.2 Chemical conjugation of CF555 dye on TBSV NPs

TBSV-WT^{CF555} and TBSV-RPAR^{CF555} NPs were generated by coupling CF555-maleimide (Sigma) to exposed cysteine residues on the virus capsid. TBSV-WT and TBSV-RPAR at a concentration of 1 mg/mL in KP buffer (0.1 M potassium phosphate buffer pH 7.0) were mixed with 2,000 molar excess of CF555-maleimide in DMSO per particle (~10 CF555 molecules/CP unit). The final DMSO concentration was adjusted to 10% (v/v). The reaction was left to proceed at RT with agitation in the dark overnight. After labeling, the TBSV NPs were purified by ultracentrifugation over a 25% (w/v) sucrose cushion at 28,000 rpm using a SW 60 Ti rotor (Beckman Coulter Inc.) for 3 h. The TBSV pellet was resuspended in 0.1 M potassium phosphate buffer pH 7. Virus concentration and number of conjugated dyes were determined through UV/Visible spectroscopy by a Nanodrop 2000c spectrophotometer (Thermo Scientific) using the Lambert-Beer law

considering the TBSV and CF555 molar extinction coefficients ($4.5 \text{ mL mg}^{-1} \text{ cm}^{-1}$ at 260 nm and $150,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 555 nm, respectively). For SDS-PAGE, denatured TBSV samples ($4 \text{ }\mu\text{g}$ per lane) were loaded on 4–20% Tris-glycine gels (Bio-Rad) and ran in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Gels were imaged using Li-Cor Odyssey M scanner. Fluorescence channel (520 nm) was used to detect CF555 TBSV NPs and colorimetric channel (630 nm) was used after staining with Coomassie Blue.

5.2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

TBSV-RPAR/NRP-1 binding was assessed by ELISA. The high-binding polystyrene multiwell plates were coated with $2 \text{ }\mu\text{g/ml}$ of recombinant NRP-1 (NRP-1-WT) receptor or with b1/b2 mutated NRP-1 (NRP-1-Mut) in $100 \text{ }\mu\text{L}$ of Phosphate Buffered Saline (PBS, pH 7.4) at 4°C overnight. Wells were washed and blocked with 5% low fat milk in PBS at 37°C for 2 h. After blocking, the wells coated with NRP-1-WT were incubated with $8 \text{ }\mu\text{g/ml}$ of in-house developed mouse monoclonal antibody [2] at 37°C for 30 min, then TBSV-WT or TBSV-RPAR samples in PBS ($100 \text{ }\mu\text{L}$ at $1.5 \text{ }\mu\text{g/ml}$) were added to each well and incubated at 37°C for 2 h. Wells were washed with 0.1 % (v/v) Tween-20 in PBS to remove free TBSV NPs. The polyclonal rabbit anti-TBSV antibody (Agdia) diluted at 1:200 in PBS containing 2% low fat milk was incubated at 37°C for 90 min. The secondary antibody (polyclonal HRP-Donkey anti-rabbit IgG, Biolegend) was used at a dilution of 1:3000 in PBS containing 2% low fat milk and incubated at 37°C for 1 h. TMB Reagent (3,3',5,5'-tetramethylbenzidine, 1-Step™ Ultra TMB-Elisa Thermo Scientific) was added to the wells, the colorimetric reaction was stopped with $100 \text{ }\mu\text{L}$ of stop solution ($2 \text{ M H}_2\text{SO}_4$) and the signal was measured at 450 nm using microplate reader (Tecan).

5.2.4 Flow cytometry

PPC-1 human prostate carcinoma cells and M21 human melanoma cells were grown in DMEM containing 4.5 g/L glucose w/o L-Gln (Lonza) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and $100 \text{ }\mu\text{g/mL}$ streptomycin (Gibco). Cells were grown in the presence of 5% CO_2 in a humidified environment at 37°C . M21 and

PPC-1 cells were seeded in a 24-well plate at 100,000 cells/well and grown overnight. TBSV-WT^{CF555} or TBSV-RPAR^{CF555} NPs (10 µg/well) were added to the cells and incubated at 37° C for 1 h, washed three times with PBS, detached with non-enzymatic cell dissociation solution (Cellstripper®, Corning) and collected in 1.5 ml tube. Cells were subsequently washed, resuspended in PBS, and analyzed using a BD Accuri™ C6 plus flow cytometer. Triplicates of each sample were examined, and 10,000 events (gated for live cells) were recorded. Data were analyzed using the BD Accuri C6 software. For the NRP-1 blocking, incubation with TBSV NPs (5 µg/well) was preceded by incubation with in-house generated mouse anti-human mAb (at 10 µg/ml and RT, 30 min).

5.2.5 Confocal imaging of cultured cells

For confocal microscope imaging, 100,000 cells/well in 500 µl of DMEM were seeded on glass cover slips in 24-well plates. TBSV-WT^{CF555} and TBSV-RPAR^{CF555} NPs (10 µg/well) were added and the mixtures were incubated at 37° C for 1 h. Cells were washed 3 times with PBS to remove unbound NPs and then fixed with 4% paraformaldehyde in PBS at RT for 10 min. Cell nuclei were stained with DAPI (1µg/ml) at RT for 8 min, followed by mounting the coverslips in aqueous mounting medium Fluoromount-G (Electron Microscopy Sciences). For PPC-1-GFP/M21 co-culture experiment, the mixed suspension of cells (100,000/well) were seeded on a glass coverslip as mentioned above and exposed to TBSV-RPAR^{CF555} NPs (10 µg/well) at 37° C for 1 h. After fixation with 4% paraformaldehyde in PBS at RT for 10 min, the cells were incubated with blocking buffer (5% w/v BSA, 5% v/v FBS and 5% v/v goat serum in PBS with 0.05% Tween-20) at RT for 30 min, followed by incubation with primary in-house generated rabbit anti-human p32 antibody (diluted 1:200 in 0.2X blocking buffer) at RT for 30 min, then washed and incubated with goat anti-rabbit Alexa 647-conjugated antibody (Invitrogen) diluted 1:200 in 0.2X blocking buffer at RT for 30 min. After cell nuclei staining the coverslips were mounted on glass slides using Fluoromount-G. For the pulse-chase co-localization experiment, cells were incubated with TBSV-RPAR NPs (10 µg/well) at 37 °C for 30 min, washed 3

times with fresh medium to remove unbound NPs, and chased for 30, 90, and 270 min. After chase, cells were fixed in 4% paraformaldehyde in PBS pH 7.2 at RT for 10 min, then permeabilized with 0.2% Triton-X 100 in PBS at RT for 10 min, washed 3 times in PBS and incubated with blocking buffer at RT for 30 min. TBSV NPs were stained using rabbit anti-TBSV antibody (Agdia) diluted 1:200 in 0.2X blocking buffer at RT for 30 min followed by a goat anti-rabbit Alexa Fluor 546-conjugated secondary antibody (Invitrogen) diluted 1:200 in 0.2X blocking buffer at RT for 30 min. Endolysosomes were identified using mouse anti-human CD107a (LAMP-1) antibody (Biolengend) diluted 1:200 in 0.2X blocking buffer at RT for 30 min followed by Alexa Fluor 647-conjugated goat anti-mouse antibody (Invitrogen) diluted 1:200 in 0.2X blocking buffer at RT for 30 min. Finally, cell nuclei were stained using DAPI (1 μ g/ml) at RT for 8 min and coverslips were mounted on glass slides using Fluoromount-G. Olympus FV1200MPE confocal microscope (Olympus Europa SE & Co. KG) was used for imaging. Acquired images were analyzed using FluoView FV10-ASW 4.0 software (Olympus Europa SE & Co. KG). For co-localization analysis, Pearson's R correlation coefficient using Fiji ImageJ Coloc 2 plugin (<https://imagej.net/plugins/coloc-2>, accessed on March 2022) was used. For each time point, ten different images were analyzed.

5.2.6 Doxorubicin loading of TBSV NPs

Approximately 1mg/ml of purified TBSV NPs were incubated in swelling buffer (0.1 M Tris, 50 mM EDTA, pH 8.0) at RT in agitation for 1 hour. A 5000 molar excess of DOX (0.324 μ g DOX/ μ g virus) was added, followed by incubation on a bench-top tube rotator at 4° C overnight. To re-seal the virus cages, the samples were mixed in association buffer (0.2 M Na Acetate, 25 mM CaCl₂, 25 mM MgCl₂, pH 5.2) at RT for 1 h. DOX excess was removed by ultracentrifugation on 25% sucrose cushion using a SW 60 Ti swing-out rotor (Beckman Coulter Inc.) at 28,000 rpm at 4° C for 3 h. Pellets were resuspended in association buffer overnight and stored at 4° C until needed. TBSV^{DOX} NPs were analysed by UV/VIS using Nanodrop 2000c (Thermo scientific) at 488 nm to quantitate the

DOX loading. A calibration curve was generated and the Lambert-Beer law used to determine the concentration of the TBSV-loaded DOX. The drug encapsulation efficiency (EE) and loading capacity (LC) of DOX in NPs were also determined according to the formula:

$EE(\%) = \text{Amount of DOX in the NPs} / \text{total amount of DOX added} (\times 100)$

$LC(\%) = \text{Amount of DOX in the NPs} / \text{NPs weight} (\times 100)$

5.2.7 Cell viability assay

PPC-1 and M21 cells were plated in a white opaque 96-well plate (5,000 cells/well) and incubated at 37° C in 5% CO₂ atmosphere for 24 h. TBSV-RPAR^{DOX}, TBSV-WT^{DOX} and free DOX were then added to the wells at a final concentration of 25, 5, 1, 0.2 or 0.04 μmol/ml of drug. Empty TBSV NPs were also used at the same concentration than for DOX-loaded NPs. After 30 min incubation at 37° C, the medium was carefully removed and the wells were washed twice with fresh medium and let them grow at 37° C in 5% CO₂ atmosphere. Cell viability was measured after a period of 48 h using the CellTiter-Glo® reagent (Promega) following the manufacturer protocol. The EnSight® multimode plate reader was used to measure the chemoluminescence of the samples. Data were presented as mean ± SD of biological replicates (N=6).

5.2.8 *In vivo* biodistribution analysis of TBSV

The animal experiments were done according to protocols approved by Estonian Ministry of Agriculture, Committee of Animal Experimentation (project #159). TBSV-RPAR or TBSV-WT (200 μg in PBS) were injected into the tail vein of 8-10 weeks-old female Balb/c mice. Twenty-four h post i.v. administration the mice were perfused via the left ventricle using 20 mL of PBS. Organs were collected and fixed in cold 4% w/v paraformaldehyde in PBS at 4° C for 24 h, washed in PBS at room temperature for 1 h and cryoprotected in 15% w/v and 30% w/v sucrose (Sigma Life Science) at 4° C overnight. Cryoprotected and fixed tissues were frozen in optimal cutting temperature compound (OCT compound; Leica), cryosectioned at 10 μm on Superfrost+ slides (Thermo Fisher

Scientific) and stored at -20° C. Air-dried tissue sections were rehydrated in PBS for 10 min followed by permeabilization in PBS + 0.2% v/v Triton (Triton X-100, AppliChem) at RT for 10 min. Tissue slides were washed with PBST [PBS + 0.05% v/v Tween-20 (Sigma-Aldrich)] and blocked with 1X blocking buffer (5% w/v BSA, 5% v/v FBS and 5% v/v goat serum in PBS with 0.05% Tween-20) at RT for 1 h. Primary antibodies diluted in 0.2X blocking buffer were added and incubated at 4° C overnight. Incubation with secondary antibodies was performed at RT for 30 min, followed by washing and nuclear counterstaining with DAPI (1µg/mL in PBS) at RT for 8 min. TBSV NPs were detected using rabbit anti-TBSV (dilution 1:200) and Alexa Fluor 647 goat anti-rabbit antibody (dilution 1:500, Invitrogen by Thermo Fisher Scientific). The coverslips were mounted in Fluoromount-G™ and imaged using Olympus FV1200MPE confocal microscope (Olympus Europa SE & Co. KG).

5.2.9 Statistical Analysis

All quantitative data were presented as mean ± SD and statistical significance (p) was calculated by a two-tailed Student's t-test. All analyses were carried out using GraphPad Software.

5.3 RESULTS

5.3.1 Construction, Production and Purification of wild type (WT) and Chimeric TBSV NPs

TBSV was genetically engineered to display the RPAR peptide as a fusion to the C-terminus of the viral CP. To avoid potential interference with CP folding and virus assembly, a GGPGG linker was inserted between the CP and the peptide (Figure 1a). The linker may also provide flexibility to facilitate engagement of the peptide with the cell surface receptors [1][3]. Mechanical inoculation of *N. benthamiana* plants with the in vitro transcribed viral RNA resulted in chlorotic vein clearing, a typical symptom of infection (Figure 1b). The RNA extracted from infected plants was retro-transcribed, and sequencing of cDNA PCR fragments confirmed the presence of RPAR peptide and linker- in frame with the genomic RNA encoded CP. A second round of infection with tissue extracts from infected plants was carried out to confirm the genetic stability of the engineered virus. The yield of purified TBSV-RPAR and wild type TBSV (TBSV-WT) NPs was ~1 mg/g wet weight. SDS-PAGE and Coomassie Blue staining were used to confirm the presence of the viral CP and verify the purity of preparations (Figure 1c). These results show that the exogenous CP-fused RPAR peptide does not compromise the fitness and in planta propagation of the TBSV.

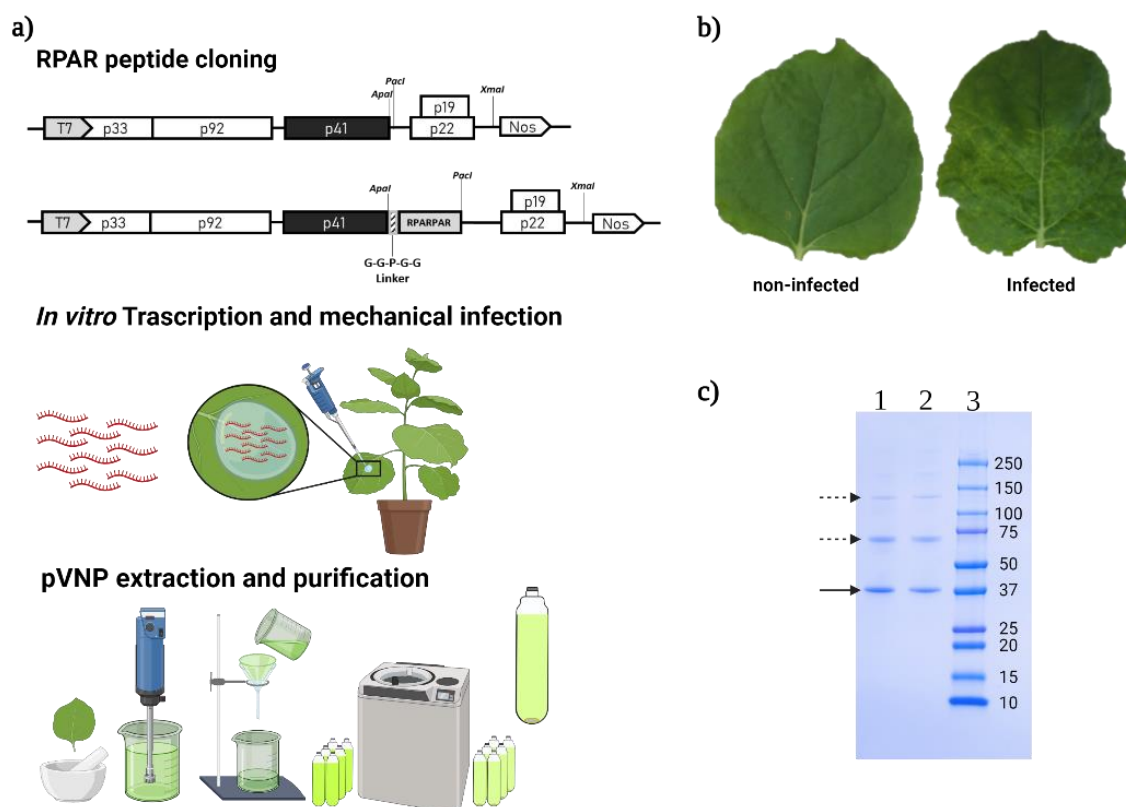


Figure 1. Preparation of TBSV-RPAR expression construct and recombinant virus production. (a) Experimental flow-chart: map of the TBSV-WT/RPAR plasmids, RNA inoculum in planta, and pVNPs purification procedure. The five ORFs [p33 and p92 (RNA-dependent RNA polymerase), p41 (coat protein, CP), p22 (movement protein), p19 (silencing inhibitor)] are shown. T7, promoter sequence from T7 phage; Nos, terminator sequence of the *Agrobacterium tumefaciens* nopaline synthase encoding gene; ApaI and PacI, restriction sites used for RPAR CP genetic fusion; XmaI, restriction site used for vector linearization; striped box, GGPGG linker. (b) *N. benthamiana* leaves from non-infected plants (left) and after infection with TBSV-RPAR (right). (c) SDS-PAGE of TBSV-RPAR NPs. Purified TBSV samples (4 µg per lane) were denatured and electrophoretically separated on 4–20% Tris-glycine gel (Bio-Rad), stained with Coomassie Blue. TBSV-WT (1), TBSV-RPAR (2), Precision Plus Protein Dual Color Standards (Bio-Rad) (3). The molecular masses of the marker bands are indicated. Solid arrow indicates monomeric coat protein bands, dashed arrows indicate CP dimers and aggregates.

5.3.2 Fluorophore labeling of TBSV NPs

To design a strategy for covalent coupling of fluorophores to the TBSV capsid the crystal structure of TBSV was examined. The lysine residues, suitable for N-hydroxysuccinimide (NHS)-ester-based bioconjugation, are on the non-accessible inner surface of the capsid and unavailable for conjugation [4][5]. In contrast, 5 cysteine residues, C168, C223, C320, C376, and C353, (Figure 2a) are present on the surface of the TBSV capsid. Whereas cysteines C356 and C376 form a disulfide bridge, C168, C320 and C223 have

free sulfhydryl groups available for bioconjugation. Therefore, fluorescent dye functionalization was addressed by thiol-maleimide bioconjugation using CF555-maleimide, TBSV NPs were incubated in the presence of ~3x molar excess of the dye relative to available free sulfhydryl groups, followed by ultracentrifugation to remove the free CF555 and quantitation of the degree of labeling by UV-visible spectroscopy (Figure 2b). Highly efficient labeling was observed for both TBSV-WT^{CF555} (504 ± 24 dye molecules/particle) and TBSV-RPAR^{CF555} NPs (540 ± 10 dye molecules/particle). SDS-PAGE followed by fluorescence gel imaging and subsequent Coomassie blue staining confirmed the covalent binding of the CF555 fluorophore to the NPs (Figure 2c).

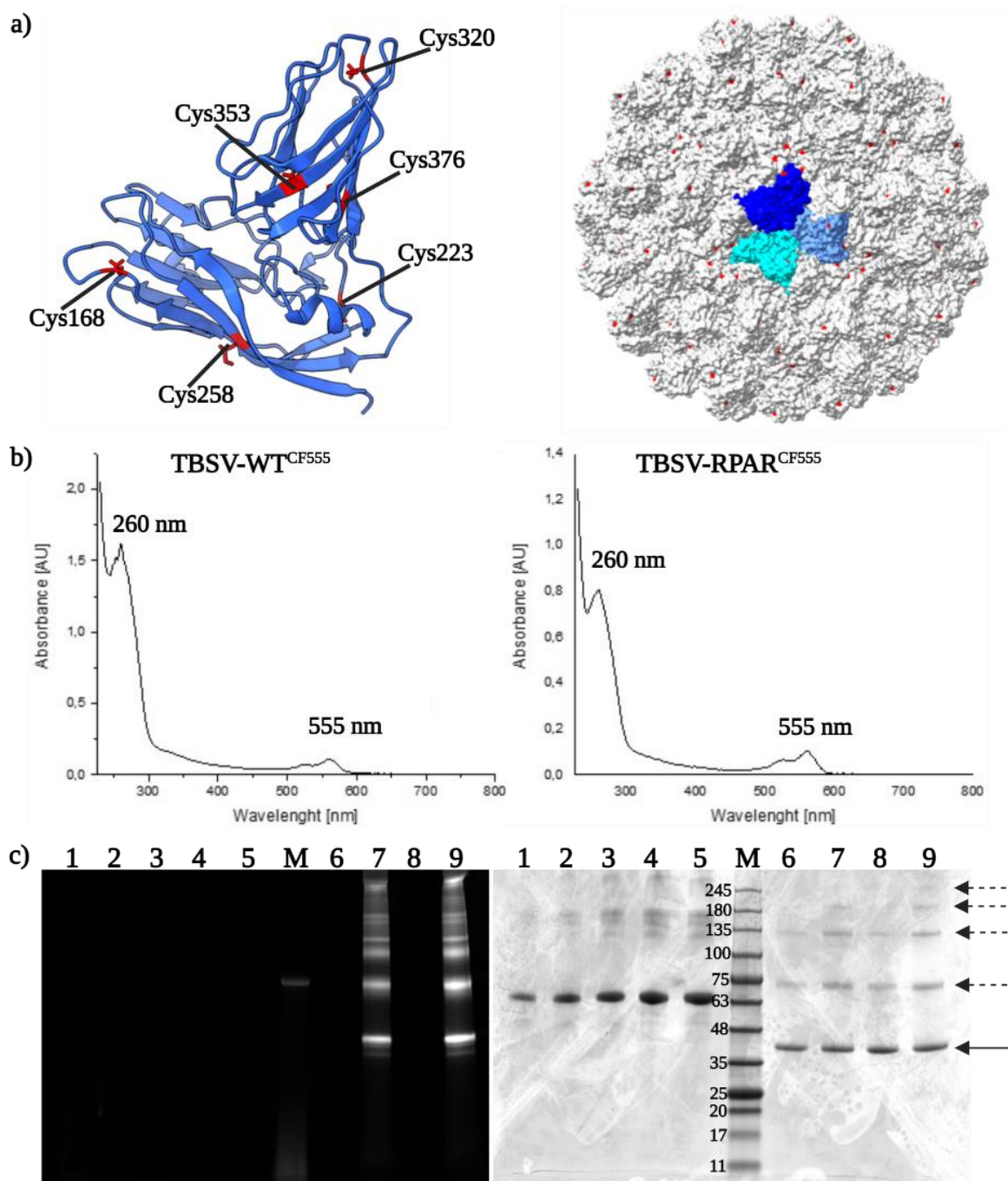


Figure 2. CF555 labeling of the TBSV NPs. (a) Structure of the CP and the TBSV virions. Left: ribbon model of the TBSV CP subunit (GeneBank U80935.1) with cysteine residues indicated. Surface-exposed cysteine residues with free sulfhydryl groups (C168, C320 and C223) are highlighted in red. Right: crystal structure of the TBSV particle. The 180 CP units are arranged in a T = 3 symmetry with the CP units in a representative trimer highlighted in shades of blue. The structural information was retrieved from <https://www.rcsb.org/>, Protein Data Bank file PDB:2TBV. The images were created using UCSF ChimeraX software. (b) UV-visible spectra of TBSV-WT^{CF555} and TBSV-RPAR^{CF555} after conjugation with CF555-maleimide. (c) SDS-PAGE of TBSV NPs following CF555 labeling. Gel was imaged under fluorescence channel (left) and, after Coomassie blue staining, under white light (right). M: Opti-Protein XL molecular weight marker; lanes 1-5: 1, 2, 3, 4 and 5 μg/lane BSA; lanes 6 and 7: unlabeled and CF555-labeled TBSV-WT; lanes 8 and 9: unlabeled and CF555-labeled TBSV-RPAR. Solid arrow indicates monomeric coat protein bands, dashed arrows indicate CP dimers and aggregates.

5.3.3 *In vitro* binding of TBSV-RPAR to NRP-1

We next tested the ability of RPAR-functionalized TBSV NPs to interact with NRP-1 under cell free conditions and with NRP-1 expressed on the cell surface. Recombinant b1b2 domain of human NRP-1 (wt-NRP-1), or triple mutant b1b2 with nonfunctional CendR binding pocket (mut-NRP-1), were coated on multiwell plates and binding of TBSV-RPAR and TBSV-WT was assessed by sandwich ELISA. RPAR functionalization of TBSV NPs increased wt-NRP-1 binding ~11 fold. In contrast, in the wells coated with the mut-NRP-1, only baseline TBSV-RPAR binding was observed. Furthermore, pre-incubation of wells with a monoclonal anti-NRP-1 antibody, that interferes with the binding of the CendR ligands to NRP-1 [2], resulted in ~5.5 fold decrease of binding of TBSV-RPAR (Figure 3a). These cell-free binding studies confirmed the CendR-dependent NRP-1 binding of TBSV NPs. Next, we studied the binding of WT and RPAR-coated CF555-labeled TBSV NPs to cultured NRP-1 positive PPC-1 prostate cancer cells and NRP-1-negative M21 melanoma cells. Flow cytometry demonstrated increased RPAR-dependent binding of TBSV NPs to PPC1 cells and a very low signal for both peptide-guided and control NPs in the NRP-1-negative M21 cells (Figure 3b). Preincubation of PPC1 cells with the CendR-blocking antibody decreased binding of TBSV-RPAR ~4-fold (Figure 3c).

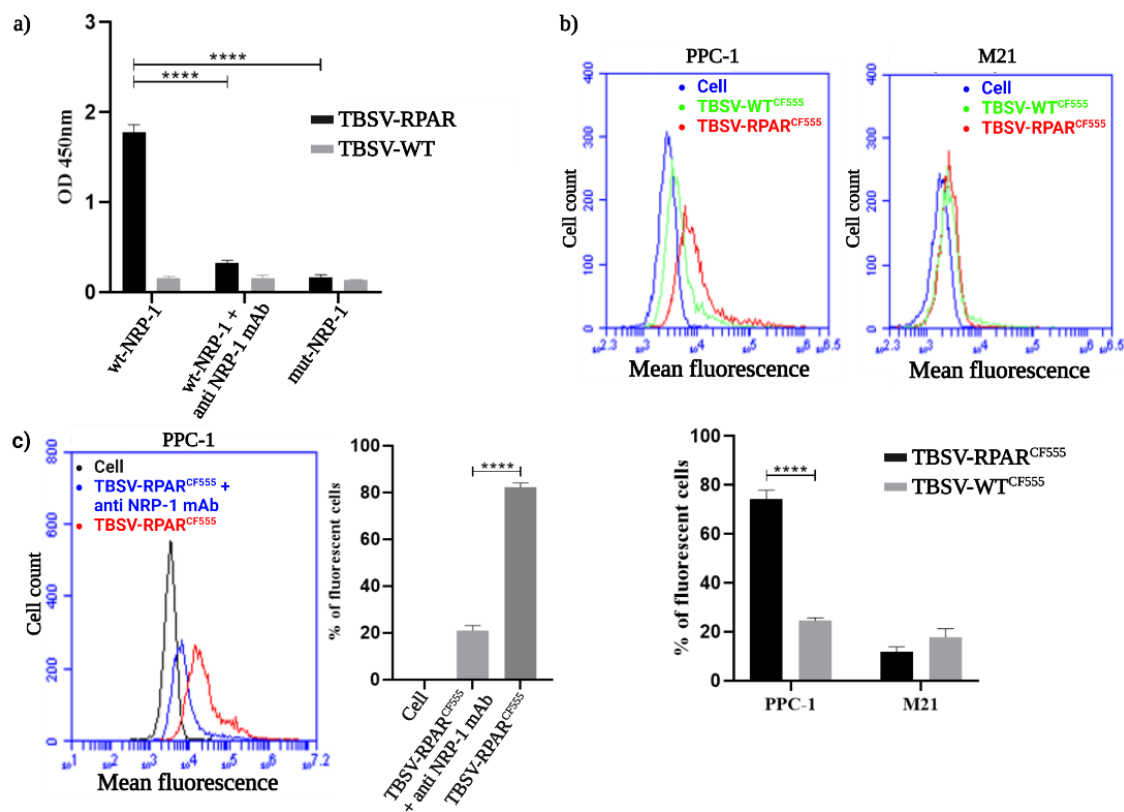


Figure 3. TBSV-RPAR NPs interact with recombinant NRP-1 and with cell surface NRP-1. (a) Binding of TBSV-RPAR and TBSV-WT to wt-NRP-1 or mut-NRP-1. For TBSV detection using sandwich ELISA, primary rabbit anti-TBSV antibody followed by HRP-conjugated anti-rabbit antibody were used, followed by chromogenic reaction and quantitation of signal at 450 nm. CendR-blocking anti NRP-1 antibody reduced TBSV-RPAR binding. **** $p < 0.0001$ Student's t-test. (b) Flow cytometry of CF555-labeled TBSV-WT and TBSV-RPAR incubated with NRP-1-expressing PPC-1 and NRP-1 negative M21 cells. Cell count vs. mean fluorescence and percentage of fluorescent cells are shown. **** $p < 0.0001$. (c) Effect of anti NRP-1 antibody on binding of CF555-labeled TBSV-RPAR to PPC-1 cells. Cell count vs. mean fluorescence and percentage of fluorescent cells after incubation with TBSV-RPAR^{CF555} are shown. **** $p < 0.0001$ Student's t-test.

Binding and internalization of TBSV-WT^{CF555} and TBSV-RPAR^{CF555} in PPC-1 and M21 cells was further investigated by confocal microscope imaging. PPC-1 cells showed uptake of TBSV-RPAR^{CF555} NPs and no binding of TBSV-WT^{CF555} (Figure 4 a and b). In M21 cells, no labeling was detected following incubation with both TBSV-WT^{CF555} and TBSV-RPAR^{CF555} (Figure 4 c and d). After addition of TBSV-RPAR^{CF555} to the mixed culture of Green Fluorescent Protein (GFP)-expressing PPC-1 cells (PPC-1-GFP) and “dark” M21 cells, the binding of NPs was seen only in NRP-1 positive PPC-1 cells (Figure 4e).

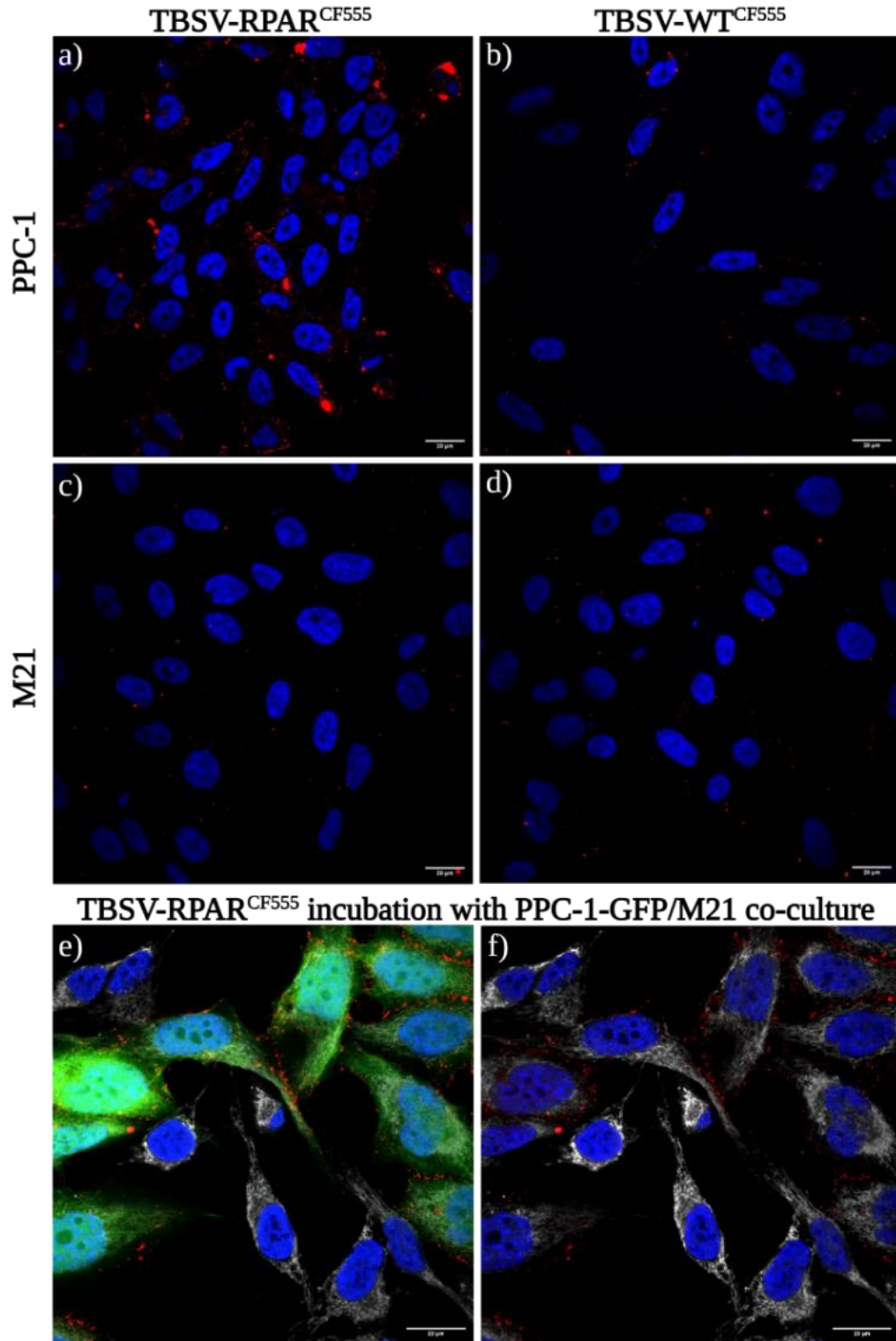


Figure 4. TBSV-RPAR NPs target NRP-1-expressing cells. Confocal microscopy images of PPC-1 or M21 cells incubated at 37°C for 1h with TBSV-RPAR^{CF555} (panels a and c) or TBSV-WT^{CF555} (panels b and d). Red = TBSV NP NPs; blue = DAPI (nuclei). (e, f) TBSV-RPAR^{CF555} binding to co-culture of PPC-1-GFP and M21 cells. TBSV-RPAR^{CF555} are taken up only by GFP-positive PPC-1 cells. Red = TBSV-NP NPs; blue = DAPI (nuclei); green = GFP expressed in PPC-1 cells; white = p32. Panel f is not showing the GFP fluorescence channel. Scale bars = 20 μm.

In PPC-1 cells, pulse-chase co-localization studies of TBSV-RPAR with the lysosomal marker LAMP-1 showed the time-dependent entry of the peptide-guided NPs in the endolysosomal pathway (Figure 5).

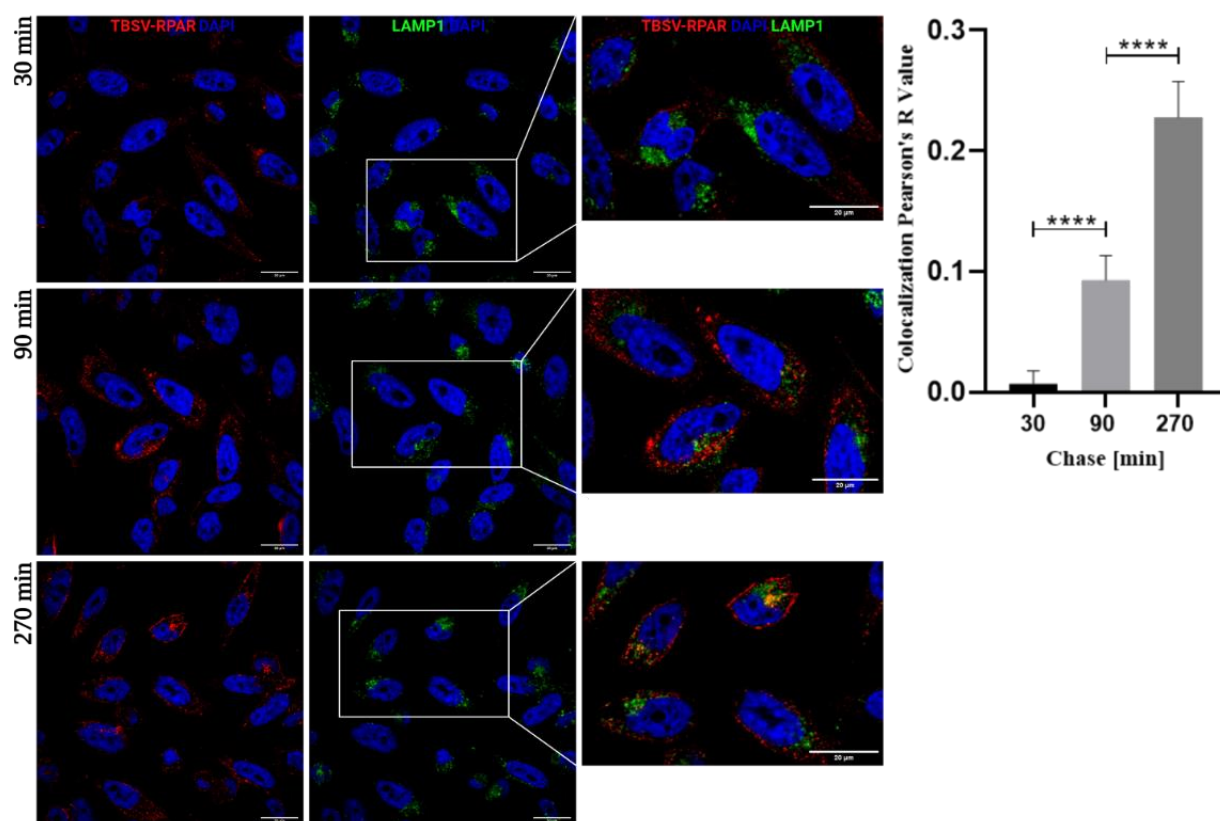


Figure 5. Internalized TBSV-RPAR NPs are routed to LAMP-1-positive lysosomal compartment. PPC-1 cells were incubated with TBSV-RPAR for 30 min, washed to remove unbound NPs, and imaged at 30, 90, and 270 min time points. The cells were stained with lysosomal marker LAMP-1 (green), and anti-TBSV antibody (red). Blue: DAPI (nuclei). Scale bars = 20 μ m. The relative co-localization was quantified using ImageJ software with the Pearson's R value shown in the bar chart. **** $p < 0.0001$ (Student's t-test).

5.3.4 TBSV-RPAR Loading with DOX and cytotoxicity test *in vitro*

Having established that the TBSV-RPAR NPs engage with NRP-1 under cell-free conditions and are internalized in NRP-1-expressing cells, we next studied whether this selectivity translates into increased cytotoxicity for TBSV NPs loaded with a cytotoxic anticancer drug. Doxorubicin (DOX)-loaded TBSV-NPs were prepared using a previously developed protocol [3]. DOX was added at 5,000 molar excess to the TBSV-NPs in an alkaline swelling buffer containing EDTA to loosen viral capsid to enable entry of the

drug. To entrap the drug, the compact state of the viral capsid was re-established by restoring the pH and adjusting the Ca^{2+} and Mg^{2+} concentration. Free DOX was removed from the TBSV samples by a sucrose cushion ultracentrifugation. Similar degree of DOX loading was achieved for both types of NPs: 48 ± 12 ng DOX/ μg TBSV-WT and 58 ± 6 ng DOX/ μg TBSV-RPAR, corresponding to 729 ± 181 and 892 ± 92 DOX molecules/virion, respectively. The encapsulation efficacy (EE) was 29 and 34%, while the loading capacity (LC) was 4.8 and 5.8, respectively. For cellular viability assessment, PPC-1 and M21 cells were incubated with DOX-loaded TBSV NPs and free DOX and after 48 h incubation the cells were subjected to MTT assay (Figure 6). In PPC1 cells, TBSV-RPAR^{DOX} and free DOX were significantly more toxic than TBSV-WT^{DOX}. In contrast, in M21 cells the effect of TBSV-WT^{DOX} and TBSV-RPAR^{DOX} on cell viability was similar and significantly lower than the effect of free DOX. These data show that functionalization of the cytotoxic compound-carrying TBSV with the CendR peptide increases the toxic effect the the particles on NRP-1 positive cells.

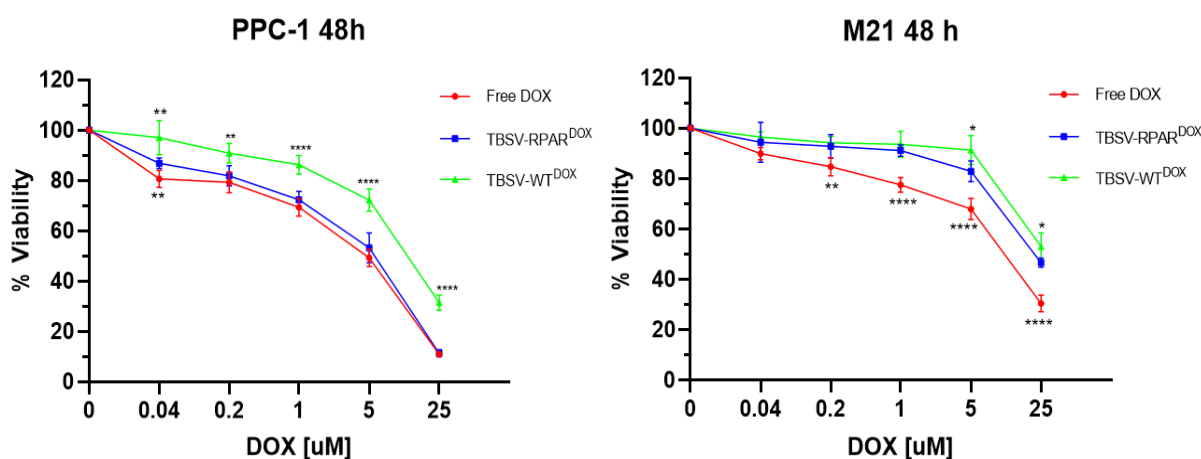


Figure 6. Cytotoxic effect of drug-loaded TBSV-NPs. Viability of PPC-1 and M21 cells after 48 h of incubation with different concentration of free DOX or DOX-loaded TBSV-NPs was estimated by MTT assay. Data are presented as mean $\pm$ SD of biological replicates, N=6. * $p = 0.018$; ** $p = 0.0051$; **** $p < 0.0001$. Student's t-test (TBSV-RPAR^{DOX} compared with TBSV-WT^{DOX} and TBSV-RPAR^{DOX} compared with free DOX).

5.3.5 Systemic TBSV-RPAR NPs home to pulmonary tissue

NRP-1 is widely expressed in normal tissues and organs, including on the luminal side of the vascular endothelium. Published studies have shown that after systemic injection, NPs guided with CendR peptides show a robust homing to the first-met microvasculature in the pulmonary and cardiac tissues [6][7]. To study whether CendR peptides have similar activity when present on TBSV NPs, the biodistribution of TBSV-WT and TBSV-RPAR in the Balb/c mice following intravenous (iv) administration and 24 h circulation was studied. Confocal imaging of TBSV immunoreactivity showed a robust signal in pulmonary tissue of TBSV-RPAR-injected mice, and a lower amount of NPs in the lungs of TBSV-WT-injected mice (Fig. 7). These data show that RPAR targeted TBSV NPs show a lung tropism similar to that of other classes of NPs tested in the past.

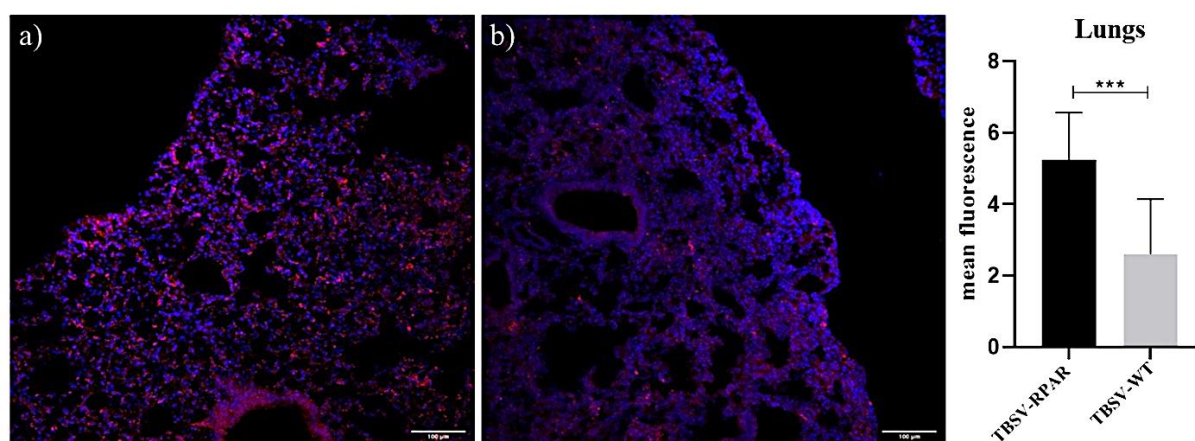


Figure 7. TBSV-RPAR accumulation in the pulmonary tissue following i.v. administration. Immuno-fluorescent localization of TBSV in lung sections of mice i.v. injected with (a) TBSV-RPAR and (b) TBSV-WT. Scale bars = 100 μ m. TBSV fluorescence signal (red) was quantified using ImageJ software and expressed as mean of fluorescence. Five randomly chosen fields from 3 different animals per group were analyzed. Data represent mean $\pm$ SD, *** $p < 0.001$ (Student's t-test).

5.4 DISCUSSION

Here, we report proof-of-concept studies on targeting of TBSV NPs with prototypic CendR peptide, RPAR. We show that the display of RPAR as fusion to the viral CP increases binding of TBSV NPs to recombinant NRP-1, results in their uptake in cultured NRP-1 positive cells, and, following systemic administration, promotes their accumulation in the lung microvasculature. We also show cargo loading of the TBSV NPs using two strategies: chemical conjugation to the exposed free cysteine residues on the surface of the viral capsid via sulfhydryl-maleimide coupling, and a diffusion-based strategy for drug loading into the internal cavity of the viral NPs. Collectively, our studies show the feasibility of the TBSV-derived precision guided nanocarriers for delivery of imaging agents and therapeutic compounds. In recent years, TBSV has emerged as a versatile drug delivery platform. The TBSV capsid is stable and remains intact after incubation with human serum at 37° C [8]. Uniform batches of TBSV NPs can be purified from infected plants using a well-established protocol. Upon systemic administration, TBSV is present in the bloodstream for a longer time (still detectable at 7 days) as compared to PVX (disappeared after 48 h) [9]. TBSV NPs can be functionalized through chemical modifications, osmotic loading techniques, and genetic engineering [1]. TBSV has been employed as a drug delivery platform to medulloblastoma [3]. In this preclinical study, doxorubicin-loaded TBSV NPs genetically modified to display tumor homing peptides on their external surface were shown to exert antitumor activity *in vitro* and accumulate in the malignant lesions. A potential disadvantage of all plant viruses is that they are generally immunogenic, a challenge that can be addressed by coating the carriers with shielding compounds such as polyethylene glycol (PEG) [10][11]. We chose to use homing peptides as TBSV-guiding modules due to their emerging translational relevance. Homing peptides are small (<9 amino acids), show low immunogenicity, are not species specific, and have affinity in the range avoiding “binding site barrier” known to limit extravasation and tissue penetration of high affinity ligands such as antibodies [12]. In particular, CendR targeting module used in the current study for TBSV targeting is pre-

sent in all tumor penetrating peptides (TPPs), an emerging class of tumor homing peptides with ability to extravasate and penetrate throughout tumor parenchyma. Whereas the CendR peptides have been demonstrated to enable targeting of biological phage NPs and different synthetic nanoscale scaffolds [13][14][15][16], the structural and physico-chemical properties of NPs can have a dramatic effect on targetability with affinity ligands. Here, we show that RPAR peptide remains active when displayed on the TBSV surface as a C-terminal fusion to the CP. TBSV-RPAR binds to recombinant NRP-1 under cell free conditions, is internalized in cultured NRP-1 positive cells, and homes to lung microvessels following i.v. administration. The implication of these observations is the applicability of the entire TPP family for precision targeting of TBSV NPs, a possibility that remains to be rigorously tested in follow-up studies. The homing peptides were displayed on the TBSV NPs at 180 copies/32 nm particle (~3.4-fold higher density than in the case of 60 nm T7 bacteriophage used for homing peptide discovery at ~200 peptides/60 nm particle). Targeting ligand density is a critical factor affecting the NP recruitment to the target cells and a key determinant of the choice of cellular internalization pathway [17]. Somewhat counterintuitively, higher ligand density does not always result in a more effective targeting [18]. The effect of the relationship of the density of the targeting peptides on the TBSV surface and its targeting selectivity remains to be tested in the follow-up studies. A majority of nanocarriers are taken up by cells via endocytosis and are primarily routed towards lysosomes [19]. In our study, TBSV-RPAR NPs were also internalized via the endolysosomal uptake pathway. Lysosomal trafficking has been reported for other plant viruses such as the CPMV [20], Physalis Mottle Virus (PhMV) [21] and Sesbania Mosaic Virus (SeMV) [22], which, even if belonging to different taxonomic families, have similar shape and size as TBSV. Whereas the lysosomal delivery of nanocarriers may be useful in the case of certain classes of therapeutics (i.e., drugs for treatment of lysosomal storage disorders), the harsh milieu of the lysosomal compartment due to low pH and an array of hydrolases capable of breaking down all types of biological polymers (proteins, nucleic acids, carbohydrates, and lipids) represents a barrier to drug delivery. Therefore, strategies aimed at facilitating the endosomal

escape of the NPs [23], or their routing to different compartments, are favored. Recently, we showed that NRP-1 interacts with endosomal SNX-BAR sorting complex promoting exit 1 (ESCPE-1) and that this interaction mediates retrograde trafficking of NPs functionalized with a CendR peptide [24]. Optimization of the TBSV-CendR NPs for preferential use of retrograde trafficking pathway (i.e., by modulating the density of surface peptides) and/or employing endosomal escape promoting strategies (such as adding poly-histidines as endosolytic buffering agents) may prove useful in preventing lysosomal routing.

5.5 CONCLUSIONS

Our study demonstrates that CendR peptide-displaying TBSV NPs can specifically interact with NRP-1 receptor and actuate cellular binding and internalization in cultured cells, and lung homing and penetration *in vivo*. CendR-mediated targeting opens applications for the TBSV-based diagnostic and therapeutic platforms for pulmonary targeting and solid tumor delivery.

References are listed in Appendix IV

Figures have been Created with BioRender.com

6. GENERAL CONCLUSIONS AND PERSPECTIVES

In this PhD project a number of strategies to engineer and characterize TBSV NPs for targeted drug delivery were successfully implemented for the treatment of Shh-MB, a difficult-to-treat pediatric brain tumor. Specifically, we genetically engineered the viral surface of TBSV with various tumor-targeting peptides and produced the resulting cTBSV NPs on a large scale using *N. benthamiana* plants. We have also carefully characterized the stability of these particles using several techniques, including SDS gels, transmission electron microscopy, zeta potential analysis, and dynamic light scattering. Importantly, we assessed that genetic modifications do not compromise the integrity or behavior of viral particles, thus demonstrating the suitability of these procedures for generating cTBSV NPs with tumor targeting properties. We have also developed an innovative drug loading protocol that allows for the efficient and controlled internalization of DOX molecules into the inner cavity of the viral capsid. This approach was very effective in all the different TBSV viral particles tested, demonstrating the reproducibility and efficiency of the methodology in generating the drug delivery system. *In silico* and *in vitro* studies confirmed the efficacy of cTBSV viral particles in targeting MB cells and their precursors, with stable interactions established between the peptides on the viral surface and the overexpressed receptors on tumor cells. Furthermore, by delivering the drug through the viral particles, we also observed a significant reduction of DOX dose necessary to induce MB cells death in comparison to the dose necessary to obtain the same result with free DOX. Moving onto *in vivo* studies, we tested the most promising viral chimeras in mice with clear tumor symptoms, observing that among these, TBSV-CooP possesses a greater and specific ability to target the MB tumor. These results provide further validation of the efficacy of using tumor targeting peptides to guide TBSV particles to the tumor site. Finally, we conducted a preclinical study to evaluate the therapeutic efficacy of doxorubicin-loaded TBSV-CooP particles. Importantly, we

observed that a single dose of DOX-TBSV-CooP can activate the mechanisms of reduction of cells proliferation and apoptosis, underlying an antitumor effect, whereas multiple doses can delay the progression of MB PNLs, and does not exert any systemic toxic side effect. These findings provide a solid basis for future studies and highlight how the TBSV plant virus-based targeting and drug delivery platform may be a promising therapeutic approach for the treatment of Shh-MB.

We also validated the targeting and delivery platform based on a new genetic engineered cTBSV on tumor cells different from MB. In this light a chemical conjugation protocol to produce fluorescent TBSV particles was developed and used to investigate the mechanisms of cellular internalization. The presence of exposed thiol groups on the viral surface allowed the maleimide-based cross-linking reaction to be exploited efficiently; while the cellular internalization process, as already demonstrated for other types of plant viruses, seems to follow the endolysosomal pathway.

Collectively, this project underlines the importance of a multidisciplinary approach, bringing together different scientific fields to address complex medical challenges and provides valuable insights into the potential use of the TBSV virus to develop an efficient drug delivery and targeting platform, not only for the treatment of pediatric brain tumors. In fact, in addition to the ease and speed of production, TBSV offers a large margin of manipulation that allows modifications to be made through different strategies, paving the way for other possible applications.

Appendix I

References of Chapter 1

1. Thorbinson, C.; Kilday, J.P. Childhood Malignant Brain Tumors: Balancing the Bench and Bedside. *Cancers (Basel)*. **2021**, *13*, doi:10.3390/CANCERS13236099.
2. Johnson, K.J.; Cullen, J.; Barnholtz-Sloan, J.S.; Ostrom, Q.T.; Langer, C.E.; Turner, M.C.; McKean-Cowdin, R.; Fisher, J.L.; Lupo, P.J.; Partap, S.; et al. Childhood Brain Tumor Epidemiology: A Brain Tumor Epidemiology Consortium Review. *Cancer Epidemiol. Biomarkers Prev.* **2014**, *23*, 2716, doi:10.1158/1055-9965.EPI-14-0207.
3. Ostrom, Q.T.; Fahmideh, M.A.; Cote, D.J.; Muskens, I.S.; Schraw, J.M.; Scheurer, M.E.; Bondy, M.L. Risk Factors for Childhood and Adult Primary Brain Tumors. *Neuro. Oncol.* **2019**, *21*, 1357, doi:10.1093/NEUONC/NOZ123.
4. Vienne-Jumeau, A.; Tafani, C.; Ricard, D. Environmental Risk Factors of Primary Brain Tumors: A Review. *Rev. Neurol. (Paris)*. **2019**, *175*, 664–678, doi:10.1016/J.NEUROL.2019.08.004.
5. Hawkins, C.; Croul, S. Viruses and Human Brain Tumors: Cytomegalovirus Enters the Fray. *J. Clin. Invest.* **2011**, *121*, 3831, doi:10.1172/JCI60005.
6. Frühwald, M.C.; Rutkowski, S. Tumors of the Central Nervous System in Children and Adolescents. *Dtsch. Arztebl. Int.* **2011**, *108*, 390, doi:10.3238/ARZTEBL.2011.0390.
7. Walker, C.; Baborie, A.; Crooks, D.; Wilkins, S.; Jenkinson, M.D. Biology, Genetics and Imaging of Glial Cell Tumours. *Br. J. Radiol.* **2011**, *84*, S090, doi:10.1259/BJR/23430927.
8. Millard, N.E.; De Braganca, K.C. Medulloblastoma. *J. Child Neurol.* **2016**, *31*, 1341, doi:10.1177/0883073815600866.
9. Tandel, G.S.; Biswas, M.; Kakde, O.G.; Tiwari, A.; Suri, H.S.; Turk, M.; Laird, J.R.; Asare, C.K.; Ankrah, A.A.; Khanna, N.N.; et al. A Review on a Deep Learning Perspective in Brain Cancer Classification. *Cancers* **2019**, *Vol. 11*, Page 111 **2019**, *11*, 111, doi:10.3390/CANCERS11010111.
10. Gritsch, S.; Batchelor, T.T.; Gonzalez Castro, L.N. Diagnostic, Therapeutic, and Prognostic Implications of the 2021 World Health Organization Classification of Tumors of the Central Nervous System. *Cancer* **2022**, *128*, 47–58, doi:10.1002/CNCR.33918.
11. Louis, D.N.; Perry, A.; Wesseling, P.; Brat, D.J.; Cree, I.A.; Figarella-Branger, D.; Hawkins, C.; Ng,

- H.K.; Pfister, S.M.; Reifenberger, G.; et al. The 2021 WHO Classification of Tumors of the Central Nervous System: A Summary. *Neuro. Oncol.* **2021**, *23*, 1231, doi:10.1093/NEUONC/NOAB106.
12. Torp, S.H.; Solheim, O.; Skjulsvik, A.J. The WHO 2021 Classification of Central Nervous System Tumours: A Practical Update on What Neurosurgeons Need to Know —a Minireview. *Acta Neurochir. (Wien)*. **2022**, *164*, 2453, doi:10.1007/S00701-022-05301-Y.
 13. Patel, A. Benign vs Malignant Tumors. *JAMA Oncol.* **2020**, *6*, 1488–1488, doi:10.1001/JAMAONCOL.2020.2592.
 14. Chung, C.; Laperriere, N. Radiation Therapy and Grade II/III Oligodendroglial Tumors. *CNS Oncol.* **2015**, *4*, 325, doi:10.2217/CNS.15.25.
 15. Strowd, R.E.; Holdhoff, M.; Grossman, S.A. Chemotherapy for Treatment of Grade II Gliomas. *Oncology (Williston Park)*. **2014**, *28*, 1036–1043.
 16. Hanif, F.; Muzaffar, K.; Perveen, K.; Malhi, S.M.; Simjee, S.U. Glioblastoma Multiforme: A Review of Its Epidemiology and Pathogenesis through Clinical Presentation and Treatment. *Asian Pac. J. Cancer Prev.* **2017**, *18*, 3, doi:10.22034/APJCP.2017.18.1.3.
 17. Mueller, S.; Chang, S. Pediatric Brain Tumors: Current Treatment Strategies and Future Therapeutic Approaches. *Neurotherapeutics* **2009**, *6*, 570–586, doi:10.1016/J.NURT.2009.04.006.
 18. Wooley, J.R.; Penas-Prado, M. Pediatric versus Adult Medulloblastoma: Towards a Definition That Goes beyond Age. *Cancers (Basel)*. **2021**, *13*, doi:10.3390/CANCERS13246313.
 19. Mahapatra, S.; Amsbaugh, M.J. Medulloblastoma. *StatPearls* **2022**.
 20. Roussel, M.F.; Hatten, M.E. Cerebellum: Development and Medulloblastoma. *Curr. Top. Dev. Biol.* **2011**, *94*, 235, doi:10.1016/B978-0-12-380916-2.00008-5.
 21. Kline, C.N.; Packer, R.J.; Hwang, E.I.; Raleigh, D.R.; Braunstein, S.; Raffel, C.; Bandopadhyay, P.; Solomon, D.A.; Aboian, M.; Cha, S.; et al. Case-Based Review: Pediatric Medulloblastoma. *Neuro-Oncology Pract.* **2017**, *4*, 138, doi:10.1093/NOP/NPX011.
 22. Franceschi, E.; Giannini, C.; Furtner, J.; Pajtler, K.W.; Ascoli, S.; Guzman, R.; Seidel, C.; Gatto, L.; Hau, P. Adult Medulloblastoma: Updates on Current Management and Future Perspectives. *Cancers (Basel)*. **2022**, *14*, 3708, doi:10.3390/CANCERS14153708.
 23. Louis, D.N.; Perry, A.; Reifenberger, G.; von Deimling, A.; Figarella-Branger, D.; Cavenee, W.K.; Ohgaki, H.; Wiestler, O.D.; Kleihues, P.; Ellison, D.W. The 2016 World Health Organization

- Classification of Tumors of the Central Nervous System: A Summary. *Acta Neuropathol.* **2016**, *131*, 803–820, doi:10.1007/S00401-016-1545-1.
24. Gibson, P.; Tong, Y.; Robinson, G.; Thompson, M.C.; Currle, D.S.; Eden, C.; Kranenburg, T.A.; Hogg, T.; Poppleton, H.; Martin, J.; et al. Subtypes of Medulloblastoma Have Distinct Developmental Origins. *Nature* **2010**, *468*, 1095, doi:10.1038/NATURE09587.
 25. Gilbertson, R.J.; Ellison, D.W. The Origins of Medulloblastoma Subtypes. *Annu. Rev. Pathol.* **2008**, *3*, 341–365, doi:10.1146/ANNUREV.PATHMECHDIS.3.121806.151518.
 26. Coluccia, D.; Figueredo, C.; Isik, S.; Smith, C.; Rutka, J.T. Medulloblastoma: Tumor Biology and Relevance to Treatment and Prognosis Paradigm. *Curr. Neurol. Neurosci. Rep.* **2016**, *16*, 1–11, doi:10.1007/S11910-016-0644-7/TABLES/2.
 27. Juraschka, K.; Taylor, M.D. Medulloblastoma in the Age of Molecular Subgroups: A Review. *J. Neurosurg. Pediatr.* **2019**, *24*, 353–363, doi:10.3171/2019.5.PEDS18381.
 28. El Doussouki, M.; Gajjar, A.; Chamdine, O. Molecular Genetics of Medulloblastoma in Children: Diagnostic, Therapeutic and Prognostic Implications. *Future Neurol.* **2019**, *14*, doi:10.2217/FNL-2018-0030/ASSET/IMAGES/LARGE/FIGURE1.JPEG.
 29. Gajjar, A.; Bowers, D.C.; Karajannis, M.A.; Leary, S.; Witt, H.; Gottardo, N.G. Pediatric Brain Tumors: Innovative Genomic Information Is Transforming the Diagnostic and Clinical Landscape. *J. Clin. Oncol.* **2015**, *33*, 2986–2998, doi:10.1200/JCO.2014.59.9217.
 30. Northcott, P.A.; Hielscher, T.; Dubuc, A.; Mack, S.; Shih, D.; Remke, M.; Al-Halabi, H.; Albrecht, S.; Jabado, N.; Eberhart, C.G.; et al. Pediatric and Adult Sonic Hedgehog Medulloblastomas Are Clinically and Molecularly Distinct. *Acta Neuropathol.* **2011**, *122*, 231, doi:10.1007/S00401-011-0846-7.
 31. Tao, R.; Murad, N.; Xu, Z.; Zhang, P.; Okonechnikov, K.; Kool, M.; Rivero-Hinojosa, S.; Lazarski, C.; Zheng, P.; Liu, Y.; et al. MYC Drives Group 3 Medulloblastoma through Transformation of Sox2+ Astrocyte Progenitor Cells. *Cancer Res.* **2019**, *79*, 1967–1980, doi:10.1158/0008-5472.CAN-18-1787.
 32. Taylor, M.D.; Northcott, P.A.; Korshunov, A.; Remke, M.; Cho, Y.J.; Clifford, S.C.; Eberhart, C.G.; Parsons, D.W.; Rutkowski, S.; Gajjar, A.; et al. Molecular Subgroups of Medulloblastoma: The Current Consensus. *Acta Neuropathol.* **2012**, *123*, 465–472, doi:10.1007/S00401-011-0922-Z.

33. Dubuc, A.M.; Remke, M.; Korshunov, A.; Northcott, P.A.; Zhan, S.H.; Mendez-Lago, M.; Kool, M.; Jones, D.T.W.; Unterberger, A.; Morrissy, A.S.; et al. Aberrant Patterns of H3K4 and H3K27 Histone Lysine Methylation Occur across Subgroups in Medulloblastoma. *Acta Neuropathol.* **2013**, *125*, 373–384, doi:10.1007/S00401-012-1070-9.
34. Juraschka, K.; Taylor, M.D. Medulloblastoma in the Age of Molecular Subgroups: A Review. *J. Neurosurg. Pediatr.* **2019**, *24*, 353–363, doi:10.3171/2019.5.PEDS18381.
35. Yoon, J.W.; Gilbertson, R.; Iannaccone, S.; Iannaccone, P.; Walterhouse, D. Defining a Role for Sonic Hedgehog Pathway Activation in Desmoplastic Medulloblastoma by Identifying GLI1 Target Genes. *Int. J. Cancer* **2009**, *124*, 109, doi:10.1002/IJC.23929.
36. Wu, S.M.; Choo, A.B.H.; Yap, M.G.S.; Chan, K.K.K. Role of Sonic Hedgehog Signaling and the Expression of Its Components in Human Embryonic Stem Cells. *Stem Cell Res.* **2010**, *4*, 38–49, doi:10.1016/J.SCR.2009.09.002.
37. Wang, W.; Shiraishi, R.; Kawauchi, D. Sonic Hedgehog Signaling in Cerebellar Development and Cancer. *Front. Cell Dev. Biol.* **2022**, *10*, doi:10.3389/FCELL.2022.864035.
38. Hatten, M.E.; Heintz, N. Mechanisms of Neural Patterning and Specification in the Developing Cerebellum. *Annu. Rev. Neurosci.* **1995**, *18*, 385–408, doi:10.1146/ANNUREV.NE.18.030195.002125.
39. Sillitoe, R. V.; Joyner, A.L. Morphology, Molecular Codes, and Circuitry Produce the Three-Dimensional Complexity of the Cerebellum. *Annu. Rev. Cell Dev. Biol.* **2007**, *23*, 549–577, doi:10.1146/ANNUREV.CELLBIO.23.090506.123237.
40. Tamayo-Orrego, L.; Charron, F. Recent Advances in SHH Medulloblastoma Progression: Tumor Suppressor Mechanisms and the Tumor Microenvironment. *F1000Research* **2019**, *8*, doi:10.12688/F1000RESEARCH.20013.1.
41. Goodrich, L. V.; Scott, M.P. Hedgehog and Patched in Neural Development and Disease. *Neuron* **1998**, *21*, 1243–1257, doi:10.1016/S0896-6273(00)80645-5.
42. Kimonis, V.E.; Goldstein, A.M.; Pastakia, B.; Yang, M.L.; Kase, R.; Digiovanna, J.J.; Bale, A.E.; Bale, S.J. Clinical Manifestations in 105 Persons With Nevroid Basal Cell Carcinoma Syndrome. *J. Med. Genet* **1997**, *69*, 299–308.
43. Goodrich, L. V.; Milenković, L.; Higgins, K.M.; Scott, M.P. Altered Neural Cell Fates and Medulloblastoma in Mouse Patched Mutants. *Science* **1997**, *277*, 1109–1113,

doi:10.1126/SCIENCE.277.5329.1109.

44. Raleigh, D.R.; Reiter, J.F. Misactivation of Hedgehog Signaling Causes Inherited and Sporadic Cancers. *J. Clin. Invest.* **2019**, *129*, 465, doi:10.1172/JCI120850.
45. Doheny, D.; Manore, S.G.; Wong, G.L.; Lo, H.W. Hedgehog Signaling and Truncated GLI1 in Cancer. *Cells* **2020**, *9*, doi:10.3390/CELLS9092114.
46. Yauch, R.L.; Dijkgraaf, G.J.P.; Alicke, B.; Januário, T.; Ahn, C.P.; Holcomb, T.; Pujara, K.; Stinson, J.; Callahan, C.A.; Tang, T.; et al. Smoothed Mutation Confers Resistance to a Hedgehog Pathway Inhibitor in Medulloblastoma. *Science* **2009**, *326*, 572–574, doi:10.1126/SCIENCE.1179386.
47. Northcott, P.A.; Jones, D.T.W.; Kool, M.; Robinson, G.W.; Gilbertson, R.J.; Cho, Y.J.; Pomeroy, S.L.; Korshunov, A.; Lichter, P.; Taylor, M.D.; et al. Medulloblastomics: The End of the Beginning. *Nat. Rev. Cancer* **2012**, *12*, 818, doi:10.1038/NRC3410.
48. Roussel, M.F.; Robinson, G.W. Role of MYC in Medulloblastoma. *Cold Spring Harb. Perspect. Med.* **2013**, *3*, doi:10.1101/CSHPERSPECT.A014308.
49. Ramaswamy, V.; Nör, C.; Taylor, M.D. P53 and Medulloblastoma. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a026278, doi:10.1101/CSHPERSPECT.A026278.
50. Taylor, M.D.; Liu, L.; Raffel, C.; Hui, C. chung; Mainprize, T.G.; Zhang, X.; Agatep, R.; Chiappa, S.; Gao, L.; Lowrance, A.; et al. Mutations in SUFU Predispose to Medulloblastoma. *Nat. Genet.* **2002**, *31*, 306–310, doi:10.1038/NG916.
51. Castellino, R.C.; Barwick, B.G.; Schniederjan, M.; Buss, M.C.; Becher, O.; Hambardzumyan, D.; Macdonald, T.J.; Brat, D.J.; Durden, D.L. Heterozygosity for Pten Promotes Tumorigenesis in a Mouse Model of Medulloblastoma. *PLoS One* **2010**, *5*, doi:10.1371/JOURNAL.PONE.0010849.
52. Wu, X.; Northcott, P.A.; Croul, S.; Taylor, M.D. Mouse Models of Medulloblastoma. *Chin. J. Cancer* **2011**, *30*, 442, doi:10.5732/CJC.011.10040.
53. Hahn, H.; Wojnowski, L.; Zimmer, A.M.; Hall, J.; Miller, G.; Zimmer, A. Rhabdomyosarcomas and Radiation Hypersensitivity in a Mouse Model of Gorlin Syndrome. *Nat. Med.* **1998**, *4*, 619–622, doi:10.1038/NM0598-619.
54. Uziel, T.; Zindy, F.; Xie, S.; Lee, Y.; Forget, A.; Magdaleno, S.; Rehg, J.E.; Calabrese, C.; Solecki, D.; Eberhart, C.G.; et al. The Tumor Suppressors Ink4c and P53 Collaborate Independently with Patched to Suppress Medulloblastoma Formation. *Genes Dev.* **2005**, *19*, 2656–2667,

doi:10.1101/GAD.1368605.

55. Ayrault, O.; Zindy, F.; Rehg, J.; Sherr, C.J.; Roussel, M.F. Two Tumor Suppressors, P27Kip1 and Patched-1, Collaborate to Prevent Medulloblastoma. *Mol. Cancer Res.* **2009**, *7*, 33–40, doi:10.1158/1541-7786.MCR-08-0369.
56. Briggs, K.J.; Corcoran-Schwartz, I.M.; Zhang, W.; Harcke, T.; Devereux, W.L.; Baylin, S.B.; Eberhart, C.G.; Watkins, D.N. Cooperation between the Hic1 and Ptch1 Tumor Suppressors in Medulloblastoma. *Genes Dev.* **2008**, *22*, 770, doi:10.1101/GAD.1640908.
57. Yang, Z.J.; Ellis, T.; Markant, S.L.; Read, T.A.; Kessler, J.D.; Bourboulas, M.; Schüller, U.; Machold, R.; Fishell, G.; Rowitch, D.H.; et al. Medulloblastoma Can Be Initiated by Deletion of Patched in Lineage-Restricted Progenitors or Stem Cells. *Cancer Cell* **2008**, *14*, 135–145, doi:10.1016/J.CCR.2008.07.003.
58. Lee, Y.; Kawagoe, R.; Sasai, K.; Li, Y.; Russell, H.R.; Curran, T.; McKinnon, P.J. Loss of Suppressor-of-Fused Function Promotes Tumorigenesis. *Oncogene* **2007**, *26*, 6442–6447, doi:10.1038/SJ.ONC.1210467.
59. Hallahan, A.R.; Pritchard, J.I.; Hansen, S.; Benson, M.; Stoeck, J.; Hatton, B.A.; Russell, T.L.; Ellenbogen, R.G.; Bernstein, I.D.; Beachy, P.A.; et al. The SmoA1 Mouse Model Reveals That Notch Signaling Is Critical for the Growth and Survival of Sonic Hedgehog-Induced Medulloblastomas. *Cancer Res.* **2004**, *64*, 7794–7800, doi:10.1158/0008-5472.CAN-04-1813.
60. Hatton, B.A.; Villavicencio, E.H.; Tsuchiya, K.D.; Pritchard, J.I.; Ditzler, S.; Pullar, B.; Hansen, S.; Knoblaugh, S.E.; Lee, D.; Eberhart, C.G.; et al. The Smo/Smo Model: Hedgehog-Induced Medulloblastoma with 90% Incidence and Leptomeningeal Spread. *Cancer Res.* **2008**, *68*, 1768–1776, doi:10.1158/0008-5472.CAN-07-5092.
61. Marino, S.; Vooijs, M.; Van Der Gulden, H.; Jonkers, J.; Berns, A. Induction of Medulloblastomas in P53-Null Mutant Mice by Somatic Inactivation of Rb in the External Granular Layer Cells of the Cerebellum. *Genes Dev.* **2000**, *14*, 994, doi:10.1101/gad.14.8.994.
62. Yan, C.T.; Kaushal, D.; Murphy, M.; Zhang, Y.; Datta, A.; Chen, C.; Monroe, B.; Mostoslavsky, G.; Coakley, K.; Gao, Y.; et al. XRCC4 Suppresses Medulloblastomas with Recurrent Translocations in P53-Deficient Mice. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 7378–7383, doi:10.1073/PNAS.0601938103.

63. Frappart, P.O.; Lee, Y.; Russell, H.R.; Chalhoub, N.; Wang, Y.D.; Orii, K.E.; Zhao, J.; Kondo, N.; Baker, S.J.; McKinnon, P.J. Recurrent Genomic Alterations Characterize Medulloblastoma Arising from DNA Double-Strand Break Repair Deficiency. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 1880–1885, doi:10.1073/PNAS.0806882106.
64. Swartling, F.J.; Grimmer, M.R.; Hackett, C.S.; Northcott, P.A.; Fan, Q.W.; Goldenberg, D.D.; Lau, J.; Masic, S.; Nguyen, K.; Yakovenko, S.; et al. Pleiotropic Role for MYCN in Medulloblastoma. *Genes Dev.* **2010**, *24*, 1059–1072, doi:10.1101/GAD.1907510.
65. Pazzaglia, S. Ptc1 Heterozygous Knockout Mice as a Model of Multi-Organ Tumorigenesis. *Cancer Lett.* **2006**, *234*, 124–134, doi:10.1016/J.CANLET.2005.03.047.
66. Pazzaglia, S.; Mancuso, M.; Atkinson, M.J.; Tanori, M.; Rebessi, S.; Di Majo, V.; Covelli, V.; Hahn, H.; Saran, A. High Incidence of Medulloblastoma Following X-Ray-Irradiation of Newborn Ptc1 Heterozygous Mice. *Oncogene* **2002**, *21*, 7580–7584, doi:10.1038/SJ.ONC.1205973.
67. Pazzaglia, S.; Mancuso, M.; Tanori, M.; Atkinson, M.J.; Merola, P.; Rebessi, S.; Di Majo, V.; Covelli, V.; Hahn, H.; Saran, A. Modulation of Patched-Associated Susceptibility to Radiation Induced Tumorigenesis by Genetic Background. *Cancer Res.* **2004**, *64*, 3798–3806, doi:10.1158/0008-5472.CAN-03-3716.
68. Mancuso, M.; Pazzaglia, S.; Tanori, M.; Hahn, H.; Merola, P.; Rebessi, S.; Atkinson, M.J.; Di Majo, V.; Covelli, V.; Saran, A. Basal Cell Carcinoma and Its Development: Insights from Radiation-Induced Tumors in Ptch1-Deficient Mice. *Cancer Res.* **2004**, *64*, 934–941, doi:10.1158/0008-5472.CAN-03-2460.
69. Pazzaglia, S.; Tanori, M.; Mancuso, M.; Gessi, M.; Pasquali, E.; Leonardi, S.; Oliva, M.A.; Rebessi, S.; Di Majo, V.; Covelli, V.; et al. Two-Hit Model for Progression of Medulloblastoma Preneoplasia in Patched Heterozygous Mice. *Oncogene* **2006**, *25*, 5575–5580, doi:10.1038/SJ.ONC.1209544.
70. Thomas, A.; Noël, G. Medulloblastoma: Optimizing Care with a Multidisciplinary Approach. *J. Multidiscip. Healthc.* **2019**, *12*, 335, doi:10.2147/JMDH.S167808.
71. Chen, Z. he; Zhang, X. heng; Lin, F. hua; Li, C.; Jin, J. tian; Zhou, Z. huan; Zhu, S. han; Cheng, Z. qing; Zhong, S.; He, Z. qiang; et al. The Application of Fluorescein Sodium for the Resection of Medulloblastoma. *J. Neurooncol.* **2022**, *158*, 463–470, doi:10.1007/S11060-022-04035-2.
72. Rae, A.I.; Mehta, A.; Cloney, M.; Kinslow, C.J.; Wang, T.J.C.; Bhagat, G.; Canoll, P.D.; Zanazzi, G.J.;

- Sisti, M.B.; Sheth, S.A.; et al. Craniotomy and Survival for Primary Central Nervous System Lymphoma. *Neurosurgery* **2019**, *84*, 935, doi:10.1093/NEUROS/NYY096.
73. Albright, A.L.; Wisoff, J.H.; Zeltzer, P.M.; Boyett, J.M.; Rorke, L.B.; Stanley, P. Effects of Medulloblastoma Resections on Outcome in Children: A Report from the Children's Cancer Group. *Neurosurgery* **1996**, *38*, 265–271, doi:10.1097/00006123-199602000-00007.
 74. Baskar, R.; Lee, K.A.; Yeo, R.; Yeoh, K.W. Cancer and Radiation Therapy: Current Advances and Future Directions. *Int. J. Med. Sci.* **2012**, *9*, 193, doi:10.7150/IJMS.3635.
 75. Halperin, E.C. Impact of Radiation Technique upon the Outcome of Treatment for Medulloblastoma. *Int. J. Radiat. Oncol.* **1996**, *36*, 233–239, doi:10.1016/S0360-3016(96)00276-3.
 76. Christopherson, K.M.; Rotondo, R.L.; Bradley, J.A.; Pincus, D.W.; Wynn, T.T.; Fort, J.A.; Morris, C.G.; Mendenhall, N.P.; Marcus, R.B.; Indelicato, D.J. Late Toxicity Following Craniospinal Radiation for Early-Stage Medulloblastoma. *Acta Oncol.* **2014**, *53*, 471–480, doi:10.3109/0284186X.2013.862596.
 77. Lafay-Cousin, L.; Bouffet, E.; Hawkins, C.; Amid, A.; Huang, A.; Mabbott, D.J. Impact of Radiation Avoidance on Survival and Neurocognitive Outcome in Infant Medulloblastoma. *Curr. Oncol.* **2009**, *16*, 21, doi:10.3747/CO.V16I6.435.
 78. Zhang, R.; Howell, R.M.; Taddei, P.J.; Giebeler, A.; Mahajan, A.; Newhauser, W.D. A Comparative Study on the Risks of Radiogenic Second Cancers and Cardiac Mortality in a Set of Pediatric Medulloblastoma Patients Treated with Photon or Proton Craniospinal Irradiation. *Radiother. Oncol.* **2014**, *113*, 84–88, doi:10.1016/J.RADONC.2014.07.003.
 79. Seidel, C.; Heider, S.; Hau, P.; Glasow, A.; Dietzsch, S.; Kortmann, R.D. Radiotherapy in Medulloblastoma—Evolution of Treatment, Current Concepts and Future Perspectives. *Cancers (Basel)*. **2021**, *13*, doi:10.3390/CANCERS13235945.
 80. Pompe, R.S.; Von Bueren, A.O.; Mynarek, M.; Von Hoff, K.; Friedrich, C.; Kwiecien, R.; Treulieb, W.; Lindow, C.; Deinlein, F.; Fleischhack, G.; et al. Intraventricular Methotrexate as Part of Primary Therapy for Children with Infant and/or Metastatic Medulloblastoma: Feasibility, Acute Toxicity and Evidence for Efficacy. *Eur. J. Cancer* **2015**, *51*, 2634–2642, doi:10.1016/j.ejca.2015.08.009.
 81. Gavrilovic, I.T.; Hormigo, A.; Yahalom, J.; DeAngelis, L.M.; Abrey, L.E. Long-Term Follow-up of High-Dose Methotrexate-Based Therapy with and without Whole Brain Irradiation for Newly

- Diagnosed Primary CNS Lymphoma. *J. Clin. Oncol.* **2006**, *24*, 4570–4574, doi:10.1200/JCO.2006.06.6910.
82. Vezmar, S.; Becker, A.; Bode, U.; Jaehde, U. Biochemical and Clinical Aspects of Methotrexate Neurotoxicity. *Chemotherapy* **2003**, *49*, 92–104, doi:10.1159/000069773.
 83. Price, P.; Thompson, H.; Bessell, E.M.; Bloom, H.J.G. Renal Impairment Following the Combined Use of High-Dose Methotrexate and Procarbazine. *Cancer Chemother. Pharmacol.* **1988**, *21*, 265–267, doi:10.1007/BF00262783/METRICS.
 84. Macdonald, T.J.; Aguilera, D.; Castellino, R.C. The Rationale for Targeted Therapies in Medulloblastoma. *Neuro. Oncol.* **2014**, *16*, 9, doi:10.1093/NEUONC/NOT147.
 85. Caimano, M.; Lospinoso Severini, L.; Loricchio, E.; Infante, P.; Di Marcotullio, L. Drug Delivery Systems for Hedgehog Inhibitors in the Treatment of SHH-Medulloblastoma. *Front. Chem.* **2021**, *9*, 389, doi:10.3389/FCHEM.2021.688108/BIBTEX.
 86. Buczkowicz, P.; Ma, J.; Hawkins, C. GLI2 Is a Potential Therapeutic Target in Pediatric Medulloblastoma. *J. Neuropathol. Exp. Neurol.* **2011**, *70*, 430–437, doi:10.1097/NEN.0B013E31821B94DB.
 87. Huang, S.Y.; Yang, J.Y. Targeting the Hedgehog Pathway in Pediatric Medulloblastoma. *Cancers (Basel)*. **2015**, *7*, 2110, doi:10.3390/CANCERS7040880.
 88. Zhong, L.; Li, Y.; Xiong, L.; Wang, W.; Wu, M.; Yuan, T.; Yang, W.; Tian, C.; Miao, Z.; Wang, T.; et al. Small Molecules in Targeted Cancer Therapy: Advances, Challenges, and Future Perspectives. *Signal Transduct. Target. Ther.* **2021**, *6*, 1–48, doi:10.1038/s41392-021-00572-w.
 89. Kieran, M.W. Targeted Treatment for Sonic Hedgehog-Dependent Medulloblastoma. *Neuro. Oncol.* **2014**, *16*, 1037, doi:10.1093/NEUONC/NOU109.
 90. Kian, W.; Roisman, L.C.; Goldstein, I.M.; Abo-Quider, A.; Samueli, B.; Wallach, N.; Alguayn, F.; Shalata, W.; Levitas, D.; Belochitski, O.; et al. Vismodegib as First-Line Treatment of Mutated Sonic Hedgehog Pathway in Adult Medulloblastoma. *JCO Precis. Oncol.* **2020**, *4*, 437–441, doi:10.1200/PO.19.00264.
 91. Carpenter, R.L.; Ray, H. Efficacy and Safety of Sonic Hedgehog Pathway Inhibitors in Cancer. *Drug Saf.* **2019**, *42*, 263, doi:10.1007/S40264-018-0777-5.
 92. Gajjar, A.; Stewart, C.F.; Ellison, D.W.; Kaste, S.; Kun, L.E.; Packer, R.J.; Goldman, S.

- Chintagumpala, M.; Wallace, D.; Takebe, N.; et al. Phase I Study of Vismodegib in Children with Recurrent or Refractory Medulloblastoma: A Pediatric Brain Tumor Consortium Study. *Clin. Cancer Res.* **2013**, *19*, 6305–6312, doi:10.1158/1078-0432.CCR-13-1425/86043/AM/PHASE-I-STUDY-OF-VISMODEGIB-IN-CHILDREN-WITH.
93. Robinson, G.W.; Orr, B.A.; Wu, G.; Gururangan, S.; Lin, T.; Qaddoumi, I.; Packer, R.J.; Goldman, S.; Prados, M.D.; Desjardins, A.; et al. Vismodegib Exerts Targeted Efficacy Against Recurrent Sonic Hedgehog–Subgroup Medulloblastoma: Results From Phase II Pediatric Brain Tumor Consortium Studies PBTC-025B and PBTC-032. *J. Clin. Oncol.* **2015**, *33*, 2646, doi:10.1200/JCO.2014.60.1591.
 94. Yuile, A.K.; Kastelan, M.; Lee, A.P.S.; Back, M.; Drummond, J.; Wheeler, H.R. The Use of Sonidegib in the Adjuvant and Advanced Phases of Sonic Hedge Hog Mutant Medulloblastomas. *Oxford Med. case reports* **2022**, *2022*, 92–95, doi:10.1093/OMCR/OMAC019.
 95. Burness, C.B. Sonidegib: First Global Approval. *Drugs* **2015**, *75*, 1559–1566, doi:10.1007/S40265-015-0458-Y/METRICS.
 96. Li, Y.; Song, Q.; Day, B.W. Phase I and Phase II Sonidegib and Vismodegib Clinical Trials for the Treatment of Paediatric and Adult MB Patients: A Systemic Review and Meta-Analysis. *Acta Neuropathol. Commun.* **2019**, *7*, 123, doi:10.1186/S40478-019-0773-8.
 97. Villani, A.; Fabbrocini, G.; Costa, C.; Scalvenzi, M. Sonidegib: Safety and Efficacy in Treatment of Advanced Basal Cell Carcinoma. *Dermatol. Ther. (Heidelb).* **2020**, *10*, 401, doi:10.1007/S13555-020-00378-8.
 98. Lewandowicz, G.M.; Harding, B.; Harkness, W.; Hayward, R.; Thomas, D.G.T.; Darling, J.L. Chemosensitivity in Childhood Brain Tumours in Vitro: Evidence of Differential Sensitivity to Lomustine (CCNU) and Vincristine. *Eur. J. Cancer* **2000**, *36*, 1955–1964, doi:10.1016/S0959-8049(00)00245-8.
 99. Presutti, D.; Ceccarelli, M.; Micheli, L.; Papoff, G.; Santini, S.; Samperna, S.; Lalli, C.; Zentilin, L.; Ruberti, G.; Tirone, F. Tis21-Gene Therapy Inhibits Medulloblastoma Growth in a Murine Allograft Model. *PLoS One* **2018**, *13*, doi:10.1371/JOURNAL.PONE.0194206.
 100. Wang, C.; Liang, Q.; Chen, G.; Jing, J.; Wang, S. Inhibition of GPR137 Suppresses Proliferation of Medulloblastoma Cells in Vitro. *Biotechnol. Appl. Biochem.* **2015**, *62*, 868–873, doi:10.1002/BAB.1331.

101. Wu, X.; Xiao, S.; Zhang, M.; Yang, L.; Zhong, J.; Li, B.; Li, F.; Xia, X.; Li, X.; Zhou, H.; et al. A Novel Protein Encoded by Circular SMO RNA Is Essential for Hedgehog Signaling Activation and Glioblastoma Tumorigenicity. *Genome Biol.* **2021**, *22*, 1–29, doi:10.1186/S13059-020-02250-6/FIGURES/6.
102. Vo, B.H.T.; Kwon, J.A.; Li, C.; Finkelstein, D.; Xu, B.; Orr, B.A.; Sherr, C.J.; Roussel, M.F. Mouse Medulloblastoma Driven by CRISPR Activation of Cellular Myc. *Sci. Rep.* **2018**, *8*, doi:10.1038/S41598-018-24956-1.
103. Zhang, J.; Wang, T. Immune Cell Landscape and Immunotherapy of Medulloblastoma. *Pediatr. Investig.* **2021**, *5*, 299, doi:10.1002/PED4.12261.
104. Kabir, T.F.; Kunos, C.A.; Villano, J.L.; Chauhan, A. Immunotherapy for Medulloblastoma: Current Perspectives. *ImmunoTargets Ther.* **2020**, *9*, 57–77, doi:10.2147/ITT.S198162.
105. Voskamp, M.J.; Li, S.; van Daalen, K.R.; Crnko, S.; Ten Broeke, T.; Bovenschen, N. Immunotherapy in Medulloblastoma: Current State of Research, Challenges, and Future Perspectives. *Cancers (Basel)*. **2021**, *13*, doi:10.3390/CANCERS13215387.
106. Pham, C.D.; Flores, C.; Yang, C.; Pinheiro, E.M.; Yearley, J.H.; Sayour, E.J.; Pei, Y.; Moore, C.; McLendon, R.E.; Huang, J.; et al. Differential Immune Microenvironments and Response to Immune Checkpoint Blockade among Molecular Subtypes of Murine Medulloblastoma. *Clin. Cancer Res.* **2016**, *22*, 582–595, doi:10.1158/1078-0432.CCR-15-0713.
107. Allen, F.; Dorand, R.D.; Rauhe, P.; Petrosiute, A.; Huang, A.Y. The Efficacy of PD-L1 Blockade on PD-L1 Negative Medulloblastoma Is Dependent on Timing and the Tumor Microenvironment. *J. Immunol.* **2018**, *200*, 178.14–178.14, doi:10.4049/JIMMUNOL.200.SUPP.178.14.
108. Ghoulzani, A.; Kandoussi, S.; Tall, M.; Reddy, K.P.; Rafii, S.; Badou, A. Immune Checkpoint Inhibitors in Human Glioma Microenvironment. *Front. Immunol.* **2021**, *12*, 2664, doi:10.3389/FIMMU.2021.679425/BIBTEX.
109. Morotti, M.; Albukhari, A.; Alsaadi, A.; Artibani, M.; Brenton, J.D.; Curbishley, S.M.; Dong, T.; Dustin, M.L.; Hu, Z.; McGranahan, N.; et al. Promises and Challenges of Adoptive T-Cell Therapies for Solid Tumours. *Br. J. Cancer* **2021**, *124*, 1759–1776, doi:10.1038/s41416-021-01353-6.
110. Nellan, A.; Rota, C.; Majzner, R.; Lester-McCully, C.M.; Griesinger, A.M.; Mulcahy Levy, J.M.;

- Foreman, N.K.; Warren, K.E.; Lee, D.W. Durable Regression of Medulloblastoma after Regional and Intravenous Delivery of Anti-HER2 Chimeric Antigen Receptor T Cells. *J. Immunother. cancer* **2018**, *6*, doi:10.1186/S40425-018-0340-Z.
111. Orlando, D.; Miele, E.; De Angelis, B.; Guercio, M.; Boffa, I.; Sinibaldi, M.; Po, A.; Caruana, I.; Abballe, L.; Carai, A.; et al. Adoptive Immunotherapy Using PRAME-Specific T Cells in Medulloblastoma. *Cancer Res.* **2018**, *78*, 3337–3349, doi:10.1158/0008-5472.CAN-17-3140.
 112. Donovan, L.K.; Delaidelli, A.; Joseph, S.K.; Bielamowicz, K.; Fousek, K.; Holgado, B.L.; Manno, A.; Srikanthan, D.; Gad, A.Z.; Van Ommeren, R.; et al. Locoregional Delivery of CAR T Cells to the Cerebrospinal Fluid for Treatment of Metastatic Medulloblastoma and Ependymoma. *Nat. Med.* **2020**, *26*, 720–731, doi:10.1038/S41591-020-0827-2.
 113. Ardon, H.; De Vleeschouwer, S.; Van Calenbergh, F.; Claes, L.; Kramm, C.M.; Rutkowski, S.; Wolff, J.E.A.; Van Gool, S.W. Adjuvant Dendritic Cell-Based Tumour Vaccination for Children with Malignant Brain Tumours. *Pediatr. Blood Cancer* **2010**, *54*, 519–525, doi:10.1002/PBC.22319.
 114. Hutzen, B.; Bid, H.K.; Houghton, P.J.; Pierson, C.R.; Powell, K.; Bratasz, A.; Raffel, C.; Studebaker, A.W. Treatment of Medulloblastoma with Oncolytic Measles Viruses Expressing the Angiogenesis Inhibitors Endostatin and Angiostatin. *BMC Cancer* **2014**, *14*, doi:10.1186/1471-2407-14-206.
 115. Varela-Guruceaga, M.; Tejada-Solís, S.; García-Moure, M.; Fueyo, J.; Gomez-Manzano, C.; Patiño-García, A.; Alonso, M.M. Oncolytic Viruses as Therapeutic Tools for Pediatric Brain Tumors. *Cancers (Basel)*. **2018**, *10*, doi:10.3390/CANCERS10070226.
 116. Rossi, A.; Caracciolo, V.; Russo, G.; Reiss, K.; Giordano, A. Medulloblastoma: From Molecular Pathology to Therapy. *Clin. Cancer Res.* **2008**, *14*, 971–976, doi:10.1158/1078-0432.CCR-07-2072.
 117. Packer, R.J.; Cogen, P.; Vezina, G.; Rorke, L.B. Medulloblastoma: Clinical and Biologic Aspects. *Neuro. Oncol.* **1999**, *1*, 232–250, doi:10.1093/NEUONC/1.3.232.
 118. Mulhern, R.K.; Palmer, S.L.; Merchant, T.E.; Wallace, D.; Kocak, M.; Brouwers, P.; Krull, K.; Chintagumpala, M.; Stargatt, R.; Ashley, D.M.; et al. Neurocognitive Consequences of Risk-Adapted Therapy for Childhood Medulloblastoma. *J. Clin. Oncol.* **2005**, *23*, 5511–5519, doi:10.1200/JCO.2005.00.703.
 119. Packer, R.J.; Vezina, G. Management of and Prognosis with Medulloblastoma: Therapy at a Crossroads. *Arch. Neurol.* **2008**, *65*, 1419–1424, doi:10.1001/ARCHNEUR.65.11.1419.

120. Samkari, A.; Hwang, E.; Packer, R.J. Medulloblastoma/Primitive Neuroectodermal Tumor and Germ Cell Tumors: The Uncommon but Potentially Curable Primary Brain Tumors. *Hematol. Oncol. Clin. North Am.* **2012**, *26*, 881–895, doi:10.1016/J.HOC.2012.04.002.
121. Bartlett, F.; Kortmann, R.; Saran, F. Medulloblastoma. *Clin. Oncol. (R. Coll. Radiol.)*. **2013**, *25*, 36–45, doi:10.1016/J.CLON.2012.09.008.
122. Sasai, K.; Romer, J.T.; Kimura, H.; Eberhart, D.E.; Rice, D.S.; Curran, T. Medulloblastomas Derived from Cxcr6 Mutant Mice Respond to Treatment with a Smoothed Inhibitor. *Cancer Res.* **2007**, *67*, 3871–3877, doi:10.1158/0008-5472.CAN-07-0493/654422/P/MEDULLOBLASTOMAS-DERIVED-FROM-CXCR6-MUTANT-MICE.
123. Freitas, R.A. What Is Nanomedicine? *Nanomedicine Nanotechnology, Biol. Med.* **2005**, *1*, 2–9, doi:10.1016/J.NANO.2004.11.003.
124. Jabir, N.R.; Tabrez, S.; Ashraf, G.M.; Shakil, S.; Damanhour, G.A.; Kamal, M.A. Nanotechnology-Based Approaches in Anticancer Research. *Int. J. Nanomedicine* **2012**, *7*, 4391, doi:10.2147/IJN.S33838.
125. Lee Ventola, C. The Nanomedicine Revolution: Part 1: Emerging Concepts. *Pharm. Ther.* **2012**, *37*, 512.
126. Khan, I.; Saeed, K.; Khan, I. Nanoparticles: Properties, Applications and Toxicities. *Arab. J. Chem.* **2019**, *12*, 908–931, doi:10.1016/J.ARABJC.2017.05.011.
127. Su, Y.L.; Hu, S.H. Functional Nanoparticles for Tumor Penetration of Therapeutics. *Pharmaceutics* **2018**, *10*, doi:10.3390/PHARMACEUTICS10040193.
128. Albanese, A.; Tang, P.S.; Chan, W.C.W. The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems. <https://doi.org/10.1146/annurev-bioeng-071811-150124> **2012**, *14*, 1–16, doi:10.1146/ANNUREV-BIOENG-071811-150124.
129. Singh, N.; Marets, C.; Boudon, J.; Millot, N.; Saviot, L.; Maurizi, L. In Vivo Protein Corona on Nanoparticles: Does the Control of All Material Parameters Orient the Biological Behavior? *Nanoscale Adv.* **2021**, *3*, 1209–1229, doi:10.1039/D0NA00863J.
130. Friedman, A.D.; Claypool, S.E.; Liu, R. The Smart Targeting of Nanoparticles. *Curr. Pharm. Des.* **2013**, *19*, 6315, doi:10.2174/13816128113199990375.
131. Liu, Y.; Hardie, J.; Zhang, X.; Rotello, V.M. Effects of Engineered Nanoparticles on the Innate

- Immune System. *Semin. Immunol.* **2017**, *34*, 25, doi:10.1016/j.SMIM.2017.09.011.
132. Lee, M.S.; Dees, E.C.; Wang, A.Z. Nanoparticle-Delivered Chemotherapy: Old Drugs in New Packages. *Oncology (Williston Park)*. **2017**, *31*, 198–208.
 133. Babaei, M.; Ganjalikhani, M. The Potential Effectiveness of Nanoparticles as Radio Sensitizers for Radiotherapy. *Bioimpacts* **2014**, *4*, 15, doi:10.5681/BI.2014.003.
 134. Key, J.; Leary, J.F. Nanoparticles for Multimodal in Vivo Imaging in Nanomedicine. *Int. J. Nanomedicine* **2014**, *9*, 711–726, doi:10.2147/IJN.S53717.
 135. Aikins, M.E.; Xu, C.; Moon, J.J. Engineered Nanoparticles for Cancer Vaccination and Immunotherapy. *Acc. Chem. Res.* **2020**, *53*, 2094–2105, doi:10.1021/ACS.ACCOUNTS.0C00456/ASSET/IMAGES/LARGE/AR0C00456_0006.JPEG.
 136. Mukalel, A.J.; Riley, R.S.; Zhang, R.; Mitchell, M.J. Nanoparticles for Nucleic Acid Delivery: Applications in Cancer Immunotherapy. *Cancer Lett.* **2019**, *458*, 102, doi:10.1016/j.CANLET.2019.04.040.
 137. Jaque, D.; Martínez Maestro, L.; Del Rosal, B.; Haro-Gonzalez, P.; Benayas, A.; Plaza, J.L.; Martín Rodríguez, E.; García Solé, J. Nanoparticles for Photothermal Therapies. *Nanoscale* **2014**, *6*, 9494–9530, doi:10.1039/C4NR00708E.
 138. Danhier, F.; Feron, O.; Préat, V. To Exploit the Tumor Microenvironment: Passive and Active Tumor Targeting of Nanocarriers for Anti-Cancer Drug Delivery. *J. Control. Release* **2010**, *148*, 135–146, doi:10.1016/j.JCONREL.2010.08.027.
 139. Attia, M.F.; Anton, N.; Wallyn, J.; Omran, Z.; Vandamme, T.F. An Overview of Active and Passive Targeting Strategies to Improve the Nanocarriers Efficiency to Tumour Sites. *J. Pharm. Pharmacol.* **2019**, *71*, 1185–1198, doi:10.1111/JPHP.13098.
 140. Matsumura, Y.; Maeda, H. A New Concept for Macromolecular Therapeutics in Cancer Chemotherapy: Mechanism of Tumoritropic Accumulation of Proteins and the Antitumor Agent Smancs. *Cancer Res.* **1986**, *46*, 6387–6392.
 141. Kobayashi, H.; Watanabe, R.; Choyke, P.L. Improving Conventional Enhanced Permeability and Retention (EPR) Effects; What Is the Appropriate Target? *Theranostics* **2013**, *4*, 81–89, doi:10.7150/THNO.7193.
 142. Van Vlerken, L.E.; Duan, Z.; Seiden, M. V.; Amiji, M.M. Modulation of Intracellular Ceramide

- Using Polymeric Nanoparticles to Overcome Multidrug Resistance in Cancer. *Cancer Res.* **2007**, *67*, 4843–4850, doi:10.1158/0008-5472.CAN-06-1648.
143. BAZAK, R.; HOURI, M.; ACHY, S. EL; HUSSEIN, W.; REFAAT, T. Passive Targeting of Nanoparticles to Cancer: A Comprehensive Review of the Literature. *Mol. Clin. Oncol.* **2014**, *2*, 904, doi:10.3892/MCO.2014.356.
 144. Torchilin, V.P. Passive and Active Drug Targeting: Drug Delivery to Tumors as an Example. *Handb. Exp. Pharmacol.* **2010**, *197*, 3–53, doi:10.1007/978-3-642-00477-3_1/FIGURES/8.
 145. Rosenblum, D.; Joshi, N.; Tao, W.; Karp, J.M.; Peer, D. Progress and Challenges towards Targeted Delivery of Cancer Therapeutics. *Nat. Commun.* **2018**, *9*, 1–12, doi:10.1038/s41467-018-03705-y.
 146. Shipunova, V.O.; Kotelnikova, P.A.; Aghayeva, U.F.; Stremovskiy, O.A.; Novikov, I.A.; Schulga, A.A.; Nikitin, M.P.; Deyev, S.M. Self-Assembling Nanoparticles Biofunctionalized with Magnetite-Binding Protein for the Targeted Delivery to HER2/Neu Overexpressing Cancer Cells. *J. Magn. Magn. Mater.* **2019**, *469*, 450–455, doi:10.1016/J.JMMM.2018.09.015.
 147. Sun, M.; Wang, T.; Li, L.; Li, X.; Zhai, Y.; Zhang, J.; Li, W. The Application of Inorganic Nanoparticles in Molecular Targeted Cancer Therapy: EGFR Targeting. *Front. Pharmacol.* **2021**, *12*, doi:10.3389/FPHAR.2021.702445.
 148. Ambasta, R.K.; Sharma, A.; Kumar, P. Nanoparticle Mediated Targeting of VEGFR and Cancer Stem Cells for Cancer Therapy. *Vasc. Cell* **2011**, *3*, 26, doi:10.1186/2045-824X-3-26.
 149. Sercombe, L.; Veerati, T.; Moheimani, F.; Wu, S.Y.; Sood, A.K.; Hua, S. Advances and Challenges of Liposome Assisted Drug Delivery. *Front. Pharmacol.* **2015**, *6*, 286, doi:10.3389/FPHAR.2015.00286/BIBTEX.
 150. Puiggalí-Jou, A.; del Valle, L.J.; Alemán, C. Drug Delivery Systems Based on Intrinsically Conducting Polymers. *J. Control. Release* **2019**, *309*, 244–264, doi:10.1016/J.JCONREL.2019.07.035.
 151. Wang, F.; Li, C.; Cheng, J.; Yuan, Z. Recent Advances on Inorganic Nanoparticle-Based Cancer Therapeutic Agents. *Int. J. Environ. Res. Public Health* **2016**, *13*, doi:10.3390/IJERPH13121182.
 152. Fonseca-Santos, B.; Gremião, M.P.D.; Chorilli, M. Nanotechnology-Based Drug Delivery Systems for the Treatment of Alzheimer's Disease. *Int. J. Nanomedicine* **2015**, *10*, 4981, doi:10.2147/IJN.S87148.

153. Anselmo, A.C.; Mitragotri, S. Nanoparticles in the Clinic: An Update. *Bioeng. Transl. Med.* **2019**, *4*, doi:10.1002/BTM2.10143.
154. Fenton, O.S.; Olafson, K.N.; Pillai, P.S.; Mitchell, M.J.; Langer, R. Advances in Biomaterials for Drug Delivery. *Adv. Mater.* **2018**, *30*, doi:10.1002/ADMA.201705328.
155. Pulingam, T.; Foroozandeh, P.; Chuah, J.A.; Sudesh, K. Exploring Various Techniques for the Chemical and Biological Synthesis of Polymeric Nanoparticles. *Nanomaterials* **2022**, *12*, doi:10.3390/NANO12030576.
156. Rideau, E.; Dimova, R.; Schwille, P.; Wurm, F.R.; Landfester, K. Liposomes and Polymersomes: A Comparative Review towards Cell Mimicking. *Chem. Soc. Rev.* **2018**, *47*, 8572–8610, doi:10.1039/C8CS00162F.
157. SR, C.; GS, K. Polymeric Micelles for Drug Delivery. *Curr. Pharm. Des.* **2006**, *12*, 4669–4684, doi:10.2174/138161206779026245.
158. Xu, L.; Zhang, H.; Wu, Y. Dendrimer Advances for the Central Nervous System of Therapeutics. *ACS Chem. Neurosci.* **2014**, *5*, 2, doi:10.1021/CN400182Z.
159. Mendes, L.P.; Pan, J.; Torchilin, V.P. Dendrimers as Nanocarriers for Nucleic Acid and Drug Delivery in Cancer Therapy. *Molecules* **2017**, *22*, doi:10.3390/MOLECULES22091401.
160. Yang, W.; Liang, H.; Ma, S.; Wang, D.; Huang, J. Gold Nanoparticle Based Photothermal Therapy: Development and Application for Effective Cancer Treatment. *Sustain. Mater. Technol.* **2019**, *22*, e00109, doi:10.1016/J.SUSMAT.2019.E00109.
161. Arias, L.S.; Pessan, J.P.; Vieira, A.P.M.; De Lima, T.M.T.; Delbem, A.C.B.; Monteiro, D.R. Iron Oxide Nanoparticles for Biomedical Applications: A Perspective on Synthesis, Drugs, Antimicrobial Activity, and Toxicity. *Antibiot. (Basel, Switzerland)* **2018**, *7*, doi:10.3390/ANTIBIOTICS7020046.
162. Huang, K.W.; Hsu, F.F.; Qiu, J.T.; Chern, G.J.; Lee, Y.A.; Chang, C.C.; Huang, Y.T.; Sung, Y.C.; Chiang, C.C.; Huang, R.L.; et al. Highly Efficient and Tumor-Selective Nanoparticles for Dual-Targeted Immunogene Therapy against Cancer. *Sci. Adv.* **2020**, *6*, doi:10.1126/SCIADV.AAX5032.
163. Xu, C.; Nam, J.; Hong, H.; Xu, Y.; Moon, J.J. Positron Emission Tomography -Guided Photodynamic Therapy with Biodegradable Mesoporous Silica Nanoparticles for Personalized Cancer Immunotherapy. *ACS Nano* **2019**, *13*, 12148–12161, doi:10.1021/ACSNANO.9B06691.
164. Wagner, A.M.; Knipe, J.M.; Orive, G.; Peppas, N.A. Quantum Dots in Biomedical Applications.

- Acta Biomater.* **2019**, *94*, 44–63, doi:10.1016/j.ACTBIO.2019.05.022.
165. Zhang, Y.; Meng, S.; Ding, J.; Peng, Q.; Yu, Y. Transition Metal-Coordinated Graphitic Carbon Nitride Dots as a Sensitive and Facile Fluorescent Probe for β -Amyloid Peptide Detection. *Analyst* **2019**, *144*, 504–511, doi:10.1039/C8AN01620H.
 166. Jacob, J.; Haponiuk, J.T.; Thomas, S.; Gopi, S. Biopolymer Based Nanomaterials in Drug Delivery Systems: A Review. *Mater. Today Chem.* **2018**, *9*, 43–55, doi:10.1016/j.MTCHEM.2018.05.002.
 167. Martínez-López, A.L.; Pangua, C.; Reboredo, C.; Campión, R.; Morales-Gracia, J.; Irache, J.M. Protein-Based Nanoparticles for Drug Delivery Purposes. *Int. J. Pharm.* **2020**, *581*, 119289, doi:10.1016/j.IJPHARM.2020.119289.
 168. Yan, M.; Du, J.; Gu, Z.; Liang, M.; Hu, Y.; Zhang, W.; Priceman, S.; Wu, L.; Zhou, Z.H.; Liu, Z.; et al. A Novel Intracellular Protein Delivery Platform Based on Single-Protein Nanocapsules. *Nat. Nanotechnol.* **2010**, *5*, 48–53, doi:10.1038/NNANO.2009.341.
 169. Tariq, H.; Batool, S.; Asif, S.; Ali, M.; Abbasi, B.H. Virus-Like Particles: Revolutionary Platforms for Developing Vaccines Against Emerging Infectious Diseases. *Front. Microbiol.* **2021**, *12*, doi:10.3389/FMICB.2021.790121.
 170. Hiatt, A.; Cafferkey, R.; Bowdish, K. Production of Antibodies in Transgenic Plants. *Nature* **1989**, *342*, 76–78, doi:10.1038/342076A0.
 171. Gomord, V.; Faye, L. Posttranslational Modification of Therapeutic Proteins in Plants. *Curr. Opin. Plant Biol.* **2004**, *7*, 171–181, doi:10.1016/j.pbi.2004.01.015.
 172. Shanmugaraj, B.; Bulaon, C.J.I.; Phoolcharoen, W. Plant Molecular Farming: A Viable Platform for Recombinant Biopharmaceutical Production. *Plants (Basel, Switzerland)* **2020**, *9*, 1–19, doi:10.3390/PLANTS9070842.
 173. Yao, J.; Weng, Y.; Dickey, A.; Wang, K.Y. Plants as Factories for Human Pharmaceuticals: Applications and Challenges. *Int. J. Mol. Sci.* **2015**, *16*, 28549–28565, doi:10.3390/IJMS161226122.
 174. Jones, H.D.; Sparks, C.A. Stable Transformation of Plants. *Methods Mol. Biol.* **2009**, *513*, 111–130, doi:10.1007/978-1-59745-427-8_7.
 175. Potenza, C.; Aleman, L.; Sengupta-Gopalan, C. Targeting Transgene Expression in Research, Agricultural, and Environmental Applications: Promoters Used in Plant Transformation. *Vitr. Cell. Dev. Biol. - Plant* **2004**, *40*, 1–22, doi:10.1079/IVP2003477.

176. Gelvin, S.B. Integration of *Agrobacterium* T-DNA into the Plant Genome. *Annu. Rev. Genet.* **2017**, *51*, 195–217, doi:10.1146/ANNUREV-GENET-120215-035320.
177. Lau, O.S.; Sun, S.S.M. Plant Seeds as Bioreactors for Recombinant Protein Production. *Biotechnol. Adv.* **2009**, *27*, 1015–1022, doi:10.1016/J.BIOTECHADV.2009.05.005.
178. Boyhan, D.; Daniell, H. Low-Cost Production of Proinsulin in Tobacco and Lettuce Chloroplasts for Injectable or Oral Delivery of Functional Insulin and C-Peptide. *Plant Biotechnol. J.* **2011**, *9*, 585–598, doi:10.1111/J.1467-7652.2010.00582.X.
179. Gao, M.; Li, Y.; Xue, X.; Wang, X.; Long, J. Stable Plastid Transformation for High-Level Recombinant Protein Expression: Promises and Challenges. *J. Biomed. Biotechnol.* **2012**, *2012*, doi:10.1155/2012/158232.
180. Spök, A.; Twyman, R.M.; Fischer, R.; Ma, J.K.C.; Sparrow, P.A.C. Evolution of a Regulatory Framework for Pharmaceuticals Derived from Genetically Modified Plants. *Trends Biotechnol.* **2008**, *26*, 506–517, doi:10.1016/J.TIBTECH.2008.05.007.
181. Bawa, A.S.; Anilakumar, K.R. Genetically Modified Foods: Safety, Risks and Public Concerns —a Review. *J. Food Sci. Technol.* **2013**, *50*, 1035, doi:10.1007/S13197-012-0899-1.
182. Gepts, P.; Papa, R. Possible Effects of (Trans)Gene Flow from Crops on the Genetic Diversity from Landraces and Wild Relatives. *Environ. Biosafety Res.* **2003**, *2*, 89–103, doi:10.1051/EBR:2003009.
183. Pogue, G.P.; Vojdani, F.; Palmer, K.E.; Hiatt, E.; Hume, S.; Phelps, J.; Long, L.; Bohorova, N.; Kim, D.; Pauly, M.; et al. Production of Pharmaceutical-Grade Recombinant Aprotinin and a Monoclonal Antibody Product Using Plant-Based Transient Expression Systems. *Plant Biotechnol. J.* **2010**, *8*, 638–654, doi:10.1111/J.1467-7652.2009.00495.X.
184. Ma, J.K.C.; Drake, P.M.W.; Christou, P. The Production of Recombinant Pharmaceutical Proteins in Plants. *Nat. Rev. Genet.* **2003**, *4*, 794–805, doi:10.1038/NRG1177.
185. Floss, D.M.; Falkenburg, D.; Conrad, U. Production of Vaccines and Therapeutic Antibodies for Veterinary Applications in Transgenic Plants: An Overview. *Transgenic Res.* **2007**, *16*, 315–332, doi:10.1007/S11248-007-9095-X.
186. Lico, C.; Chen, Q.; Santi, L. Viral Vectors for Production of Recombinant Proteins in Plants. *J. Cell. Physiol.* **2008**, *216*, 366–377, doi:10.1002/JCP.21423.
187. Sharma, A.K.; Sharma, M.K. Plants as Bioreactors: Recent Developments and Emerging

- Opportunities. *Biotechnol. Adv.* **2009**, 27, 811–832, doi:10.1016/J.BIOTECHADV.2009.06.004.
188. Joh, L.D.; VanderGheynst, J.S. Agroinfiltration of Plant Tissues for Production of High-Value Recombinant Proteins: An Alternative to Production in Transgenic Crops. *J. Sci. Food Agric.* **2006**, 86, 2002–2004, doi:10.1002/JSFA.2572.
 189. Vézina, L.P.; Faye, L.; Lerouge, P.; D'Aoust, M.A.; Marquet-Blouin, E.; Burel, C.; Lavoie, P.O.; Bardor, M.; Gomord, V. Transient Co-Expression for Fast and High-Yield Production of Antibodies with Human-like N-Glycans in Plants. *Plant Biotechnol. J.* **2009**, 7, 442–455, doi:10.1111/J.1467-7652.2009.00414.X.
 190. Mallory, A.C.; Parks, G.; Endres, M.W.; Baulcombe, D.; Bowman, L.H.; Pruss, G.J.; Vance, V.B. The Amplicon-plus System for High-Level Expression of Transgenes in Plants. *Nat. Biotechnol.* **2002**, 20, 622–625, doi:10.1038/NBT0602-622.
 191. Hellens, R.P.; Allan, A.C.; Friel, E.N.; Bolitho, K.; Grafton, K.; Templeton, M.D.; Karunairetnam, S.; Gleave, A.P.; Laing, W.A. Transient Expression Vectors for Functional Genomics, Quantification of Promoter Activity and RNA Silencing in Plants. *Plant Methods* **2005**, 1, doi:10.1186/1746-4811-1-13.
 192. Azhakanandam, K.; Weissinger, S.M.; Nicholson, J.S.; Qu, R.; Weissinger, A.K. Amplicon-plus Targeting Technology (APTT) for Rapid Production of a Highly Unstable Vaccine Protein in Tobacco Plants. *Plant Mol. Biol.* **2007**, 63, 393–404, doi:10.1007/S11103-006-9096-9.
 193. Benchabane, M.; Saint-Jore-Dupas, C.; Faye, L.; Gomord, V.; Michaud, D. Nucleocytoplasmic Transit of Human Alpha1-Antichymotrypsin in Tobacco Leaf Epidermal Cells. *Plant Biotechnol. J.* **2009**, 7, 161–171, doi:10.1111/J.1467-7652.2008.00383.X.
 194. Sourrouille, C.; Marshall, B.; Liénard, D.; Faye, L. From Neanderthal to Nanobiotech: From Plant Potions to Pharming with Plant Factories. *Methods Mol. Biol.* **2009**, 483, 1–23, doi:10.1007/978-1-59745-407-0_1.
 195. Pogue, G.P.; Lindbo, J.A.; Garger, S.J.; Fitzmaurice, W.P. Making an Ally from an Enemy: Plant Virology and the New Agriculture. *Annu. Rev. Phytopathol.* **2002**, 40, 45–74, doi:10.1146/ANNUREV.PHYTO.40.021102.150133.
 196. Gleba, Y.; Marillonnet, S.; Klimyuk, V. Plant Virus Vectors (Gene Expression Systems). *Encycl. Virol.* **2008**, 229–237, doi:10.1016/B978-012374410-4.00572-0.

197. Streatfield, S.J. Approaches to Achieve High-Level Heterologous Protein Production in Plants. *Plant Biotechnol. J.* **2007**, *5*, 2–15, doi:10.1111/J.1467-7652.2006.00216.X.
198. Giritch, A.; Marillonnet, S.; Engler, C.; Van Eldik, G.; Botterman, J.; Klimyuk, V.; Gleba, Y. Rapid High-Yield Expression of Full-Size IgG Antibodies in Plants Coinfected with Noncompeting Viral Vectors. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 14701–14706, doi:10.1073/PNAS.0606631103.
199. Gleba, Y.; Klimyuk, V.; Marillonnet, S. Magniffection--a New Platform for Expressing Recombinant Vaccines in Plants. *Vaccine* **2005**, *23*, 2042–2048, doi:10.1016/J.VACCINE.2005.01.006.
200. Gleba, Y.; Klimyuk, V.; Marillonnet, S. Viral Vectors for the Expression of Proteins in Plants. *Curr. Opin. Biotechnol.* **2007**, *18*, 134–141, doi:10.1016/J.COPBIO.2007.03.002.
201. Fischlechner, M.; Donath, E. Viruses as Building Blocks for Materials and Devices. *Angew. Chem. Int. Ed. Engl.* **2007**, *46*, 3184–3193, doi:10.1002/ANIE.200603445.
202. Varanda, C.; Félix, M.D.R.; Campos, M.D.; Materatski, P. An Overview of the Application of Viruses to Biotechnology. *Viruses* **2021**, *13*, doi:10.3390/V13102073.
203. Pretto, C.; Van Hest, J.C.M. Versatile Reversible Cross-Linking Strategy to Stabilize CCMV Virus like Particles for Efficient siRNA Delivery. *Bioconjug. Chem.* **2019**, *30*, 3069–3077, doi:10.1021/ACS.BIOCONJCHEM.9B00731/ASSET/IMAGES/LARGE/BC9B00731_0007.JPEG.
204. Wang, Y.; Douglas, T. Bioinspired Approaches to Self-Assembly of Virus-like Particles: From Molecules to Materials. *Acc. Chem. Res.* **2022**, doi:10.1021/ACS.ACCOUNTS.2C00056/ASSET/IMAGES/LARGE/AR2C00056_0004.JPEG.
205. Biabanikhankahdani, R.; Ho, K.L.; Alitheen, N.B.; Tan, W.S. A Dual Bioconjugated Virus-Like Nanoparticle as a Drug Delivery System and Comparison with a PH-Responsive Delivery System. *Nanomater. (Basel, Switzerland)* **2018**, *8*, doi:10.3390/NANO8040236.
206. Shan, W.; Zhang, D.; Wu, Y.; Lv, X.; Hu, B.; Zhou, X.; Ye, S.; Bi, S.; Ren, L.; Zhang, X. Modularized Peptides Modified HBc Virus-like Particles for Encapsulation and Tumor-Targeted Delivery of Doxorubicin. *Nanomedicine* **2018**, *14*, 725–734, doi:10.1016/J.NANO.2017.12.002.
207. Prasuhn, D.E.; Yeh, R.M.; Obenaus, A.; Manchester, M.; Finn, M.G. Viral MRI Contrast Agents: Coordination of Gd by Native Virions and Attachment of Gd Complexes by Azide-Alkyne Cycloaddition. *Chem. Commun. (Camb)*. **2007**, 1269–1271, doi:10.1039/B615084E.
208. Pokorski, J.K.; Breitenkamp, K.; Liepold, L.O.; Qazi, S.; Finn, M.G. Functional Virus-Based

- Polymer-Protein Nanoparticles by Atom Transfer Radical Polymerization. *J. Am. Chem. Soc.* **2011**, *133*, 9242–9245, doi:10.1021/JA203286N.
209. Carrico, Z.M.; Farkas, M.E.; Zhou, Y.; Hsiao, S.C.; Marks, J.D.; Chokhawala, H.; Clark, D.S.; Francis, M.B. N-Terminal Labeling Of Filamentous Phage To Create Cancer Marker Imaging Agents. *ACS Nano* **2012**, *6*, 6675, doi:10.1021/NN301134Z.
 210. Steinmetz, N.F.; Lin, T.; Lomonossoff, G.P.; Johnson, J.E. Structure-Based Engineering of an Icosahedral Virus for Nanomedicine and Nanotechnology. *Curr. Top. Microbiol. Immunol.* **2009**, *327*, 23–58, doi:10.1007/978-3-540-69379-6_2.
 211. Aljabali, A.A.A.; Shukla, S.; Lomonossoff, G.P.; Steinmetz, N.F.; Evans, D.J. CPMV-DOX Delivers. *Mol. Pharm.* **2013**, *10*, 3, doi:10.1021/MP3002057.
 212. Zeng, Q.; Wen, H.; Wen, Q.; Chen, X.; Wang, Y.; Xuan, W.; Liang, J.; Wan, S. Cucumber Mosaic Virus as Drug Delivery Vehicle for Doxorubicin. *Biomaterials* **2013**, *34*, 4632–4642, doi:10.1016/J.BIOMATERIALS.2013.03.017.
 213. Singh, P.; Prasuhn, D.; Yeh, R.M.; Destito, G.; Rae, C.S.; Osborn, K.; Finn, M.G.; Manchester, M. Bio-Distribution, Toxicity and Pathology of Cowpea Mosaic Virus Nanoparticles in Vivo. *J. Control. Release* **2007**, *120*, 41–50, doi:10.1016/J.JCONREL.2007.04.003.
 214. Kaiser, C.R.; Flenniken, M.L.; Gillitzer, E.; Harmsen, A.L.; Harmsen, A.G.; Jutila, M.A.; Douglas, T.; Young, M.J. Biodistribution Studies of Protein Cage Nanoparticles Demonstrate Broad Tissue Distribution and Rapid Clearance in Vivo. *Int. J. Nanomedicine* **2007**, *2*, 715.
 215. Lico, C.; Giardullo, P.; Mancuso, M.; Benvenuto, E.; Santi, L.; Baschieri, S. A Biodistribution Study of Two Differently Shaped Plant Virus Nanoparticles Reveals New Peculiar Traits. *Colloids Surf. B. Biointerfaces* **2016**, *148*, 431–439, doi:10.1016/J.COLSURFB.2016.09.019.
 216. Love, A.J.; Makarov, V.; Yaminsky, I.; Kalinina, N.O.; Talianky, M.E. The Use of Tobacco Mosaic Virus and Cowpea Mosaic Virus for the Production of Novel Metal Nanomaterials. *Virology* **2014**, *449*, 133–139, doi:10.1016/J.VIROL.2013.11.002.
 217. Nooraei, S.; Bahrulolum, H.; Hoseini, Z.S.; Katalani, C.; Hajizade, A.; Easton, A.J.; Ahmadian, G. Virus-like Particles: Preparation, Immunogenicity and Their Roles as Nanovaccines and Drug Nanocarriers. *J. Nanobiotechnology* **2021**, *19*, doi:10.1186/S12951-021-00806-7.
 218. Roossinck, M.J. Plant Virus Ecology. *PLoS Pathog.* **2013**, *9*, doi:10.1371/JOURNAL.PPAT.1003304.

219. Scholthof, K.B.G.; Adkins, S.; Czosnek, H.; Palukaitis, P.; Jacquot, E.; Hohn, T.; Hohn, B.; Saunders, K.; Candresse, T.; Ahlquist, P.; et al. Top 10 Plant Viruses in Molecular Plant Pathology. *Mol. Plant Pathol.* **2011**, *12*, 938–954, doi:10.1111/J.1364-3703.2011.00752.X.
220. Balke, I.; Zeltins, A. Use of Plant Viruses and Virus-like Particles for the Creation of Novel Vaccines. *Adv. Drug Deliv. Rev.* **2019**, *145*, 119–129, doi:10.1016/J.ADDR.2018.08.007.
221. Creager, A.N.H.; Scholthof, K.B.G.; Citovsky, V.; Scholthof, H.B. Tobacco Mosaic Virus: Pioneering Research for a Century. *Plant Cell* **1999**, *11*, 301–308, doi:10.1105/TPC.11.3.301.
222. Gamper, C.; Spenlé, C.; Boscá, S.; van der Heyden, M.; Erhardt, M.; Orend, G.; Bagnard, D.; Heinlein, M. Functionalized Tobacco Mosaic Virus Coat Protein Monomers and Oligomers as Nanocarriers for Anti-Cancer Peptides. *Cancers (Basel)*. **2019**, *11*, doi:10.3390/CANCERS11101609.
223. Franke, C.E.; Czapar, A.E.; Patel, R.B.; Steinmetz, N.F. Tobacco Mosaic Virus-Delivered Cisplatin Restores Efficacy in Platinum-Resistant Ovarian Cancer Cells. *Mol. Pharm.* **2018**, *15*, 2922–2931, doi:10.1021/ACS.MOLPHARMACEUT.7B00466.
224. Czapar, A.E.; Zheng, Y.R.; Riddell, I.A.; Shukla, S.; Awuah, S.G.; Lippard, S.J.; Steinmetz, N.F. Tobacco Mosaic Virus Delivery of Phenanthriplatin for Cancer Therapy. *ACS Nano* **2016**, *10*, 4119–4126, doi:10.1021/ACSNANO.5B07360.
225. Vernekar, A.A.; Berger, G.; Czapar, A.E.; Veliz, F.A.; Wang, D.I.; Steinmetz, N.F.; Lippard, S.J. Speciation of Phenanthriplatin and Its Analogs in the Core of Tobacco Mosaic Virus. *J. Am. Chem. Soc.* **2018**, *140*, 4279–4287, doi:10.1021/JACS.7B12697.
226. Venkataraman, S.; Apka, P.; Shoeb, E.; Badar, U.; Hefferon, K. Plant Virus Nanoparticles for Anti-Cancer Therapy. *Front. Bioeng. Biotechnol.* **2021**, *9*, doi:10.3389/FBIOE.2021.642794.
227. Parhizkar, E.; Rafieipour, P.; Sepasian, A.; Alemzadeh, E.; Dehshahri, A.; Ahmadi, F. Synthesis and Cytotoxicity Evaluation of Gemcitabine-Tobacco Mosaic Virus Conjugates. *J. Drug Deliv. Sci. Technol.* **2021**, *62*, 102388, doi:10.1016/J.JDDST.2021.102388.
228. Lin, R.D.; Steinmetz, N.F. Tobacco Mosaic Virus Delivery of Mitoxantrone for Cancer Therapy. *Nanoscale* **2018**, *10*, 16307–16313, doi:10.1039/C8NR04142C.
229. Kernan, D.L.; Wen, A.M.; Pitek, A.S.; Steinmetz, N.F. Featured Article: Delivery of Chemotherapeutic VcMMAE Using Tobacco Mosaic Virus Nanoparticles. *Exp. Biol. Med. (Maywood)*. **2017**, *242*, 1405–1411, doi:10.1177/1535370217719222.

230. Adams, M.J.; Antoniw, J.F.; Bar-Joseph, M.; Brunt, A.A.; Candresse, T.; Foster, G.D.; Martelli, G.P.; Milne, R.G.; Fauquet, C.M. Virology Division News: The New Plant Virus Family Flexiviridae and Assessment of Molecular Criteria for Species Demarcation. *Arch. Virol.* **2004**, *149*, 1045–1060, doi:10.1007/S00705-004-0304-0.
231. Massumi, H.; Poormohammadi, S.; Pishyar, S.; Maddahian, M.; Heydarnejad, J.; Hosseini-Pour, A.; van Bysterveldt, K.; Varsani, A. Molecular Characterization and Field Survey of Iranian Potato Virus X Isolates. *VirusDisease* **2014**, *25*, 338–344, doi:10.1007/S13337-014-0222-Z/FIGURES/2.
232. Park, M.R.; Kwon, S.J.; Choi, H.S.; Hemenway, C.L.; Kim, K.H. Mutations That Alter a Repeated ACCA Element Located at the 5' End of the Potato Virus X Genome Affect RNA Accumulation. *Virology* **2008**, *378*, 133–141, doi:10.1016/J.VIROL.2008.05.004.
233. Parker, L.; Kendall, A.; Stubbs, G. Surface Features of Potato Virus X from Fiber Diffraction. *Virology* **2002**, *300*, 291–295, doi:10.1006/VIRO.2002.1483.
234. Röder, J.; Dickmeis, C.; Commandeur, U. Small, Smaller, Nano: New Applications for Potato Virus X in Nanotechnology. *Front. Plant Sci.* **2019**, *10*, 158, doi:10.3389/FPLS.2019.00158/BIBTEX.
235. Nemykh, M.A.; Efimov, A. V.; Novikov, V.K.; Orlov, V.N.; Arutyunyan, A.M.; Drachev, V.A.; Lukashina, E. V.; Baratova, L.A.; Dobrov, E.N. One More Probable Structural Transition in Potato Virus X Virions and a Revised Model of the Virus Coat Protein Structure. *Virology* **2008**, *373*, 61–71, doi:10.1016/J.VIROL.2007.11.024.
236. Lico, C.; Benvenuto, E.; Baschieri, S. The Two-Faced Potato Virus X: From Plant Pathogen to Smart Nanoparticle. *Front. Plant Sci.* **2015**, *6*, 1009, doi:10.3389/FPLS.2015.01009/BIBTEX.
237. Grinzato, A.; Kandiah, E.; Lico, C.; Betti, C.; Baschieri, S.; Zanotti, G. Atomic Structure of Potato Virus X, the Prototype of the Alphaflexiviridae Family. *Nat. Chem. Biol.* **2020**, *16*, 564–569, doi:10.1038/S41589-020-0502-4.
238. Kwon, S.J.; Park, M.R.; Kim, K.W.; Plante, C.A.; Hemenway, C.L.; Kim, K.H. Cis-Acting Sequences Required for Coat Protein Binding and in Vitro Assembly of Potato Virus X. *Virology* **2005**, *334*, 83–97, doi:10.1016/J.VIROL.2005.01.018.
239. Le, D.H.T.; Lee, K.L.; Shukla, S.; Commandeur, U.; Steinmetz, N.F. Potato Virus X, a Filamentous Plant Viral Nanoparticle for Doxorubicin Delivery in Cancer Therapy. *Nanoscale* **2017**, *9*, 2348, doi:10.1039/C6NR09099K.

240. Shukla, S.; Roe, A.J.; Liu, R.; Veliz, F.A.; Commandeur, U.; Wald, D.N.; Steinmetz, N.F. Affinity of Plant Viral Nanoparticle Potato Virus X (PVX) towards Malignant B Cells Enables Cancer Drug Delivery. *Biomater. Sci.* **2020**, *8*, 3935, doi:10.1039/D0BM00683A.
241. Esfandiari, N.; Arzanani, M.K.; Soleimani, M.; Kohi-Habibi, M.; Svendsen, W.E. A New Application of Plant Virus Nanoparticles as Drug Delivery in Breast Cancer. *Tumour Biol.* **2016**, *37*, 1229–1236, doi:10.1007/S13277-015-3867-3.
242. Steinmetz, N.F. Viral Nanoparticles as Platforms for Next-Generation Therapeutics and Imaging Devices. *Nanomedicine Nanotechnology, Biol. Med.* **2010**, *6*, 634–641, doi:10.1016/J.NANO.2010.04.005.
243. Meshcheriakova, Y.; Durrant, A.; Hesketh, E.L.; Ranson, N.A.; Lomonossoff, G.P. Combining High-Resolution Cryo-Electron Microscopy and Mutagenesis to Develop Cowpea Mosaic Virus for Bionanotechnology. *Biochem. Soc. Trans.* **2017**, *45*, 1263, doi:10.1042/BST20160312.
244. Shukla, S.; Wang, C.; Beiss, V.; Cai, H.; Washington, T.; Murray, A.A.; Gong, X.; Zhao, Z.; Masarapu, H.; Zlotnick, A.; et al. The Unique Potency of Cowpea Mosaic Virus (CPMV) in Situ Cancer Vaccine. *Biomater. Sci.* **2020**, *8*, 5489–5503, doi:10.1039/D0BM01219J.
245. Czapar, A.E.; Tiu, B.D.B.; Veliz, F.A.; Pokorski, J.K.; Steinmetz, N.F. Slow-Release Formulation of Cowpea Mosaic Virus for In Situ Vaccine Delivery to Treat Ovarian Cancer. *Adv. Sci.* **2018**, *5*, doi:10.1002/ADVS.201700991.
246. Yildiz, I.; Lee, K.L.; Chen, K.; Shukla, S.; Steinmetz, N.F. Infusion of Imaging and Therapeutic Molecules into the Plant Virus-Based Carrier Cowpea Mosaic Virus: Cargo-Loading and Delivery. *J. Control. Release* **2013**, *172*, 568–578, doi:10.1016/J.JCONREL.2013.04.023.
247. Lico, C.; Schoubben, A.; Baschieri, S.; Blasi, P.; Santi, L. Nanoparticles in Biomedicine: New Insights from Plant Viruses. *Curr. Med. Chem.* **2013**, *20*, 3471–3487, doi:10.2174/09298673113209990035.
248. Sugiyama, Y.; Hamamoto, H.; Takemoto, S.; Watanabe, Y.; Okada, Y. Systemic Production of Foreign Peptides on the Particle Surface of Tobacco Mosaic Virus. *FEBS Lett.* **1995**, *359*, 247–250, doi:10.1016/0014-5793(95)00054-D.
249. Lico, C.; Capuano, F.; Renzone, G.; Donini, M.; Marusic, C.; Scaloni, A.; Benvenuto, E.; Baschieri, S. Peptide Display on Potato Virus X: Molecular Features of the Coat Protein-Fused Peptide Affecting Cell-to-Cell and Phloem Movement of Chimeric Virus Particles. *J. Gen. Virol.* **2006**, *87*, 3103–3112, doi:10.1099/vir.0.82097-0.

250. Porta, C.; Spall, V.E.; Findlay, K.C.; Gergerich, R.C.; Farrance, C.E.; Lomonossoff, G.P. Cowpea Mosaic Virus-Based Chimaeras: Effects of Inserted Peptides on the Phenotype, Host Range, and Transmissibility of the Modified Viruses. *Virology* **2003**, *310*, 50–63, doi:10.1016/S0042-6822(03)00140-5.
251. Fitchen, J.; Beachy, R.N.; Hein, M.B. Plant Virus Expressing Hybrid Coat Protein with Added Murine Epitope Elicits Autoantibody Response. *Vaccine* **1995**, *13*, 1051–1057, doi:10.1016/0264-410X(95)00075-C.
252. Lin, T.; Porta, C.; Lomonossoff, G.; Johnson, J.E. Structure-Based Design of Peptide Presentation on a Viral Surface: The Crystal Structure of a Plant/Animal Virus Chimera at 2.8 Å Resolution. *Fold. Des.* **1996**, *1*, 179–187, doi:10.1016/S1359-0278(96)00030-2.
253. Porta, C.; Spall, V.E.; Loveland, J.; Johnson, J.E.; Barker, P.J.; Lomonossoff, G.P. Development of Cowpea Mosaic Virus as a High-Yielding System for the Presentation of Foreign Peptides. *Virology* **1994**, *202*, 949–955, doi:10.1006/VIRO.1994.1417.
254. Turpen, T.H.; Reinl, S.J.; Charoenvit, Y.; Hoffman, S.L.; Fallarme, V.; Grill, L.K. Malarial Epitopes Expressed on the Surface of Recombinant Tobacco Mosaic Virus. *Biotechnology. (N. Y.)* **1995**, *13*, 53–57, doi:10.1038/NBT0195-53.
255. Narayanan, K.B.; Han, S.S. Recombinant Helical Plant Virus-Based Nanoparticles for Vaccination and Immunotherapy. *Virus Genes* **2018**, *54*, 623–637, doi:10.1007/S11262-018-1583-Y.
256. Grasso, S.; Santi, L. Viral Nanoparticles as Macromolecular Devices for New Therapeutic and Pharmaceutical Approaches. *Int. J. Physiol. Pathophysiol. Pharmacol.* **2010**, *2*, 161–178.
257. Werner, S.; Marillonnet, S.; Hause, G.; Klimyuk, V.; Gleba, Y. Immunoabsorbent Nanoparticles Based on a Tobamovirus Displaying Protein A. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 17678–17683, doi:10.1073/PNAS.0608869103.
258. Ryan, M.D.; Drew, J. Foot-and-Mouth Disease Virus 2A Oligopeptide Mediated Cleavage of an Artificial Polyprotein. *EMBO J.* **1994**, *13*, 928, doi:10.1002/J.1460-2075.1994.TB06337.X.
259. Donnelly, M.L.L.; Hughes, L.E.; Luke, G.; Mendoza, H.; Ten Dam, E.; Gani, D.; Ryan, M.D. The “cleavage” Activities of Foot-and-Mouth Disease Virus 2A Site-Directed Mutants and Naturally Occurring “2A-like” Sequences. *J. Gen. Virol.* **2001**, *82*, 1027–1041, doi:10.1099/0022-1317-82-5-1027.
260. Lockney, D.M.; Guenther, R.N.; Loo, L.; Overton, W.; Antonelli, R.; Clark, J.; Hu, M.; Luft, C.;

- Lommel, S.A.; Franzen, S. The Red Clover Necrotic Mosaic Virus Capsid as a Multifunctional Cell Targeting Plant Viral Nanoparticle. *Bioconjug. Chem.* **2011**, *22*, 67–73, doi:10.1021/BC100361Z.
261. Shahgolzari, M.; Pazhouhandeh, M.; Milani, M.; Yari Khosroushahi, A.; Fiering, S. Plant Viral Nanoparticles for Packaging and in Vivo Delivery of Bioactive Cargos. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2020**, *12*, doi:10.1002/WNAN.1629.
 262. Wen, A.M.; Steinmetz, N.F. Design of Virus-Based Nanomaterials for Medicine, Biotechnology, and Energy. *Chem. Soc. Rev.* **2016**, *45*, 4074, doi:10.1039/C5CS00287G.
 263. Ren, Y.; Sek, M.W.; Lim, L.Y. Folic Acid-Conjugated Protein Cages of a Plant Virus: A Novel Delivery Platform for Doxorubicin. *Bioconjug. Chem.* **2007**, *18*, 836–843, doi:10.1021/BC060361P.
 264. González-Gamboa, I.; Caparco, A.A.; McCaskill, J.M.; Steinmetz, N.F. Bioconjugation Strategies for Tobacco Mild Green Mosaic Virus. *Chembiochem* **2022**, *23*, doi:10.1002/CBIC.202200323.
 265. Kölmel, D.K.; Kool, E.T. Oximes and Hydrazones in Bioconjugation: Mechanism and Catalysis. *Chem. Rev.* **2017**, *117*, 10358–10376, doi:10.1021/ACS.CHEMREV.7B00090.
 266. McKay, C.S.; Finn, M.G. Click Chemistry in Complex Mixtures: Bioorthogonal Bioconjugation. *Chem. Biol.* **2014**, *21*, 1075, doi:10.1016/J.CHEMBIOL.2014.09.002.
 267. Qiu, X.; Kang, X.; Zhu, J.; Yi, L. Chemical Labeling and Crosslinking of Tobacco Mosaic Virus via Multi-Diazonium Reagents: Examples, Applications, and Prospects. *Mater. Adv.* **2022**, *3*, 5248–5259, doi:10.1039/D2MA00311B.
 268. Bruckman, M.A.; Kaur, G.; Lee, A.L.; Xie, F.; Sepulveda, J.; Breitenkamp, R.; Zhang, X.; Joralemon, M.; Russell, T.P.; Emrick, T.; et al. Surface Modification of Tobacco Mosaic Virus with “Click” Chemistry. *Chembiochem* **2008**, *9*, 519–523, doi:10.1002/CBIC.200700559.
 269. Schlick, T.L.; Ding, Z.; Kovacs, E.W.; Francis, M.B. Dual-Surface Modification of the Tobacco Mosaic Virus. *J. Am. Chem. Soc.* **2005**, *127*, 3718–3723, doi:10.1021/JA046239N.
 270. Bruckman, M.A.; Hern, S.; Jiang, K.; Flask, C.A.; Yu, X.; Steinmetz, N.F. Tobacco Mosaic Virus Rods and Spheres as Supramolecular High-Relaxivity MRI Contrast Agents. *J. Mater. Chem. B. Mater. Biol. Med.* **2013**, *1*, 1482, doi:10.1039/C3TB00461A.
 271. Yildiz, I.; Shukla, S.; Steinmetz, N.F. Applications of Viral Nanoparticles in Medicine. *Curr. Opin. Biotechnol.* **2011**, *22*, 901–908, doi:10.1016/J.COPBIO.2011.04.020.
 272. Liu, H.; Qu, C.; Johnson, J.E.; Case, D.A. Pseudo-Atomic Models of Swollen CCMV from Cryo-

- Electron Microscopy Data. *J. Struct. Biol.* **2003**, *142*, 356–363, doi:10.1016/S1047-8477(03)00028-5.
273. Oda, Y.; Saeki, K.; Takahashi, Y.; Maeda, T.; Naitow, H.; Tsukihara, T.; Fukuyama, K. Crystal Structure of Tobacco Necrosis Virus at 2.25 Å Resolution. *J. Mol. Biol.* **2000**, *300*, 153–169, doi:10.1006/JMBL2000.3831.
 274. Krüse, J.; Krüse, K.M.; Witz, J.; Chauvin, C.; Jacrot, B.; Tardieu, A. Divalent Ion-Dependent Reversible Swelling of Tomato Bushy Stunt Virus and Organization of the Expanded Virion. *J. Mol. Biol.* **1982**, *162*, 393–414, doi:10.1016/0022-2836(82)90534-4.
 275. Grasso, S.; Lico, C.; Imperatori, F.; Santi, L. A Plant Derived Multifunctional Tool for Nanobiotechnology Based on Tomato Bushy Stunt Virus. *Transgenic Res.* **2013**, *22*, 519–535, doi:10.1007/S11248-012-9663-6/FIGURES/5.
 276. Loo, L.; Guenther, R.H.; Lommel, S.A.; Franzen, S. Infusion of Dye Molecules into Red Clover Necrotic Mosaic Virus. *Chem. Commun. (Camb)*. **2008**, 88–90, doi:10.1039/B714748A.
 277. Cao, J.; Guenther, R.H.; Sit, T.L.; Lommel, S.A.; Opperman, C.H.; Willoughby, J.A. Development of Abamectin Loaded Plant Virus Nanoparticles for Efficacious Plant Parasitic Nematode Control. *ACS Appl. Mater. Interfaces* **2015**, *7*, 9546–9553, doi:10.1021/ACSAMI.5B00940.
 278. Wen, A.M.; Wang, Y.; Jiang, K.; Hsu, G.C.; Gao, H.; Lee, K.L.; Yang, A.C.; Yu, X.; Simon, D.I.; Steinmetz, N.F. Shaping Bio-Inspired Nanotechnologies to Target Thrombosis for Dual Optical-Magnetic Resonance Imaging. *J. Mater. Chem. B. Mater. Biol. Med.* **2015**, *3*, 6037, doi:10.1039/C5TB00879D.
 279. ADOLPH, K.W. The Conformation of the RNA in Cowpea Chlorotic Mottle Virus: Dye-Binding Studies. *Eur. J. Biochem.* **1975**, *53*, 449–455, doi:10.1111/J.1432-1033.1975.TB04085.X.
 280. Tama, F.; Brooks, C.L. The Mechanism and Pathway of PH Induced Swelling in Cowpea Chlorotic Mottle Virus. *J. Mol. Biol.* **2002**, *318*, 733–747, doi:10.1016/S0022-2836(02)00135-3.
 281. Azizgolshani, O.; Garmann, R.F.; Cadena-Nava, R.; Knobler, C.M.; Gelbart, W.M. Reconstituted Plant Viral Capsids Can Release Genes to Mammalian Cells. *Virology* **2013**, *441*, 12–17, doi:10.1016/J.VIROL.2013.03.001.
 282. Comellas-Aragonès, M.; Engelkamp, H.; Claessen, V.I.; Sommerdijk, N.A.J.M.; Rowan, A.E.; Christianen, P.C.M.; Maan, J.C.; Verduin, B.J.M.; Cornelissen, J.J.L.M.; Nolte, R.J.M. A Virus-Based Single-Enzyme Nanoreactor. *Nat. Nanotechnol.* **2007**, *2*, 635–639, doi:10.1038/NNANO.2007.299.

283. Sánchez-Sánchez, L.; Cadena-Nava, R.D.; Palomares, L.A.; Ruiz-Garcia, J.; Koay, M.S.T.; Cornelissen, J.J.M.T.; Vazquez-Duhalt, R. Chemotherapy Pro-Drug Activation by Biocatalytic Virus-like Nanoparticles Containing Cytochrome P450. *Enzyme Microb. Technol.* **2014**, *60*, 24–31, doi:10.1016/J.ENZMICTEC.2014.04.003.
284. Martelli, G.P.; Gallitelli, D.; Russo, M. Tombusviruses. In *The Plant Viruses*; Springer US: Boston, MA, 1988;pp. 13–72.
285. Oster, S.K.; Wu, B.; White, K.A. Uncoupled Expression of P33 and P92 Permits Amplification of Tomato Bushy Stunt Virus RNAs. *J. Virol.* **1998**, *72*, 5845–5851.
286. Scholthof, K.-B.G.; Scholthof, H.B.; Jackson, A.O. The Tomato Bushy Stunt Virus Replicase Proteins Are Coordinately Expressed and Membrane Associated. *Virology* **1995**, *208*, 365–369, doi:10.1006/viro.1995.1162.
287. Choi, I.-R.; White, K.A. An RNA Activator of Subgenomic MRNA1 Transcription in Tomato Bushy Stunt Virus. *J. Biol. Chem.* **2002**, *277*, 3760–3766, doi:10.1074/jbc.M109067200.
288. Scholthof, H.B. The Tombusvirus-Encoded P19: From Irrelevance to Elegance. *Nat. Rev. Microbiol.* **2006**, *4*, 405–411, doi:10.1038/nrmicro1395.
289. Harrison, S.C.; Olson, A.J.; Schutt, C.E.; Winkler, F.K.; Bricogne, G. Tomato Bushy Stunt Virus at 2.9 Å Resolution. *Nature* **1978**, *276*, 368–373, doi:10.1038/276368a0.
290. Olson, A.J.; Bricogne, G.; Harrison, S.C. Structure of Tomato Bushy Stunt Virus IV. The Virus Particle at 2.9 Å Resolution. *J. Mol. Biol.* **1983**, *171*, 61–93.
291. Joelson, T.; Åkerblom, L.; Oxelfelt, P.; Strandberg, B.; Tomenius, K.; Morris, T.J. Presentation of a Foreign Peptide on the Surface of Tomato Bushy Stunt Virus. *J. Gen. Virol.* **1997**, *78* (Pt 6), 1213–1217, doi:10.1099/0022-1317-78-6-1213.
292. Kumar, S.; Ochoa, W.; Singh, P.; Hsu, C.; Schneemann, A.; Manchester, M.; Olson, M.; Reddy, V. Tomato Bushy Stunt Virus (TBSV), a Versatile Platform for Polyvalent Display of Antigenic Epitopes and Vaccine Design. *Virology* **2009**, *388*, 185–190, doi:10.1016/J.VIROL.2009.02.051.
293. Zampieri, R.; Brozzetti, A.; Pericolini, E.; Bartoloni, E.; Gabrielli, E.; Roselletti, E.; Lomonosoff, G.; Meshcheriakova, Y.; Santi, L.; Imperatori, F.; et al. Prevention and Treatment of Autoimmune Diseases with Plant Virus Nanoparticles. *Sci. Adv.* **2020**, *6*, doi:10.1126/SCIADV.AAZ0295.
294. Blandino, A.; Lico, C.; Baschieri, S.; Barberini, L.; Ciroto, C.; Blasi, P.; Santi, L. In Vitro and in Vivo

Toxicity Evaluation of Plant Virus Nanocarriers. *Colloids Surfaces B Biointerfaces* **2015**, *129*, 130–136,
doi:10.1016/J.COLSURFB.2015.03.039.

Appendix II

References of Chapter 3

1. Grasso, S.; Lico, C.; Imperatori, F.; Santi, L. A Plant Derived Multifunctional Tool for Nanobiotechnology Based on Tomato Bushy Stunt Virus. *Transgenic Res.* **2013**, *22*, 519–535, doi:10.1007/S11248-012-9663-6.
2. Mancuso, M.; Pasquali, E.; Leonardi, S.; Tanori, M.; Rebessi, S.; Di Majo, V.; Pazzaglia, S.; Toni, M.P.; Pimpinella, M.; Covelli, V.; et al. Oncogenic Bystander Radiation Effects in Patched Heterozygous Mouse Cerebellum. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 12445–12450, doi:10.1073/PNAS.0804186105.
3. Tanno, B.; Leonardi, S.; Babini, G.; Giardullo, P.; De Stefano, I.; Pasquali, E.; Saran, A.; Mancuso, M. Nanog-Driven Cell-Reprogramming and Self-Renewal Maintenance in Ptch1 +/- Granule Cell Precursors after Radiation Injury. *Sci. Rep.* **2017**, *7*, doi:10.1038/S41598-017-14506-6.
4. Mota, F.; Fotinou, C.; Rana, R.R.; Chan, A.W.E.; Yelland, T.; Arooz, M.T.; O'Leary, A.P.; Hutton, J.; Frankel, P.; Zachary, I.; et al. Architecture and Hydration of the Arginine-Binding Site of Neuropilin-1. *FEBS J.* **2018**, *285*, 1290–1304, doi:10.1111/FEBS.14405.
5. Howard, E.I.; Guillot, B.; Blakeley, M.P.; Haertlein, M.; Moulin, M.; Mitschler, A.; Cousido-Siah, A.; Fadel, F.; Valsecchi, W.M.; Tomizaki, T.; et al. High-Resolution Neutron and X-Ray Diffraction Room-Temperature Studies of an H-FABP-Oleic Acid Complex: Study of the Internal Water Cluster and Ligand Binding by a Transferred Multipolar Electron-Density Distribution. *IUCrJ* **2016**, *3*, 115–126, doi:10.1107/S2052252515024161/TJ5008SUP1.PDF.
6. Arcangeli, C.; Lico, C.; Baschieri, S.; Mancuso, M. Characterization Of Blood–Brain Barrier Crossing And Tumor Homing Peptides By Molecular Dynamics Simulations. *Int. J. Nanomedicine* **2019**, *14*, 10123, doi:10.2147/IJN.S225793.
7. Fiser, A.; Šali, A. MODELLER: Generation and Refinement of Homology -Based Protein Structure Models. *Methods Enzymol.* **2003**, *374*, 461–491, doi:10.1016/S0076-6879(03)74020-8.
8. Vajda, S.; Yueh, C.; Beglov, D.; Bohnuud, T.; Mottarella, S.E.; Xia, B.; Hall, D.R.; Kozakov, D. New Additions to the ClusPro Server Motivated by CAPRI. *Proteins* **2017**, *85*, 435–444, doi:10.1002/PROT.25219.

9. Kozakov, D.; Beglov, D.; Bohnuud, T.; Mottarella, S.E.; Xia, B.; Hall, D.R.; Vajda, S. How Good Is Automated Protein Docking? *Proteins* **2013**, *81*, 2159–2166, doi:10.1002/PROT.24403.
10. Kozakov, D.; Hall, D.R.; Xia, B.; Porter, K.A.; Padhorny, D.; Yueh, C.; Beglov, D.; Vajda, S. The ClusPro Web Server for Protein-Protein Docking. *Nat. Protoc.* **2017**, *12*, 255–278, doi:10.1038/NPROT.2016.169.
11. Van Zundert, G.C.P.; Rodrigues, J.P.G.L.M.; Trellet, M.; Schmitz, C.; Kastiris, P.L.; Karaca, E.; Melquiond, A.S.J.; Van Dijk, M.; De Vries, S.J.; Bonvin, A.M.J.J. The HADDOCK2.2 Web Server: User-Friendly Integrative Modeling of Biomolecular Complexes. *J. Mol. Biol.* **2016**, *428*, 720–725, doi:10.1016/J.JMB.2015.09.014.
12. Van Der Spoel, D.; Lindahl, E.; Hess, B.; Groenhof, G.; Mark, A.E.; Berendsen, H.J.C. GROMACS: Fast, Flexible, and Free. *J. Comput. Chem.* **2005**, *26*, 1701–1718, doi:10.1002/JCC.20291.
13. Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual Molecular Dynamics. *J. Mol. Graph.* **1996**, *14*, 33–38, doi:10.1016/0263-7855(96)00018-5.
14. Harrison, S.C.; Olson, A.J.; Schutt, C.E.; Winkler, F.K.; Bricogne, G. Tomato Bushy Stunt Virus at 2.9 Å Resolution. *Nature* **1978**, *276*, 368–373, doi:10.1038/276368A0.
15. Olson, A.J.; Bricogne, G.; Harrison, S.C. Structure of Tomato Bushy Stunt Virus IV. The Virus Particle at 2.9 Å Resolution. *J. Mol. Biol.* **1983**, *171*, 61–93, doi:10.1016/S0022-2836(83)80314-3.
16. Carrillo-Tripp, M.; Shepherd, C.M.; Borelli, I.A.; Venkataraman, S.; Lander, G.; Natarajan, P.; Johnson, J.E.; Brooks, C.I.; Reddy, V.S. VIPERdb2: An Enhanced and Web API Enabled Relational Database for Structural Virology. *Nucleic Acids Res.* **2009**, *37*, D436, doi:10.1093/NAR/GKN840.
17. Lindorff-Larsen, K.; Piana, S.; Palmo, K.; Maragakis, P.; Klepeis, J.L.; Dror, R.O.; Shaw, D.E. Improved Side-Chain Torsion Potentials for the Amber Ff99SB Protein Force Field. *Proteins* **2010**, *78*, 1950–1958, doi:10.1002/PROT.22711.
18. Jorgensen, W.L.; Tirado-Rives, J. Potential Energy Functions for Atomic-Level Simulations of Water and Organic and Biomolecular Systems. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 6665–6670, doi:10.1073/PNAS.0408037102.
19. Dodda, L.S.; De Vaca, I.C.; Tirado-Rives, J.; Jorgensen, W.L. LigParGen Web Server: An Automatic OPLS-AA Parameter Generator for Organic Ligands. *Nucleic Acids Res.* **2017**, *45*, W331–W336, doi:10.1093/NAR/GKX312.

20. Dodda, L.S.; Vilseck, J.Z.; Tirado-Rives, J.; Jorgensen, W.L. 1.14*CM1A-LBCC: Localized Bond-Charge Corrected CM1A Charges for Condensed-Phase Simulations. *J. Phys. Chem. B* **2017**, *121*, 3864–3870, doi:10.1021/ACS.JPCB.7B00272.
21. Jorgensen, W.L.; Chandrasekhar, J.; Madura, J.D.; Impey, R.W.; Klein, M.L. Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys.* **1998**, *79*, 926, doi:10.1063/1.445869.
22. Petrova, S.S.; Solov'Ev, A.D. The Origin of the Method of Steepest Descent. *Hist. Math.* **1997**, *24*, 361–375, doi:10.1006/HMAT.1996.2146.
23. Bussi, G.; Donadio, D.; Parrinello, M. Canonical Sampling through Velocity Rescaling. *J. Chem. Phys.* **2007**, *126*, doi:10.1063/1.2408420.
24. Parrinello, M.; Rahman, A. Polymorphic Transitions in Single Crystals: A New Molecular Dynamics Method. *J. Appl. Phys.* **1998**, *52*, 7182, doi:10.1063/1.328693.
25. Hess, B.; Bekker, H.; Berendsen, H.J.C.; Fraaije, J.. E.M. LINCS: A Linear Constraint Solver for Molecular Simulations. *J. Comput. Chem.* **1997**, *18*, 1463–1472.
26. Daura, X.; Gademann, K.; Jaun, B.; Seebach, D.; Gunsteren, W.F. va.; Mark, A.E. Peptide Folding: When Simulation Meets Experiment. *Angew. Chemie-International Ed. English* **1999**, *38*, 236–240.
27. Baker, N.A.; Sept, D.; Joseph, S.; Holst, M.J.; McCammon, J.A. Electrostatics of Nanosystems: Application to Microtubules and the Ribosome. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 10037, doi:10.1073/PNAS.181342398.
28. Kumari, R.; Kumar, R.; Lynn, A. G_mmpbsa--a GROMACS Tool for High-Throughput MM-PBSA Calculations. *J. Chem. Inf. Model.* **2014**, *54*, 1951–1962, doi:10.1021/CI500020M.
29. Alemzadeh, E.; Izadpanah, K.; Ahmadi, F. Generation of Recombinant Protein Shells of Johnson Grass Chlorotic Stripe Mosaic Virus in Tobacco Plants and Their Use as Drug Carrier. *J. Virol. Methods* **2017**, *248*, 148–153, doi:10.1016/J.JVIROMET.2017.07.003.
30. Alipour, M.; Baneshi, M.; Hosseinkhani, S.; Mahmoudi, R.; Jabari Arabzadeh, A.; Akrami, M.; Mehrzad, J.; Bardania, H. Recent Progress in Biomedical Applications of RGD-Based Ligand: From Precise Cancer Theranostics to Biomaterial Engineering: A Systematic Review. *J. Biomed. Mater. Res. Part A* **2020**, *108*, 839–850, doi:10.1002/JBM.A.36862.
31. Roth, L.; Agemy, L.; Kotamraju, V.R.; Braun, G.; Teesalu, T.; Sugahara, K.N.; Hamzah, J.; Ruoslahti, E. Transtumoral Targeting Enabled by a Novel Neuropilin-Binding Peptide. *Oncogene* **2012**, *31*,

- 3754–3763, doi:10.1038/ONC.2011.537.
32. Feng, X.; Gao, X.; Kang, T.; Jiang, D.; Yao, J.; Jing, Y.; Song, Q.; Jiang, X.; Liang, J.; Chen, J. Mammary-Derived Growth Inhibitor Targeting Peptide-Modified PEG-PLA Nanoparticles for Enhanced Targeted Glioblastoma Therapy. *Bioconjug. Chem.* **2015**, *26*, 1850–1861, doi:10.1021/ACS.BIOCONJCHEM.5B00379.
 33. Wang, Q.; Kumar, V.; Lin, F.; Sethi, B.; Coulter, D.W.; McGuire, T.R.; Mahato, R.I. ApoE Mimetic Peptide Targeted Nanoparticles Carrying a BRD4 Inhibitor for Treating Medulloblastoma in Mice. *J. Control. Release* **2020**, *323*, 463–474, doi:10.1016/J.JCONREL.2020.04.053.
 34. Fan, X.; Eberhart, C.G. Medulloblastoma Stem Cells. *J. Clin. Oncol.* **2008**, *26*, 2821, doi:10.1200/JCO.2007.15.2264.
 35. Hyvönen, M.; Enbäck, J.; Huhtala, T.; Lammi, J.; Sihto, H.; Weisell, J.; Joensuu, H.; Rosenthal-Aizman, K.; El-Andaloussi, S.; Langel, U.; et al. Novel Target for Peptide-Based Imaging and Treatment of Brain Tumors. *Mol. Cancer Ther.* **2014**, *13*, 996, doi:10.1158/1535-7163.MCT-13-0684.
 36. Teesalu, T.; Sugahara, K.N.; Kotamraju, V.R.; Ruoslahti, E. C-End Rule Peptides Mediate Neurophilin-1-Dependent Cell, Vascular, and Tissue Penetration. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 16157–16162, doi:10.1073/PNAS.0908201106.
 37. Northcott, P.A.; Korshunov, A.; Witt, H.; Hielscher, T.; Eberhart, C.G.; Mack, S.; Bouffet, E.; Clifford, S.C.; Hawkins, C.E.; French, P.; et al. Medulloblastoma Comprises Four Distinct Molecular Variants. *J. Clin. Oncol.* **2011**, *29*, 1408–1414, doi:10.1200/JCO.2009.27.4324.
 38. Juraschka, K.; Taylor, M.D. Medulloblastoma in the Age of Molecular Subgroups: A Review. *J. Neurosurg. Pediatr.* **2019**, *24*, 353–363, doi:10.3171/2019.5.PEDS18381.
 39. Kool, M.; Jones, D.T.W.; Jäger, N.; Northcott, P.A.; Pugh, T.J.; Hovestadt, V.; Piro, R.M.; Esparza, L.A.; Markant, S.L.; Remke, M.; et al. Genome Sequencing of SHH Medulloblastoma Predicts Genotype-Related Response to Smoothed Inhibition. *Cancer Cell* **2014**, *25*, 393–405, doi:10.1016/J.CCR.2014.02.004.
 40. Zottel, A.; Paska, A.V.; Jovčevska, I. Nanotechnology Meets Oncology: Nanomaterials in Brain Cancer Research, Diagnosis and Therapy. *Mater. (Basel, Switzerland)* **2019**, *12*, doi:10.3390/MA12101588.
 41. Catanzaro, G.; Curcio, M.; Cirillo, G.; Spizzirri, U.G.; Besharat, Z.M.; Abballe, L.; Vacca, A.; Iemma,

- F.; Picci, N.; Ferretti, E. Albumin Nanoparticles for Glutathione-Responsive Release of Cisplatin: New Opportunities for Medulloblastoma. *Int. J. Pharm.* **2017**, *517*, 168–174, doi:10.1016/J.IJPHARM.2016.12.017.
42. Chenna, V.; Hu, C.; Pramanik, D.; Aftab, B.T.; Karikari, C.; Campbell, N.R.; Hong, S.M.; Zhao, M.; Rudek, M.A.; Khan, S.R.; et al. A Polymeric Nanoparticle Encapsulated Small-Molecule Inhibitor of Hedgehog Signaling (NanoHHI) Bypasses Secondary Mutational Resistance to Smoothed Antagonists. *Mol. Cancer Ther.* **2012**, *11*, 165–173, doi:10.1158/1535-7163.MCT-11-0341.
 43. Bell, J.B.; Rink, J.S.; Eckerdt, F.; Clymer, J.; Goldman, S.; Thaxton, C.S.; Platanius, L.C. HDL Nanoparticles Targeting Sonic Hedgehog Subtype Medulloblastoma. *Sci. Rep.* **2018**, *8*, doi:10.1038/S41598-017-18100-8.
 44. Kim, J.; Dey, A.; Malhotra, A.; Liu, J.; Ahn, S.I.; Sei, Y.J.; Kenney, A.M.; MacDonald, T.J.; Kim, Y.T. Engineered Biomimetic Nanoparticle for Dual Targeting of the Cancer Stem-like Cell Population in Sonic Hedgehog Medulloblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 24205–24212, doi:10.1073/PNAS.1911229117.
 45. Hwang, D.; Dismuke, T.; Tikunov, A.; Rosen, E.P.; Kagel, J.R.; Ramsey, J.D.; Lim, C.; Zamboni, W.; Kabanov, A. V.; Gershon, T.R.; et al. Poly(2-Oxazoline) Nanoparticle Delivery Enhances the Therapeutic Potential of Vismodegib for Medulloblastoma by Improving CNS Pharmacokinetics and Reducing Systemic Toxicity. *Nanomedicine* **2021**, *32*, doi:10.1016/J.NANO.2020.102345.
 46. Chariou, P.L.; Ortega-Rivera, O.A.; Steinmetz, N.F. Nanocarriers for the Delivery of Medical, Veterinary, and Agricultural Active Ingredients. *ACS Nano* **2020**, *14*, 2678–2701, doi:10.1021/ACSNANO.0C00173.
 47. Koudelka, K.J.; Destito, G.; Plummer, E.M.; Trauger, S.A.; Siuzdak, G.; Manchester, M. Endothelial Targeting of Cowpea Mosaic Virus (CPMV) via Surface Vimentin. *PLoS Pathog.* **2009**, *5*, 1000417, doi:10.1371/JOURNAL.PPAT.1000417.
 48. Zhao, J.; Zhang, L.; Dong, X.; Liu, L.; Huo, L.; Chen, H. High Expression of Vimentin Is Associated With Progression and a Poor Outcome in Glioblastoma. *Appl. Immunohistochem. Mol. Morphol. AIMM* **2018**, *26*, 337–344, doi:10.1097/PAL0000000000000420.
 49. Nkanga, C.I.; Steinmetz, N.F. The Pharmacology of Plant Virus Nanoparticles. *Virology* **2021**, *556*, 39–61, doi:10.1016/J.VIROL.2021.01.012.

50. Cao, Y.; Wang, L.; Nandy, D.; Zhang, Y.; Basu, A.; Radisky, D.; Mukhopadhyay, D. Neuropilin-1 Upholds Dedifferentiation and Propagation Phenotypes of Renal Cell Carcinoma Cells by Activating Akt and Sonic Hedgehog Axes. *Cancer Res.* **2008**, *68*, 8667–8672, doi:10.1158/0008-5472.CAN-08-2614.
51. Osada, H.; Tokunaga, T.; Nishi, M.; Hatanaka, H.; Abe, Y.; Tsugu, A.; Kijima, H.; Yamazaki, H.; Ueyama, Y.; Nakamura, M. Overexpression of the Neuropilin 1 (NRP1) Gene Correlated with Poor Prognosis in Human Glioma. *Anticancer Res.* **2004**, *24*, 547–552.
52. Snuderl, M.; Batista, A.; Kirkpatrick, N.D.; De Almodovar, C.R.; Riedemann, L.; Walsh, E.C.; Anolik, R.; Huang, Y.; Martin, J.D.; Kamoun, W.; et al. Targeting Placental Growth Factor/Neuropilin 1 Pathway Inhibits Growth and Spread of Medulloblastoma. *Cell* **2013**, *152*, 1065–1076, doi:10.1016/J.CELL.2013.01.036.
53. Pinskey, J.M.; Franks, N.E.; McMellen, A.N.; Giger, R.J.; Allen, B.L. Neuropilin-1 Promotes Hedgehog Signaling through a Novel Cytoplasmic Motif. *J. Biol. Chem.* **2017**, *292*, 15192–15204, doi:10.1074/JBC.M117.783845.
54. Smits, M.; Van Rijn, S.; Hulleman, E.; Biesmans, D.; Van Vuurden, D.G.; Kool, M.; Haberler, C.; Aronica, E.; Vandertop, W.P.; Noske, D.P.; et al. EZH2-Regulated DAB2IP Is a Medulloblastoma Tumor Suppressor and a Positive Marker for Survival. *Clin. Cancer Res.* **2012**, *18*, 4048–4058, doi:10.1158/1078-0432.CCR-12-0399.

Appendix III

References of Chapter 4

1. Mancuso, M.; Leonardi, S.; Ceccarelli, M.; Pasquali, E.; Stefano, I. De; Prisco, M.G.; Rebessi, S.; Tanori, M.; Scambia, G.; Majo, V. Di; et al. Protective Role of 17 β -Estradiol on Medulloblastoma Development in Patched 1 Heterozygous Mice. *Int. J. cancer* **2010**, *127*, 2749–2757, doi:10.1002/IJC.25293.
2. Le, D.H.T.; Lee, K.L.; Shukla, S.; Commandeur, U.; Steinmetz, N.F. Potato Virus X, a Filamentous Plant Viral Nanoparticle for Doxorubicin Delivery in Cancer Therapy. *Nanoscale* **2017**, *9*, 2348–2357, doi:10.1039/c6nr09099k.
3. Czabotar, P.E.; Colman, P.M.; Huang, D.C.S. Bax Activation by Bim? *Cell Death Differ.* **2009**, *16*, 1187–1191, doi:10.1038/CDD.2009.83.
4. Feng, X.; Gao, X.; Kang, T.; Jiang, D.; Yao, J.; Jing, Y.; Song, Q.; Jiang, X.; Liang, J.; Chen, J. Mammary-Derived Growth Inhibitor Targeting Peptide-Modified PEG-PLA Nanoparticles for Enhanced Targeted Glioblastoma Therapy. *Bioconjug. Chem.* **2015**, *26*, 1850–1861, doi:10.1021/ACS.BIOCONJCHEM.5B00379.
5. Lico, C.; Tanno, B.; Marchetti, L.; Novelli, F.; Giardullo, P.; Arcangeli, C.; Pazzaglia, S.; Podda, M.S.; Santi, L.; Bernini, R.; et al. Tomato Bushy Stunt Virus Nanoparticles as a Platform for Drug Delivery to Shh-Dependent Medulloblastoma. *Int. J. Mol. Sci.* **2021**, *22*, doi:10.3390/IJMS221910523.
6. Hyvönen, M.; Enbäck, J.; Huhtala, T.; Lammi, J.; Sihto, H.; Weisell, J.; Joensuu, H.; Rosenthal-Aizman, K.; El-Andaloussi, S.; Langel, U.; et al. Novel Target for Peptide-Based Imaging and Treatment of Brain Tumors. *Mol. Cancer Ther.* **2014**, *13*, 996–1007, doi:10.1158/1535-7163.MCT-13-0684.
7. Rezar, R.; Jirak, P.; Gschwandtner, M.; Derler, R.; Felder, T.K.; Haslinger, M.; Kopp, K.; Seelmaier, C.; Granitz, C.; Hoppe, U.C.; et al. Heart-Type Fatty Acid-Binding Protein (H-FABP) and Its Role as a Biomarker in Heart Failure: What Do We Know So Far? *J. Clin. Med.* **2020**, *9*, doi:10.3390/JCM9010164.
8. Menyhárt, O.; Győrffy, B. Molecular Stratifications, Biomarker Candidates and New Therapeutic Options in Current Medulloblastoma Treatment Approaches. *Cancer Metastasis Rev.* **2020**, *39*, 211–233, doi:10.1007/S10555-020-09854-1.

9. Eom, Y.W.; Kim, M.A.; Park, S.S.; Goo, M.J.; Kwon, H.J.; Sohn, S.; Kim, W.H.; Yoon, G.; Choi, K.S. Two Distinct Modes of Cell Death Induced by Doxorubicin: Apoptosis and Cell Death through Mitotic Catastrophe Accompanied by Senescence-like Phenotype. *Oncogene* **2005**, *24*, 4765–4777, doi:10.1038/SJ.ONC.1208627.
10. de Lima Junior, E.A.; Yamashita, A.S.; Pimentel, G.D.; De Sousa, L.G.O.; Santos, R.V.T.; Gonçalves, C.L.; Streck, E.L.; de Lira, F.S.; Rosa Neto, J.C. Doxorubicin Caused Severe Hyperglycaemia and Insulin Resistance, Mediated by Inhibition in AMPk Signalling in Skeletal Muscle. *J. Cachexia. Sarcopenia Muscle* **2016**, *7*, 615–625, doi:10.1002/JCSM.12104.
11. C. Baiu, D.; S. Brazel, C.; Bao, Y.; Otto, M. Interactions of Iron Oxide Nanoparticles with the Immune System: Challenges and Opportunities for Their Use in Nano-Oncology. *Curr. Pharm. Des.* **2013**, *19*, 6606–6621, doi:10.2174/13816128113199990409.
12. Meng, J.; Yang, M.; Jia, F.; Xu, Z.; Kong, H.; Xu, H. Immune Responses of BALB/c Mice to Subcutaneously Injected Multi-Walled Carbon Nanotubes. *Nanotoxicology* **2011**, *5*, 583–591, doi:10.3109/17435390.2010.523483.
13. Semple, S.C.; Harasym, T.O.; Clow, K.A.; Ansell, S.M.; Klimuk, S.K.; Hope, M.J. Immunogenicity and Rapid Blood Clearance of Liposomes Containing Polyethylene Glycol-Lipid Conjugates and Nucleic Acid. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 1020–1026, doi:10.1124/JPET.104.078113.
14. Raja, K.S.; Wang, Q.; Gonzalez, M.J.; Manchester, M.; Johnson, J.E.; Finn, M.G. Hybrid Virus-Polymer Materials. 1. Synthesis and Properties of PEG-Decorated Cowpea Mosaic Virus. **2003**, doi:10.1021/BM025740.
15. Milton Harris, J.; Chess, R.B. Effect of Pegylation on Pharmaceuticals. *Nat. Rev. Drug Discov.* **2003**, *2*, 214–221, doi:10.1038/NRD1033.
16. Shimizu, T.; Ichihara, M.; Yoshioka, Y.; Ishida, T.; Nakagawa, S.; Kiwada, H. Intravenous Administration of Polyethylene Glycol-Coated (PEGylated) Proteins and PEGylated Adenovirus Elicits an Anti-PEG Immunoglobulin M Response. *Biol. Pharm. Bull.* **2012**, *35*, 1336–1342, doi:10.1248/BPB.B12-00276.
17. Ishida, T.; Wang, X.Y.; Shimizu, T.; Nawata, K.; Kiwada, H. PEGylated Liposomes Elicit an Anti-PEG IgM Response in a T Cell-Independent Manner. *J. Control. Release* **2007**, *122*, 349–355, doi:10.1016/J.JCONREL.2007.05.015.

18. Verhoef, J.J.F.; Anchordoquy, T.J. Questioning the Use of PEGylation for Drug Delivery. *Drug Deliv. Transl. Res.* **2013**, *3*, 499, doi:10.1007/S13346-013-0176-5.

Appendix IV

References of Chapter 5

1. Grasso, S.; Lico, C.; Imperatori, F.; Santi, L. A Plant Derived Multifunctional Tool for Nanobiotechnology Based on Tomato Bushy Stunt Virus. *Transgenic Res.* **2013**, *22*, 519–535, doi:10.1007/S11248-012-9663-6/FIGURES/5.
2. Cantuti-Castelvetri, L.; Ojha, R.; Pedro, L.D.; Djannatian, M.; Franz, J.; Kuivanen, S.; van der Meer, F.; Kallio, K.; Kaya, T.; Anastasina, M.; et al. Neuropilin-1 Facilitates SARS-CoV-2 Cell Entry and Infectivity. *Science* (80-.). **2020**, *370*, doi:10.1126/SCIENCE.ABD2985/SUPPL_FILE/ABD2985_MДАР_REPRODUCIBILITY_CHECKLIST.PDF.
3. Lico, C.; Tanno, B.; Marchetti, L.; Novelli, F.; Giardullo, P.; Arcangeli, C.; Pazzaglia, S.; Podda, M.S.; Santi, L.; Bernini, R.; et al. Tomato Bushy Stunt Virus Nanoparticles as a Platform for Drug Delivery to Shh-Dependent Medulloblastoma. *Int. J. Mol. Sci.* **2021**, *22*, doi:10.3390/IJMS221910523.
4. Harrison, S.C.; Olson, A.J.; Schutt, C.E.; Winkler, F.K.; Bricogne, G. Tomato Bushy Stunt Virus at 2.9 Å Resolution. *Nat.* 1978 2765686 **1978**, *276*, 368–373, doi:10.1038/276368a0.
5. Olson, A.J.; Bricogne, G.; Harrison, S.C. Structure of Tomato Bushy Stunt Virus IV: The Virus Particle at 2.9 resolution. *J. Mol. Biol.* **1983**, *171*, 61–93, doi:10.1016/S0022-2836(83)80314-3.
6. Teesalu, T.; Sugahara, K.N.; Kotamraju, V.R.; Ruoslahti, E. C-End Rule Peptides Mediate Neuropilin-1-Dependent Cell, Vascular, and Tissue Penetration. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 16157–16162, doi:10.1073/PNAS.0908201106.
7. Toome, K.; Willmore, A.M.A.; Paiste, P.; Tobi, A.; Sugahara, K.N.; Kirsimäe, K.; Ruoslahti, E.; Braun, G.B.; Teesalu, T. Ratiometric in Vivo Auditioning of Targeted Silver Nanoparticles. *Nanoscale* **2017**, *9*, 10094–10100, doi:10.1039/C7NR04056C.
8. Blandino, A.; Lico, C.; Baschieri, S.; Barberini, L.; Cirotto, C.; Blasi, P.; Santi, L. In Vitro and in Vivo Toxicity Evaluation of Plant Virus Nanocarriers. *Colloids Surfaces B Biointerfaces* **2015**, *129*, 130–136, doi:10.1016/J.COLSURFB.2015.03.039.
9. Lico, C.; Giardullo, P.; Mancuso, M.; Benvenuto, E.; Santi, L.; Baschieri, S. A Biodistribution Study of Two Differently Shaped Plant Virus Nanoparticles Reveals New Peculiar Traits. *Colloids Surfaces B Biointerfaces* **2016**, *148*, 431–439, doi:10.1016/J.COLSURFB.2016.09.019.

10. Milton Harris, J.; Chess, R.B. Effect of Pegylation on Pharmaceuticals. *Nat. Rev. Drug Discov.* **2003**, *2*, 214–221, doi:10.1038/nrd1033.
11. Wattendorf, U.; Merkle, H.P. PEGylation as a Tool for the Biomedical Engineering of Surface Modified Microparticles. *J. Pharm. Sci.* **2008**, *97*, 4655–4669, doi:10.1002/JPS.21350.
12. Rudnick, S.I.; Lou, J.; Shaller, C.C.; Tang, Y.; Klein-Szanto, A.J.P.; Weiner, L.M.; Marks, J.D.; Adams, G.P. Influence of Affinity and Antigen Internalization on the Uptake and Penetration of Anti-HER2 Antibodies in Solid Tumors. *Cancer Res.* **2011**, *71*, 2250, doi:10.1158/0008-5472.CAN-10-2277.
13. Diaz Bessone, M.I.; Simón-Gracia, L.; Scodeller, P.; Ramirez, M.D.L.A.; Lago Huvelle, M.A.; Soler-Illia, G.J.A.A.; Simian, M. IRGD-Guided Tamoxifen Polymersomes Inhibit Estrogen Receptor Transcriptional Activity and Decrease the Number of Breast Cancer Cells with Self-Renewing Capacity. *J. Nanobiotechnology* **2019**, *17*, 1–14, doi:10.1186/S12951-019-0553-4/FIGURES/8.
14. Simón-Gracia, L.; Scodeller, P.; Fuentes, S.S.; Vallejo, V.G.; Ríos, X.; Sebastián, E.S.; Sidorenko, V.; Di Silvio, D.; Suck, M.; De Lorenzi, F.; et al. Application of Polymersomes Engineered to Target P32 Protein for Detection of Small Breast Tumors in Mice. *Oncotarget* **2018**, *9*, 18682–18697, doi:10.18632/ONCOTARGET.24588.
15. Hunt, H.; Simón-Gracia, L.; Tobi, A.; Teesalu, T.; Kotamraju, V.R.; Sharma, S.; Sugahara, K.N.; Ruoslahti, E.; Teesalu, T.; Nigul, M.; et al. Targeting of P32 in Peritoneal Carcinomatosis with Intraperitoneal LinTT1 Peptide-Guided pro-Apoptotic Nanoparticles. *J. Control. Release* **2017**, *260*, 142–153, doi:10.1016/J.JCONREL.2017.06.005.
16. Säälk, P.; Lingasamy, P.; Toome, K.; Mastandrea, I.; Rouso-Noori, L.; Tobi, A.; Simón-Gracia, L.; Hunt, H.; Paiste, P.; Kotamraju, V.R.; et al. Peptide-Guided Nanoparticles for Glioblastoma Targeting. *J. Control. Release* **2019**, *308*, 109–118, doi:10.1016/J.JCONREL.2019.06.018.
17. Simón-Gracia, L.; Sidorenko, V.; Uustare, A.; Ogibalov, I.; Tasa, A.; Tshubrik, O.; Teesalu, T. Novel Anthracycline Utorubicin for Cancer Therapy. *Angew. Chem. Int. Ed. Engl.* **2021**, *60*, 17018–17027, doi:10.1002/ANIE.202016421.
18. Zern, B.J.; Chacko, A.M.; Liu, J.; Greineder, C.F.; Blankemeyer, E.R.; Radhakrishnan, R.; Muzykantov, V. Reduction of Nanoparticle Avidity Enhances the Selectivity of Vascular Targeting and PET Detection of Pulmonary Inflammation. *ACS Nano* **2013**, *7*, 2461, doi:10.1021/NN305773F.

19. Patel, S.; Kim, J.; Herrera, M.; Mukherjee, A.; Kabanov, A. V.; Sahay, G. Brief Update on Endocytosis of Nanomedicines. *Adv. Drug Deliv. Rev.* **2019**, *144*, 90, doi:10.1016/J.ADDR.2019.08.004.
20. Plummer, E.M.; Manchester, M. Endocytic Uptake Pathways Utilized by CPMV Nanoparticles. *Mol. Pharm.* **2013**, *10*, 26–32, doi:10.1021/MP300238W.
21. Masarapu, H.; Patel, B.K.; Chariou, P.L.; Hu, H.; Gulati, N.M.; Carpenter, B.L.; Ghiladi, R.A.; Shukla, S.; Steinmetz, N.F. Physalis Mottle Virus-Like Particles as Nanocarriers for Imaging Reagents and Drugs. *Biomacromolecules* **2017**, *18*, 4141–4153, doi:10.1021/ACS.BIOMAC.7B01196.
22. Vishnu Vardhan, G.P.; Hema, M.; Sushmitha, C.; Savithri, H.S.; Natraj, U.; Murthy, M.R.N. Development of Sesbania Mosaic Virus Nanoparticles for Imaging. *Arch. Virol.* **2019**, *164*, 497–507, doi:10.1007/S00705-018-4097-Y.
23. Simón-Gracia, L.; Hunt, H.; Scodeller, P.D.; Gaitzsch, J.; Braun, G.B.; Willmore, A.M.A.; Ruoslahti, E.; Battaglia, G.; Teesalu, T. Paclitaxel-Loaded Polymersomes for Enhanced Intraperitoneal Chemotherapy. *Mol. Cancer Ther.* **2016**, *15*, 670–679, doi:10.1158/1535-7163.MCT-15-0713-T.
24. Simonetti, B.; Daly, J.L.; Simón-Gracia, L.; Klein, K.; Weeratunga, S.; Antón-Plágaro, C.; Tobi, A.; Hodgson, L.; Lewis, P.A.; Heesom, K.J.; et al. ESCPE-1 Mediates Retrograde Endosomal Sorting of the SARS-CoV-2 Host Factor Neuropilin-1. *Proc. Natl. Acad. Sci. U. S. A.* **2022**, *119*, doi:10.1073/PNAS.2201980119.