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Targeted Liposomal Drug Delivery To Pediatric Sarcomas

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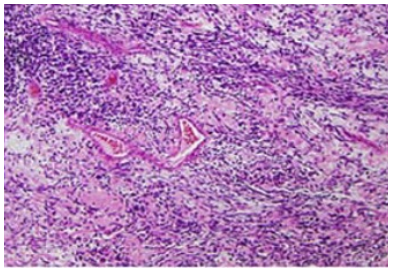
PRC symposium 2022 – 13.9.2022

tumortargeting.ch

Rhabdomyosarcoma

Rhabdo (rod) myo (muscle) sarcoma (connective tissue tumor)

Embryonal RMS

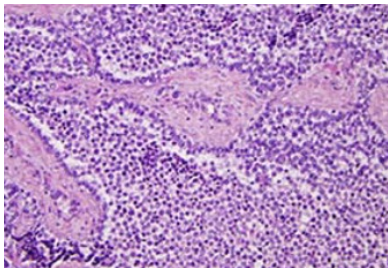


60-70%
Young children ≤ 5 years
Head and neck

Combination of diverse genetic aberrations

FUSION NEGATIVE

Alveolar RMS



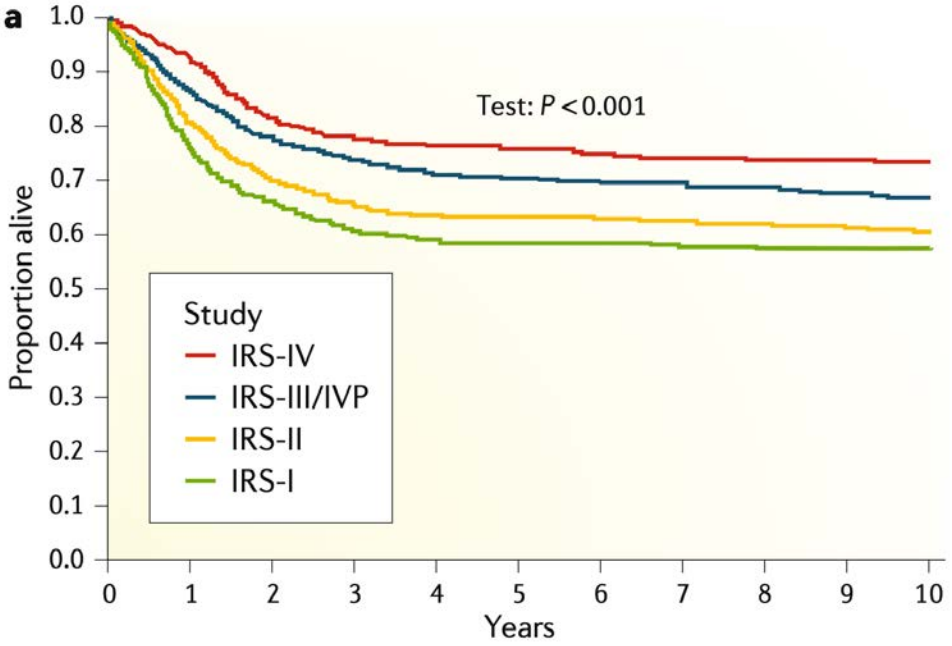
20%
Young adolescents
Trunk and extremities

Chromosomal translocation:
PAX3/7-FOXO1

FUSION POSITIVE

Rhabdomyosarcoma

- Chemotherapy
  - VAC (US)
    - Vincristine
    - Actinomycin D
    - Cyclophosphamid
  - IVADo (EU)
    - Ifosfamid
    - Vincristine
    - Actinomycin D
    - Doxorubicin
- Surgery
- Radiotherapy

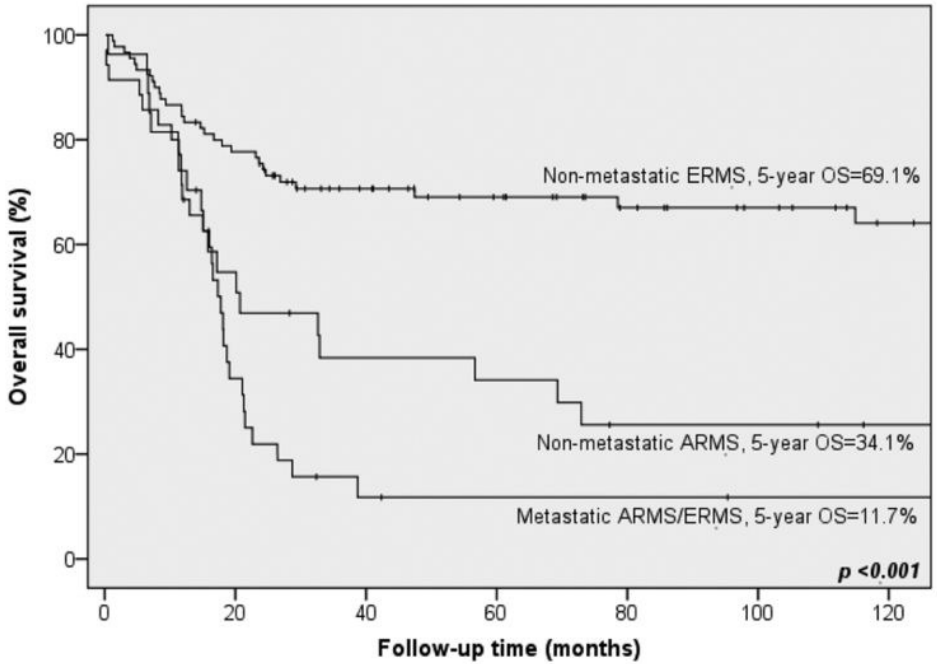


Skapek, S. X. et al. Rhabdomyosarcoma. Nat Rev Dis Primers 5, 1 (2019)

Rhabdomyosarcoma

- Chemotherapy
  - VAC (US)
    - Vincristine
    - Actinomycin D
    - Cyclophosphamid
  - IVADo (EU)
    - Ifosfamid
    - Vincristine
    - Actinomycin D
    - Doxorubicin
- Surgery
- Radiotherapy

Van Gaal et al., *Anticancer Research*, 2012



RMS targeting peptides

In vivo phage display

- RMS-P3/RR
  CMGTINTRTRRC

- Furin as target

- Furin in RMS progression

OPEN ACCESS Freely available online

PLoS one

Furin Targeted Drug Delivery for Treatment of Rhabdomyosarcoma in a Mouse Model

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Katarina Hajdin

www.impactjournals.com/oncotarget/ Oncotarget, Advance Publications 2016

The proprotein convertase furin is required to maintain viability of alveolar rhabdomyosarcoma cells

Patricia Jaaks[1], Gianmarco Meier[1], Nagjie Alijaj[1], Eva Brack[1], Peter Bode[2], Ewa Koscielniak[3], Marco Wachtel[1], Beat W. Schäfer[1], Michele Bernasconi[1]

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RESEARCH ARTICLE

The Proprotein Convertase Furin Contributes to Rhabdomyosarcoma Malignancy by Promoting Vascularization, Migration and Invasion

Patricia Jaaks[1,2], Valentina D'Alessandro[1,2], Nicole Grob[1,2], Sina Büel[1,2], Katarina Hajdin[1,2], Beat W. Schäfer[1,2], Michele Bernasconi[1,2]*

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Patricia Jaaks

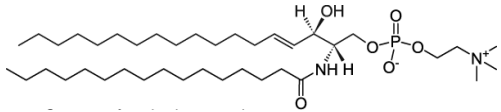
Liposomes formulation

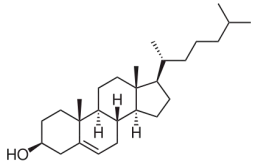
Egg-Sphingomyelin (E)
- Amide-linked aliphatic chain → low susceptibility to hydrolysis or enzymatic degradation (Webb et al., 1995)

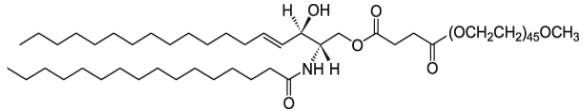
Cholesterol (C)
- Decreased permeability of the membrane
- Mechanical rigidity of fluid bilayers (Kirby et al., 1980)

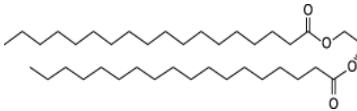
PEG-Ceramide (PEGC)
- Increased circulation lifetime of liposomes
- Slow *in vivo* drug release (Webb et al., 1998)

DSPE-PEG (DPEG)
- Coupling reaction
- Increased *in vivo* leakage of Vincristine (Webb et al., 1998)





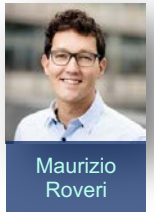




n = 3

Optimal formulation:

E	C	PEGC	DPEG
50	45	4	1



Maurizio Roveri

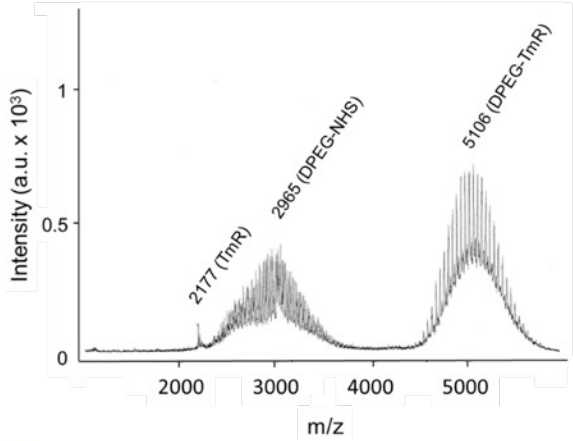
Liposomes formulation

TmR-NH_2
KRDRGGGCMGTINTRTRRC

DSPE-PEG(2000)-NHS

Optimized conditions:
– Anhydrous dimethyl sulfoxide, 3 h under constant stirring, room temperature
– DSPE-PEG(2000)-NHS : RMS-P3/RR-NH_2 (5 : 1 molar)

E	C	PEGC	DPEG	DPEG-TmR
49.8	45	4	1	0.2

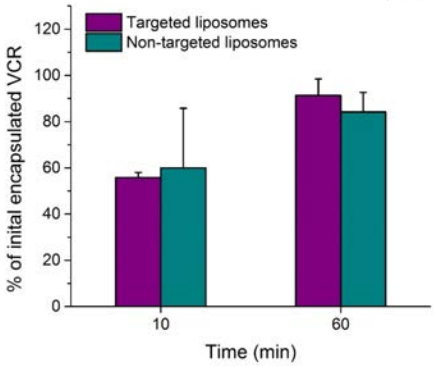


Active encapsulation by pH-gradient

- *Buffer exchange with Sephadex*

Drug loading: incubation for 60 min at 65 ºC

- *Separation of free VCR*
- *Centrifugation in Amicon-tubes*
- *Disruption of liposomes with EtOH*
- *VCR quantification with HPLC*



Roveri, M. *et al. Nanomedicine* **12**, 1135 1151 (2017)

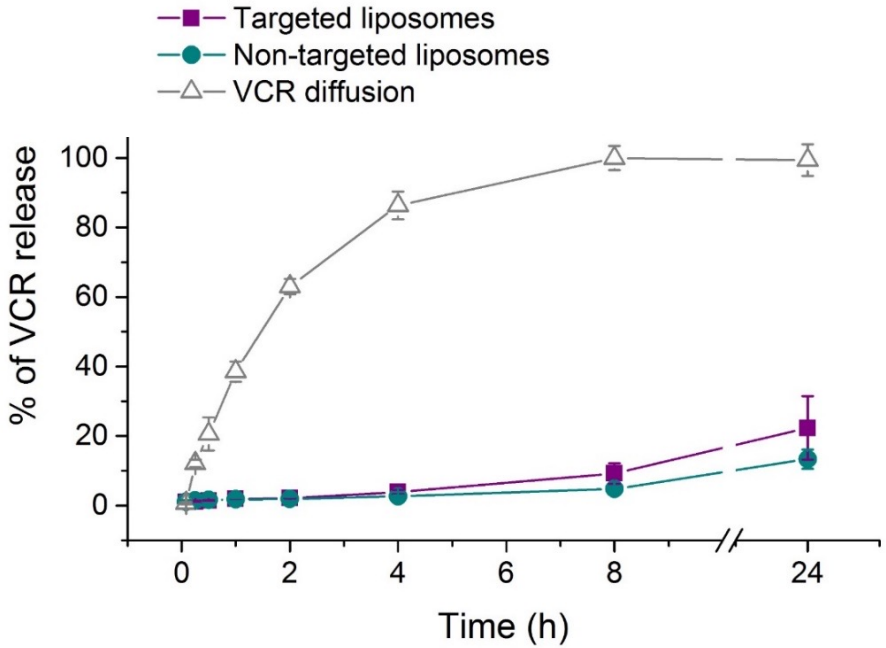
VCR release from targeted liposomes

Horizontal diffusion cells

- Semi-permeable membrane (polycarbonate, 200 nm)
- 100% serum / 37 ºC

VCR-loaded liposomes in the donor chamber

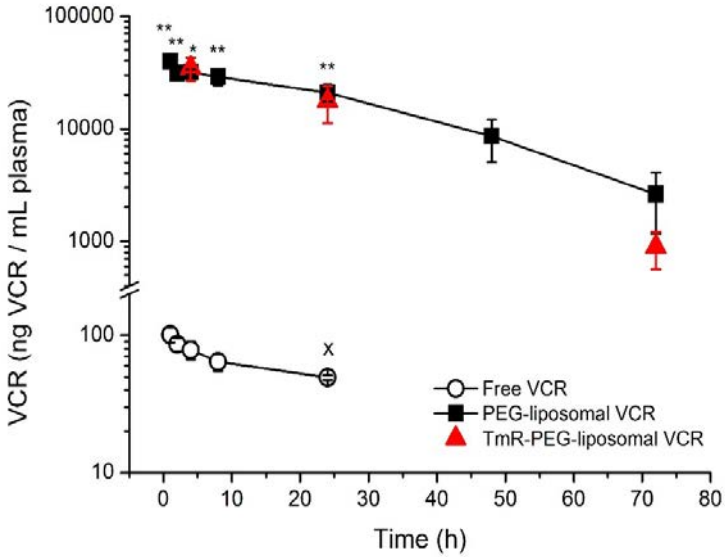
Monitoring of the free VCR in the acceptor chamber with HPLC



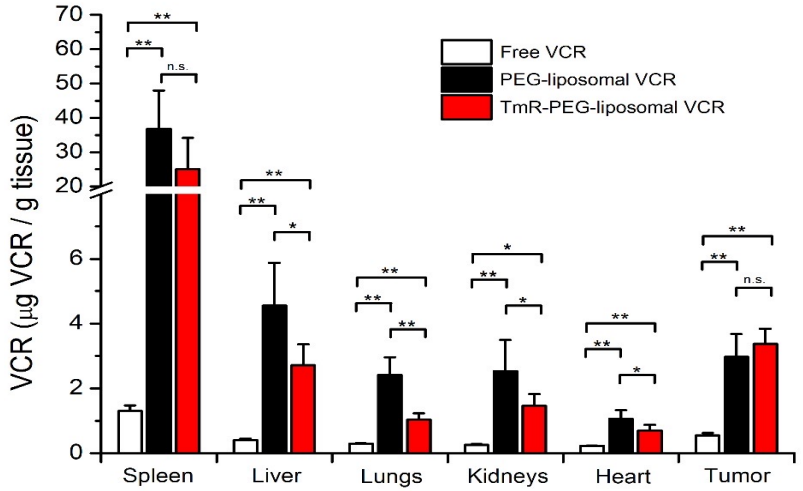
Roveri, M. *et al*. *Nanomedicine* **12**, 1135 1151 (2017)

Biodistribution of VCR-loaded Liposomes in RMS mouse xenografts

Plasma concentration of VCR

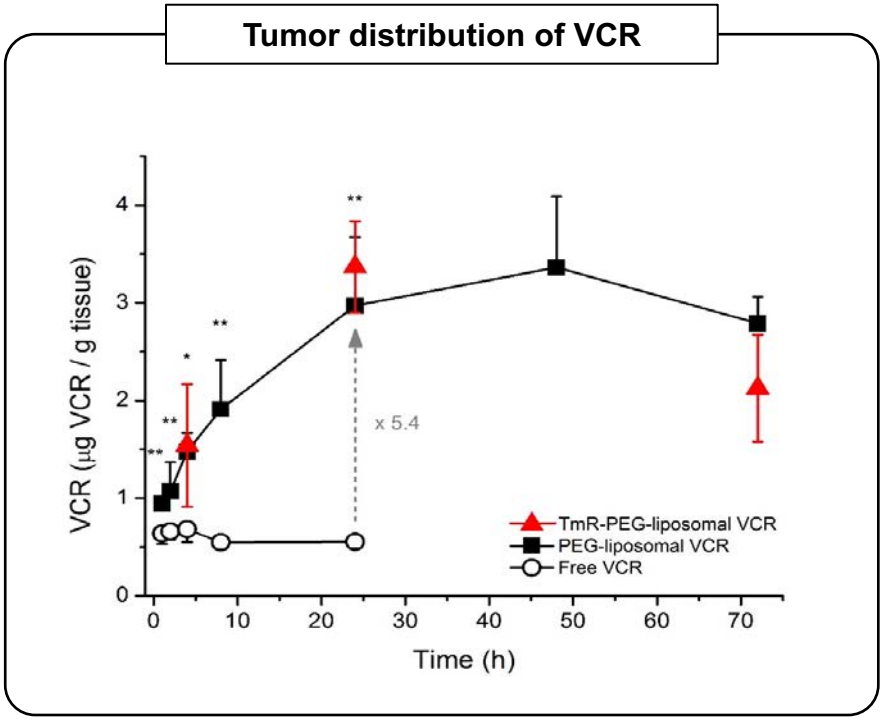


Tissue distribution of VCR after 24 h



Roveri, M. *et al*. *Nanomedicine* **12**, 1135 1151 (2017)

Biodistribution of VCR-loaded Liposomes in RMS xenograft mice



Roveri, M. *et al*. *Nanomedicine* **12**, 1135 1151 (2017)

Biodistribution of VCR-loaded Liposomes in RMS xenograft mice

Table 1. Comparison of pharmacokinetic parameters of free vincristine and PEG-liposomal vincristine.

VCR formulation (2 mg/kg)	$AUC_{0-24\,h}$ † (μg•h/ml)	$AUC_{0-\infty}$ ‡ (μg•h/ml)	C_{max} § (μg/ml)	$t_{1/2}$ ¶ (h)	λ_z # (1/h)	CL †† (l/h/kg)	V_D ‡‡ (l/kg)
Free	1.6	3.6	0.1	14.2	0.049	0.552	23.2
PEG-Liposomes	664	1672.5	39.8	46.2	0.015	0.001	0.06

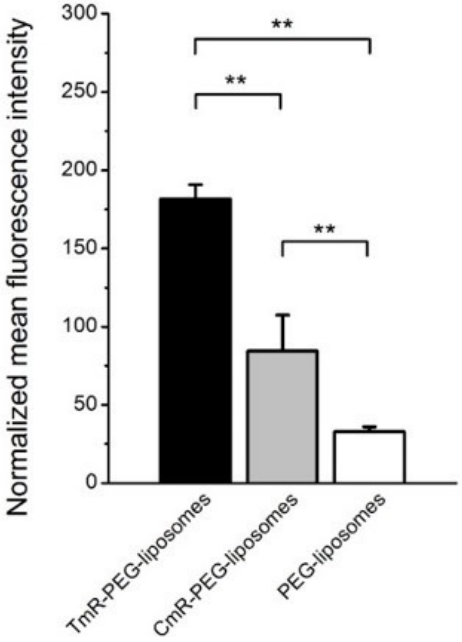
Noncompartmental pharmacokinetic analysis was used to calculate the plasma versus time curves (AUC) of free and bare PEG-liposomal VCR.
†Area under the plasma concentration–time curve for 24 h.
‡Area under the plasma concentration-time curve extrapolated for t = ∞.
§Maximum plasma drug concentration.
¶Elimination half-life calculated from 2 to 8 h, since after this time point the plasma concentration of free VCR was below the LLOQ.
#Terminal elimination rate constant.
††Total body clearance.
‡‡Volume of distribution at steady state.
AUC: Area under cover; CL: Clearance; VCR: Vincristine; LLOQ: Lower limit of quantifi

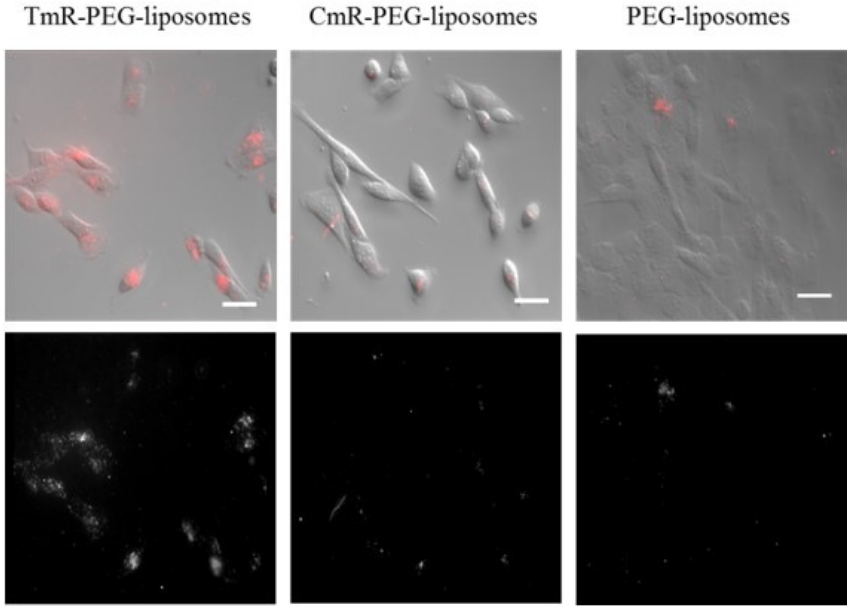
Parameter	Formulation		
	C-VINC	DSPC/Chol	SM/Chol
Vincristine dose	2 mg/kg	2 mg/kg	2 mg/kg
C_{max} (μg/ml)	0.30 (± 0.07)	26.3 (± 1.1)	22.2 (± 1.30)
Half-life (h)	1.36 (± 0.88)	4.0 (± 0.50)	6.65 (± 1.24)
MRT (h)	1.96 (± 1.26)	5.82 (± 0.72)	9.59 (± 1.79)
AUC (μg · h/ml)	0.59 (± 0.29)	153.2 (± 15.8)	213.4 (± 34.3)
Vss (ml)	145.4 (± 32.9)	1.67 (± 0.07)	1.97 (± 0.11)

Krishna, R. *et al. The Journal of pharmacology and experimental therapeutics* **298**, 1206 1212 (2001).

Roveri, M. *et al*. *Nanomedicine* **12**, 1135 1151 (2017)

In vitro binding of TmR-liposomes to RMS cells

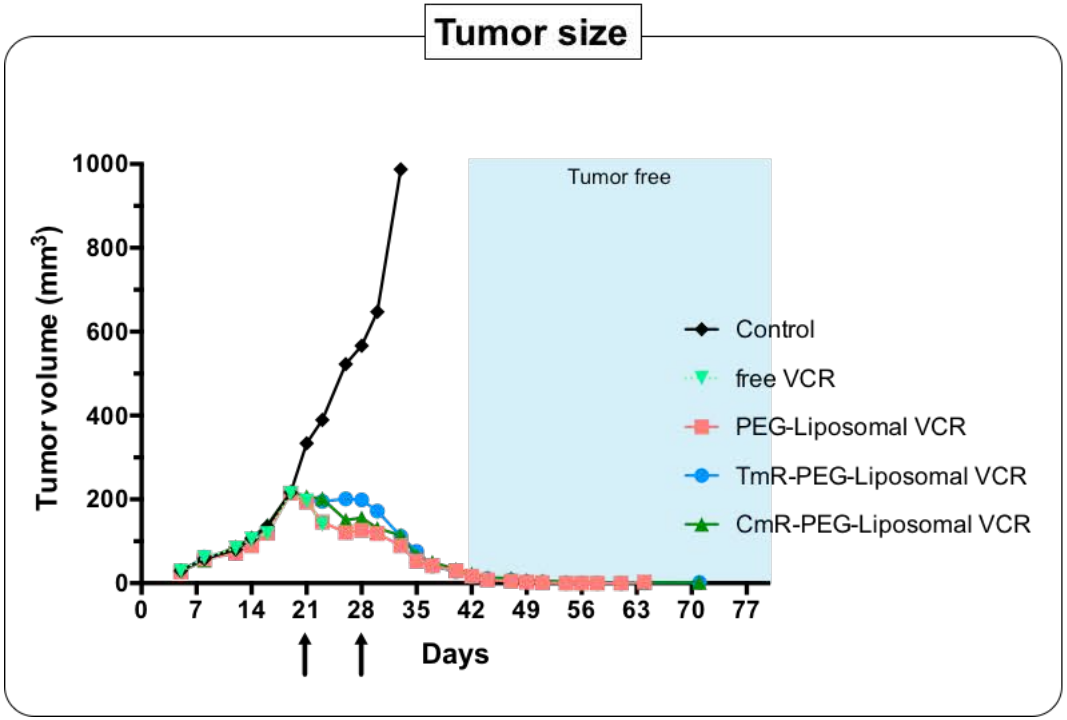




- Rh30 cells
- DiD-labelled liposomes
- 1 mM liposomes
- 2h / 37°C

Roveri, M. *et al*. *Nanomedicine* **12**, 1135 1151 (2017)

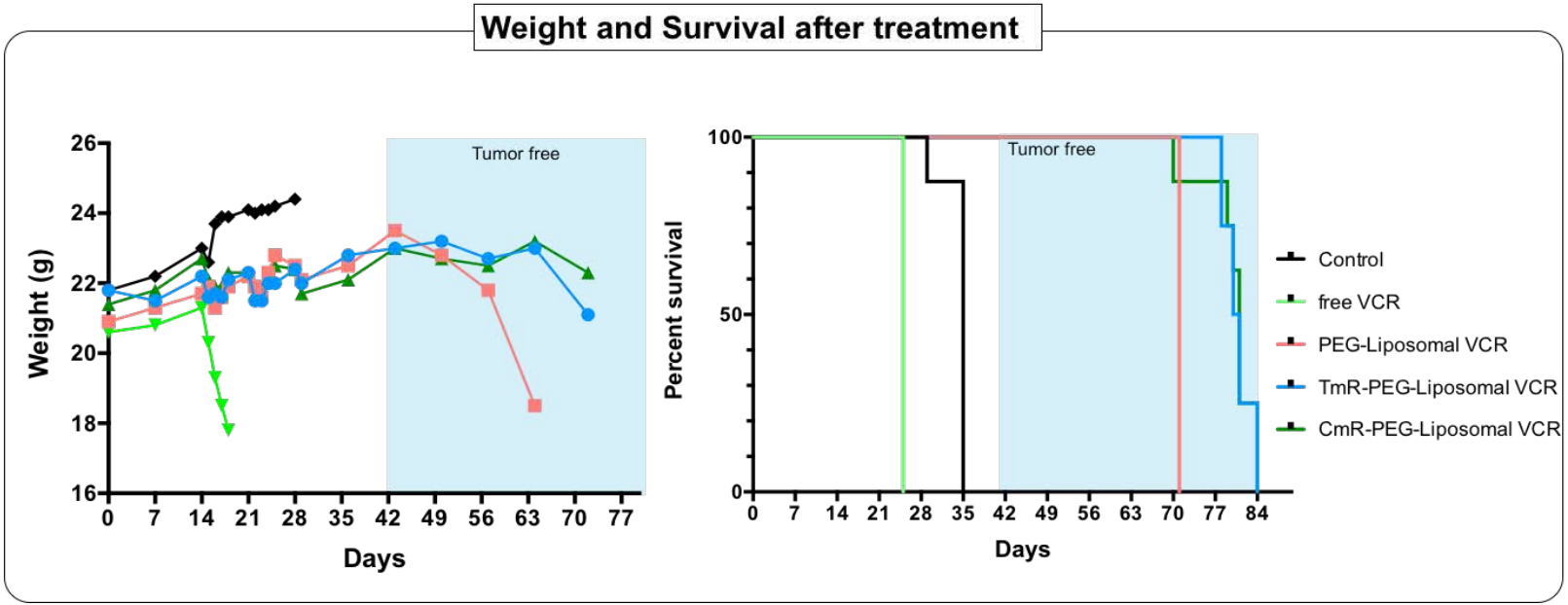
Therapeutic effect of liposomal vincristine in RMS mouse xenografts



- $5x10^6$ Rh30 cells
- Subcutaneous
- NSG mice
- 2 mg/kg VCR equivalent

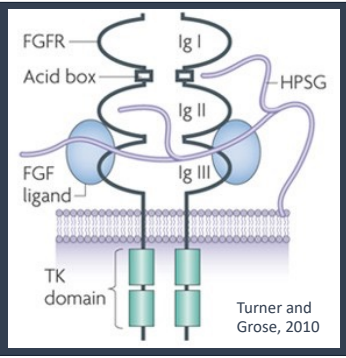
M. Römmele, Unpublished data

Therapeutic effect of liposomal vincristine in RMS mouse xenografts



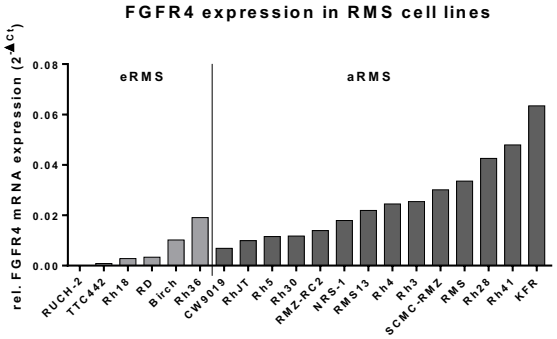
M. Römmele, Unpublished data

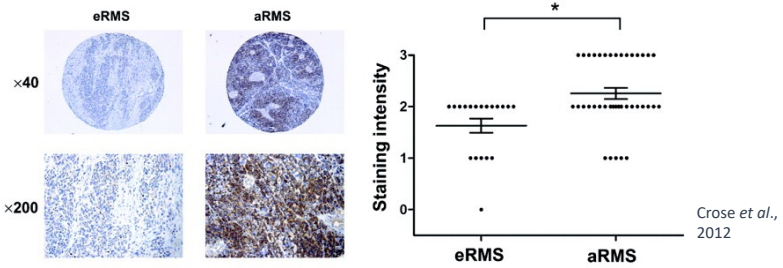
Rhabdomyosarcoma - Fibroblast growth factor receptor 4



Turner and Grose, 2010

- FGFR4: myogenesis and muscle regeneration / lipid and glucose metabolism
- Expressed in liver, kidney / absent in differentiated muscle
- **Aberrant high expression in RMS**
- ARMS: FGFR4 is target gene of PAX3-FOXO1
- ERMS: FGFR4 amplification / point mutations in 12%



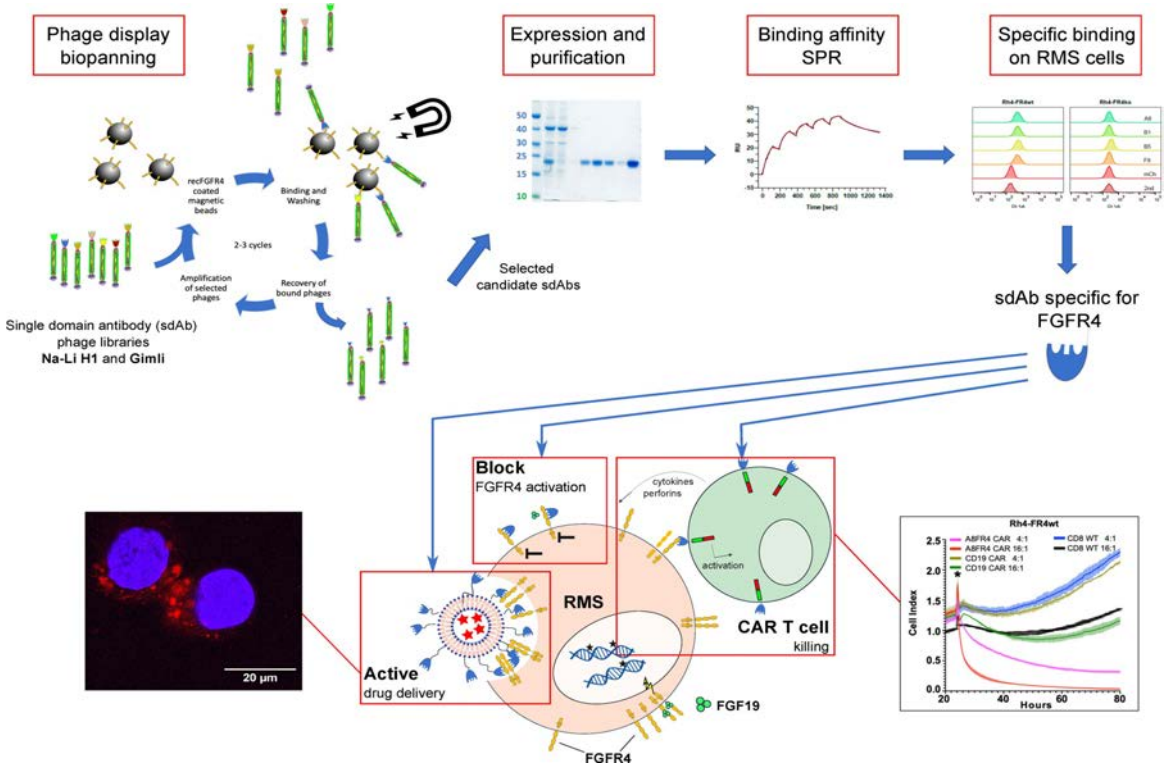


Crose *et al.*, 2012

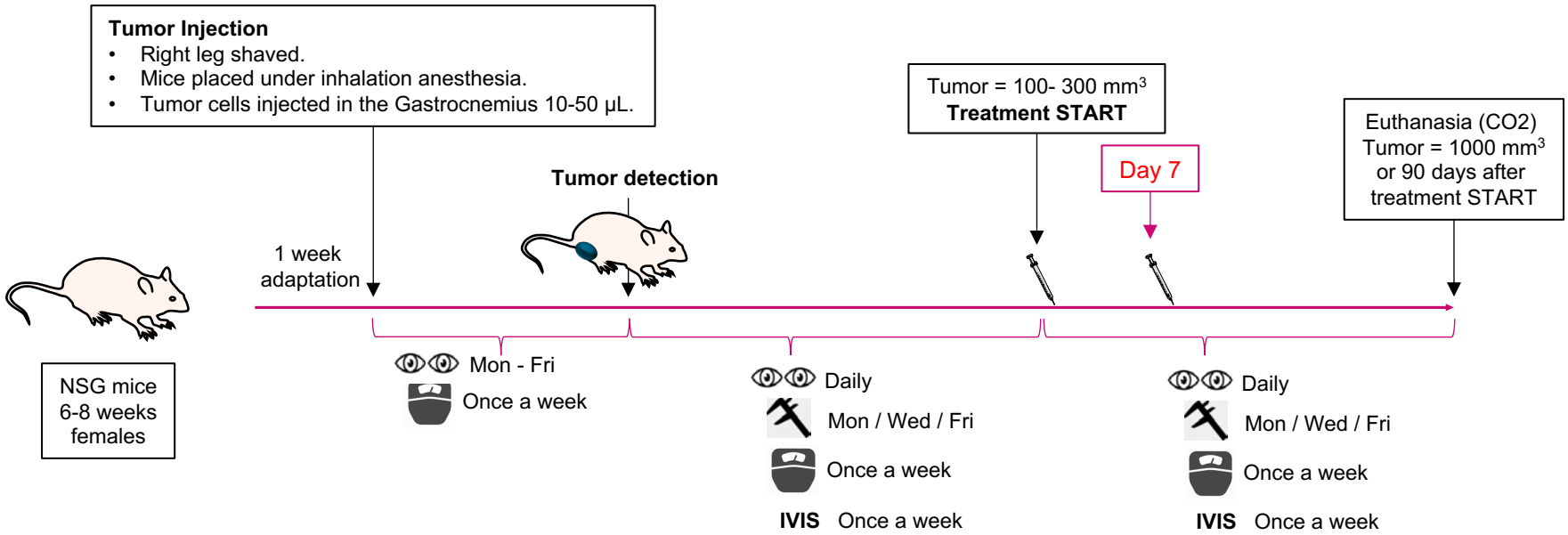
6

FGFR4 – targeting by nanobodies

Alijaj, N. *et al.* Novel FGFR4-Targeting Single-Domain Antibodies for Multiple Targeted Therapies against Rhabdomyosarcoma. *Cancers* **12**, 3313 (2020).

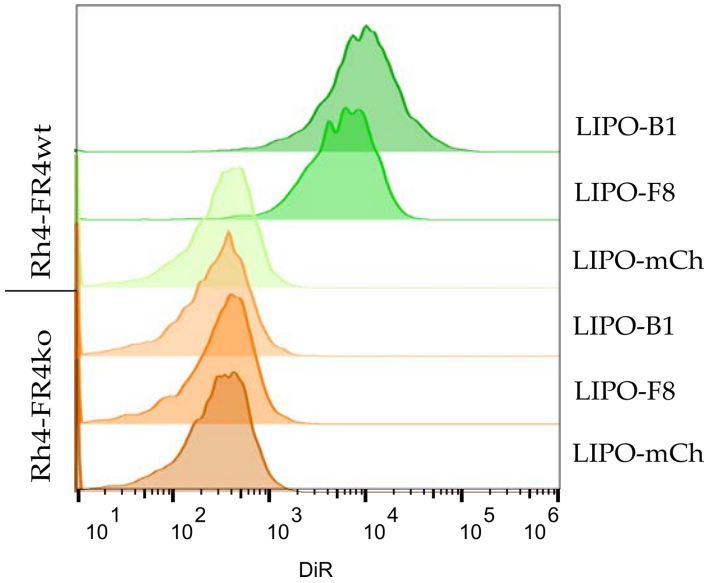


FGFR4-targeted liposomes – in vivo 0.5 mg/kg VCR

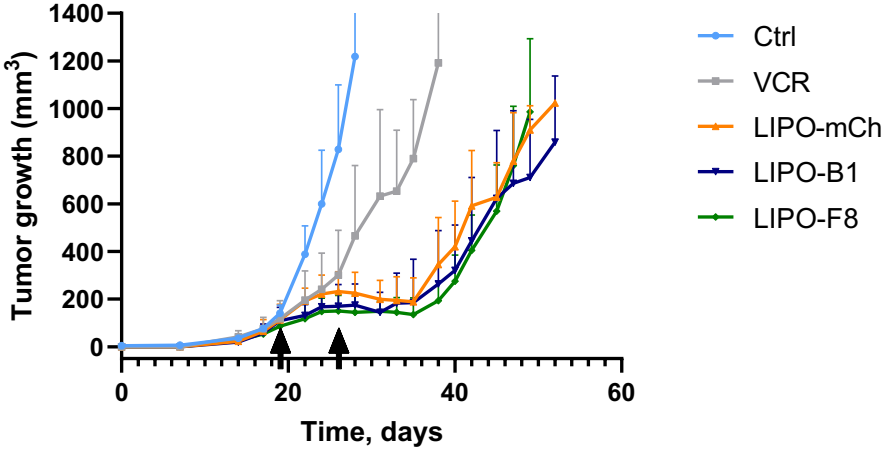


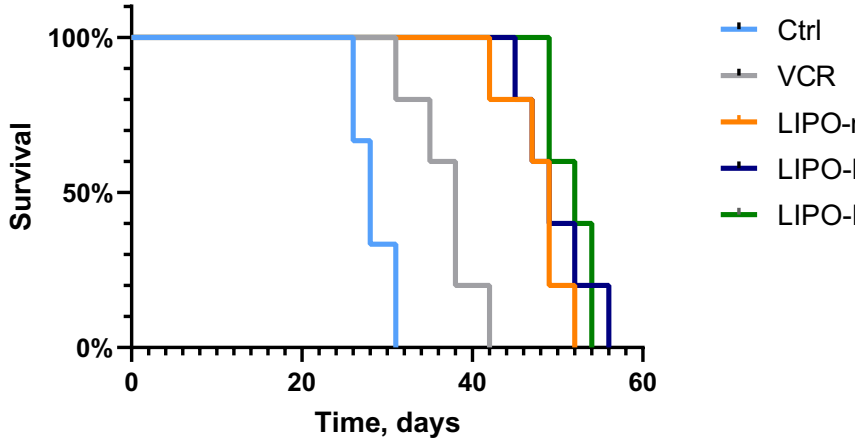
FGFR4 – targeting by nanobodies

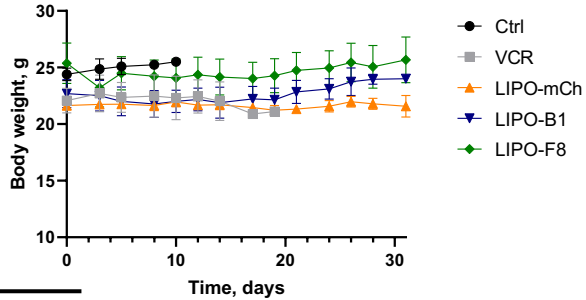
- E:C:PC:DiR:DSPE-PEG-Mal/Pep - 49.8:45:4:0.2:1
- Extrusion method
- Size: 108.3-111.5nm (109.92±1.33nm) and
- PDI below 10%
- Encapsulation efficiency over 98%



FGFR4 – targeting by nanobodies



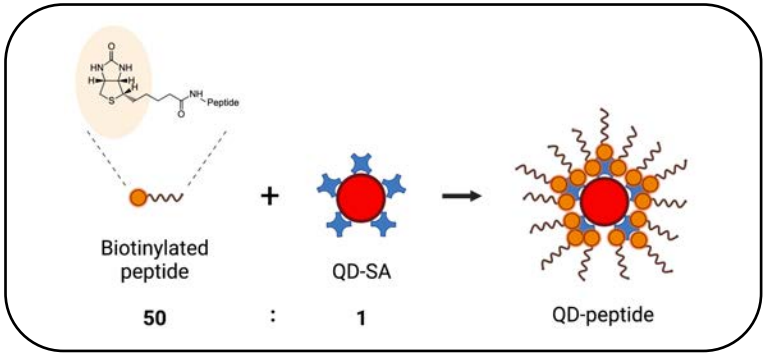


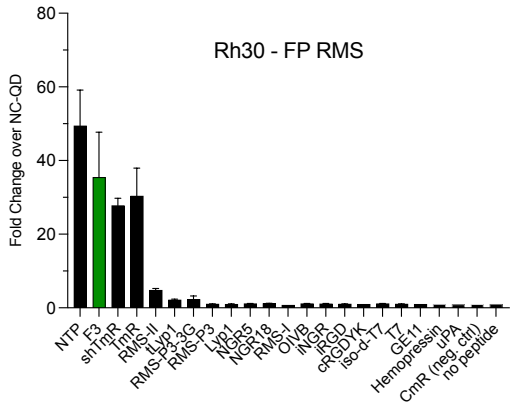


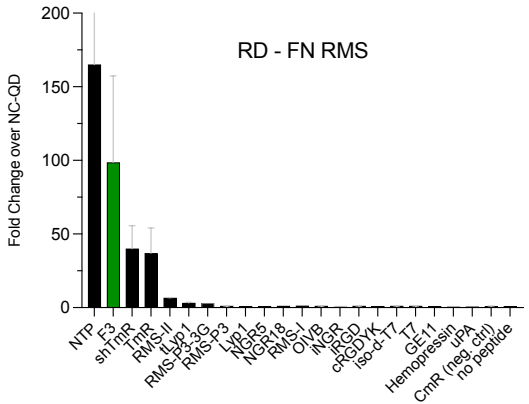
Rh4 RMS cells
n = 5 mice per group

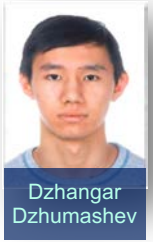
RMS targeting ligands – “new” selection

Relevance for RMS	Targeting protein	Peptide	Sequence
RMS surface molecules investigated for targeting	Integrin αvβ3	Linear RGD	CRGDS
		Cyclic RGD	cRGDyK
	Gamma-subunit fAchR	αA-conotoxins	CCGVONAACPOCVCNKTCG
	Furin	TmR	KRDRGGGCMGTINTRTRRC
		RMS-I	CQQSNRGDRKRC
		RMS-II	CMGNKRSAKRPC
		RMS-P3	CMGTINTRTRRC
		p-AGP	CAGPRTRRC
RMS specific potential binding targets	NCAM-1	NTP	ASKKPKRNIKA
	Cannabinoid Receptor 1	Hemopressin	PVNFKFLSH
	EGFR (ErbB-1)	GE11	YHWYGYTPQNVI
	Transferrin R1	T7	d-(HRPYIAH)
Surface molecules of tumor blood/lymphatic vessels	Nucleolin	F3	EPQRRSARLSAKPAPPKPEPKPKKAPAKK
	Aminopeptidase N	NGR	CNGRC
	p32	Lyp-1	CGNKRTRGC
	Neuropilin-1, p32	tLyp-1	CGNKRTR
	Neuropilin-1, Aminopeptidase N	iNGR	CRNGRGPDC
	Neuropilin-1, Integrin αvβ3	iRGD	CRGDKGPDC
Positive ctrl		TAT	YGRKKRRQRRR
Negative ctrl		CmR	KRDRGGGCMGTINTATAAC









Dzhangar Dzhumashev

Dzhumashev et al. under review

F3 peptide targeting nucleolin

KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK

A fragment of the HMGN2 protein homes to the nuclei of tumor cells and tumor endothelial cells *in vivo*

Kimmo Porkka*†, Pirjo Laakkonen*, Jason A. Hoffman*‡, Michele Bernasconi*, and Erkki Ruoslahti*§

*Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037; †Helsinki University Central Hospital, Department of Medicine, Division of Hematology, Stem Cell and Basic Science Laboratory, Haartmaninkatu 4, 00029 HUS, Helsinki, Finland; and ‡Program in Molecular Pathology, The Burnham Institute and Department of Pathology, University of California–San Diego School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093

Contributed by Erkki Ruoslahti, March 28, 2002

- Discovered by phage display of a cDNA library
- Binds to nucleolin on the cell surface (*Christian, S. et al. J Cell Biology 163, 871–878, 2003)*

- Nucleolin is an abundant nuclear non-histone protein

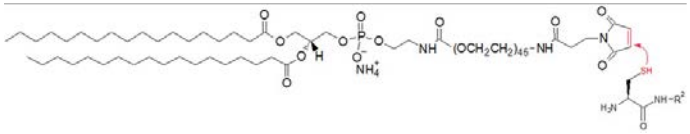
- Cell surface nucleolin is present in tumor cell lines derived from melanoma, glioblastoma, mammary and colorectal carcinoma, as well as in endothelial cells o angiogenic blood vessels (*Reviewed in Ugrinova et al., 2017)*

Novel RMS targeted liposomes – peptides conjugation

F3: KDEPQRRSARLSAKPAPPKPEPKPKKAPAKKC

DSPE-PEG-Maleimide polydisperse – 3kDa

F3 peptide 3.5kDa

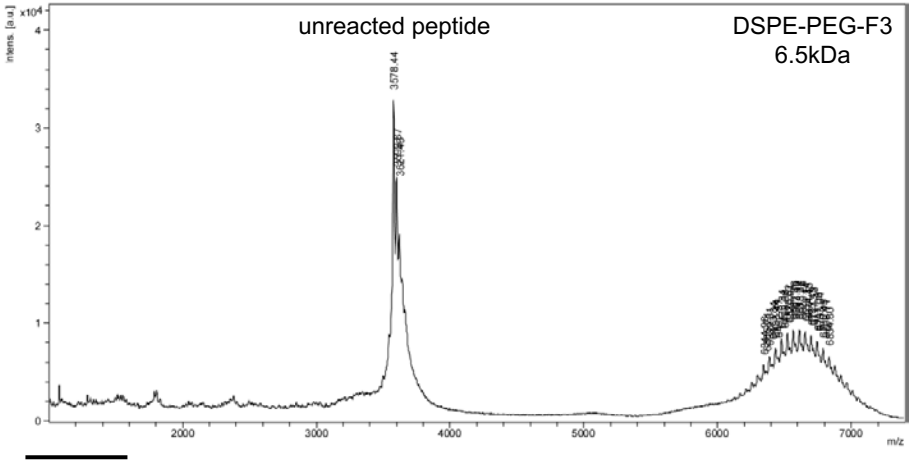


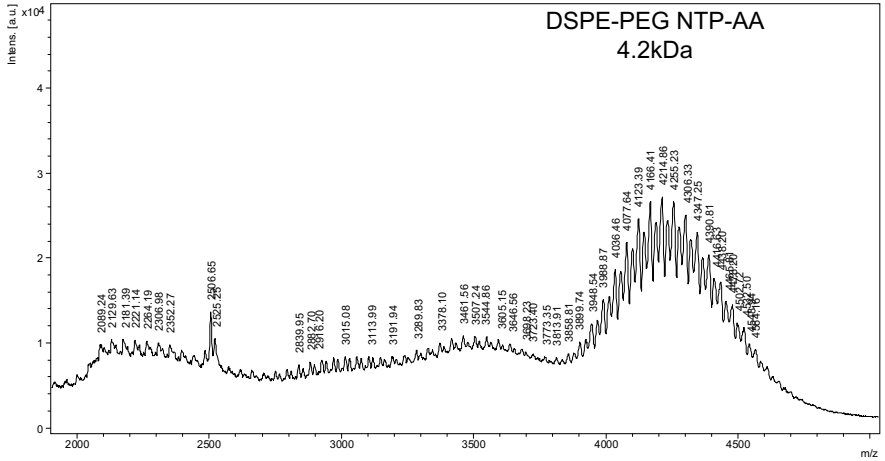
– DMSO, 24 h under constant stirring, room temperature
– DSPE-PEG(2000)-Mal : PEP-C-SH (1 : 1 molar)

Control peptide:

NTP-AA: ASKKPAANIKAC

NTP: ASKKPKRNIKAC





F3-liposomes - Microfluidic Nanoprecipitation

Solvent

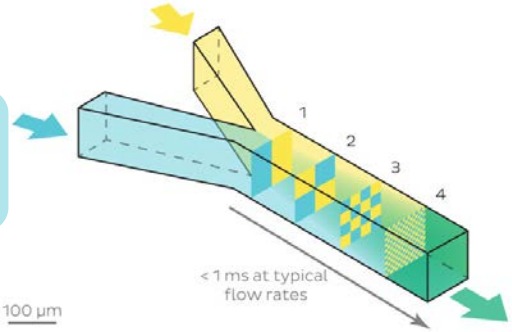
EtOH / DMSO (52 / 48 v%)

E:C:PEGC:DPEG-Pept:DiR/DiO

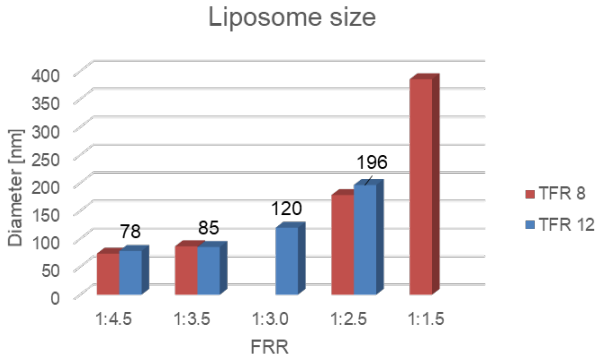
49.8:45:4:1:0.2 mol%

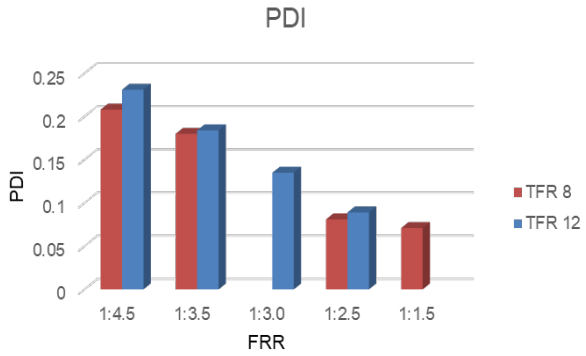
Aqueous

Citrate buffer pH 3.16



Dialysis
VCR encapsulation

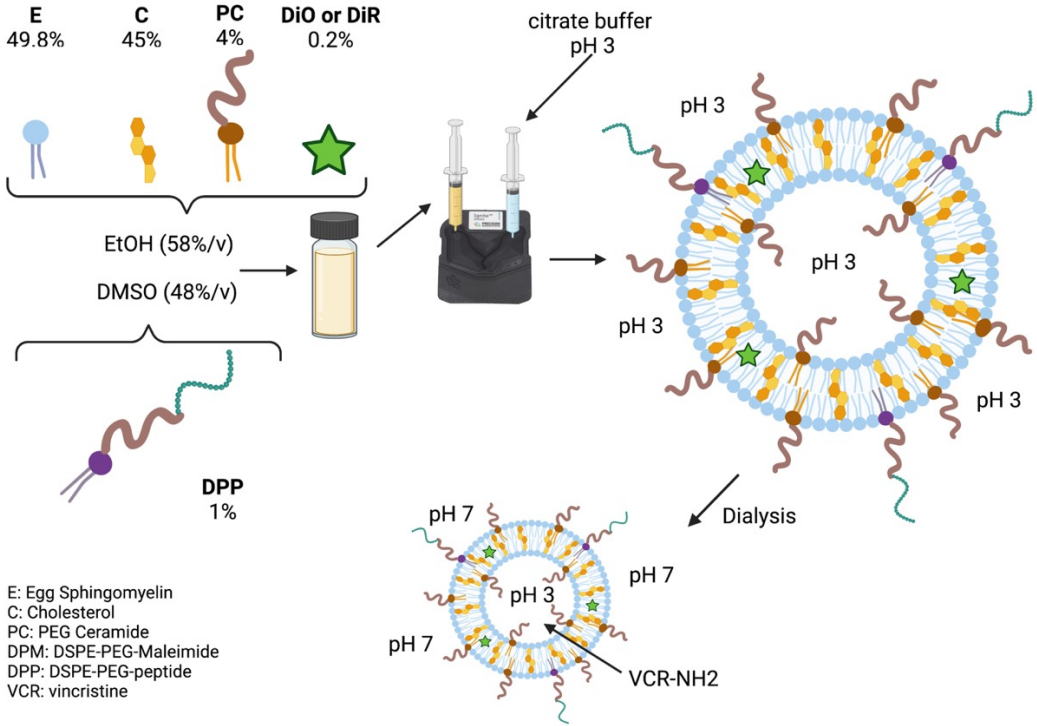


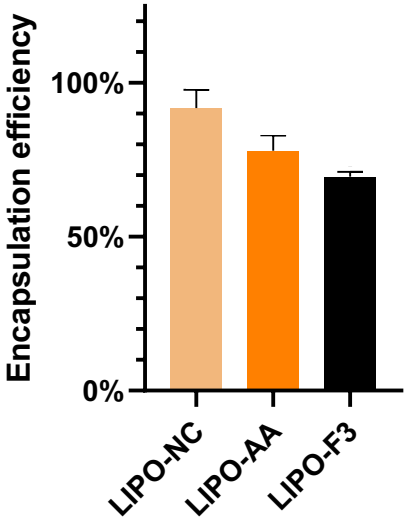


➢**FRR 3.5:1 & TFR of 12mL/min**

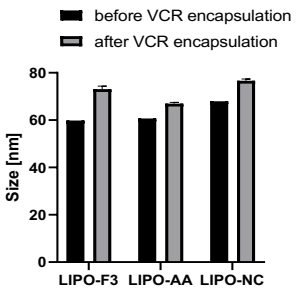
FRR: Flow Rate Ratio (organic:aqueous)
TFR: Total Flow Rate
PDI: Polydispersity Index

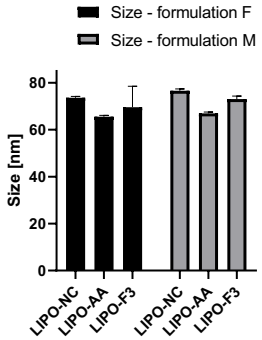
F3-Liposomes – VCR Encapsulation

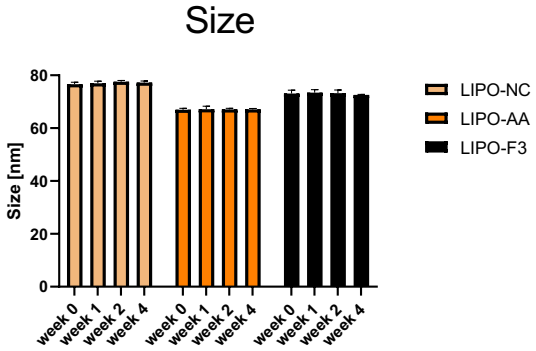


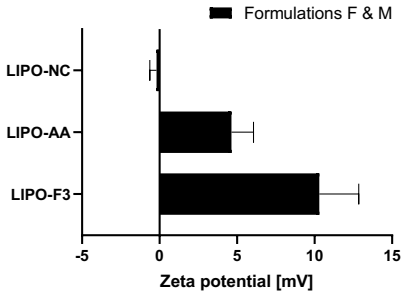


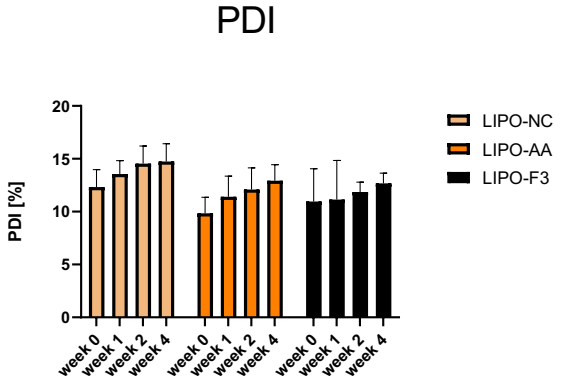
F3-Liposomes – Characterization

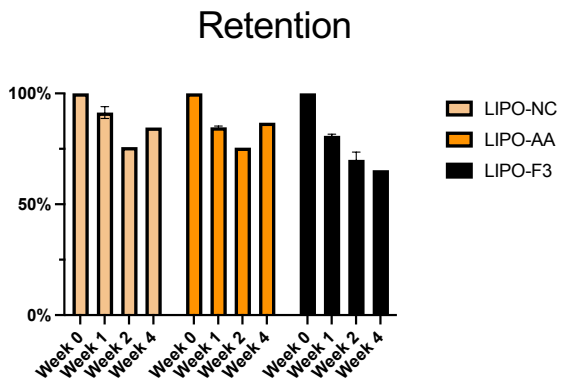




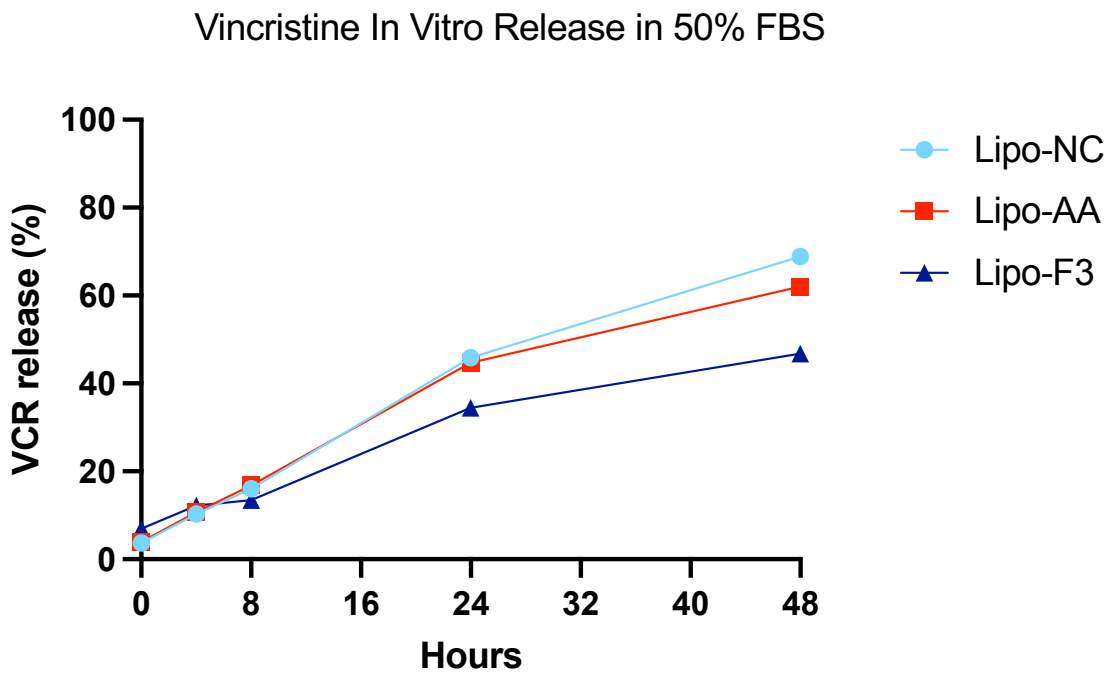




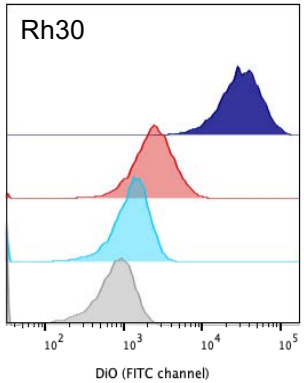




F3-Liposomes – Characterization



F3-Liposomes – Binding to RMS Cells

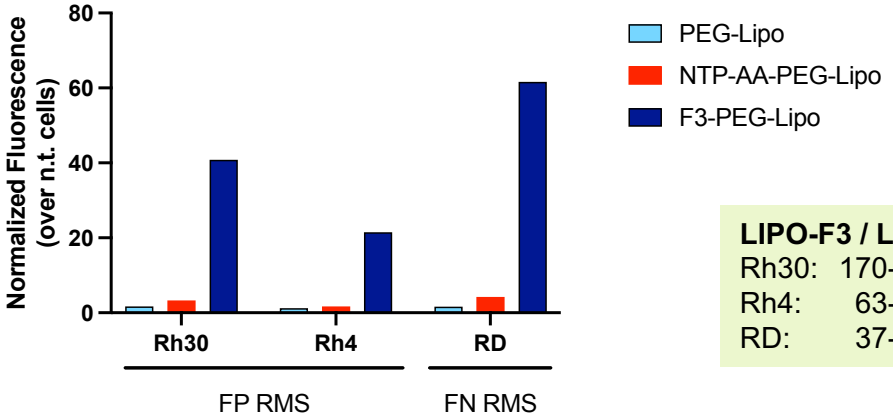


Lipo-F3

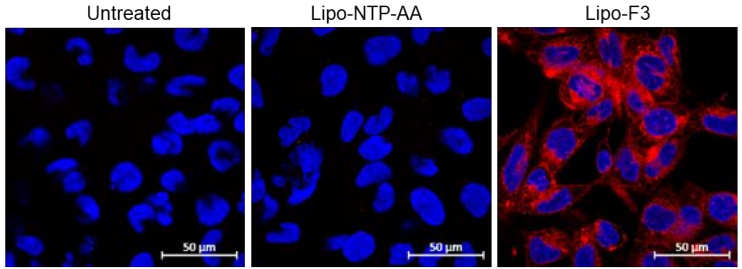
Lipo-AA

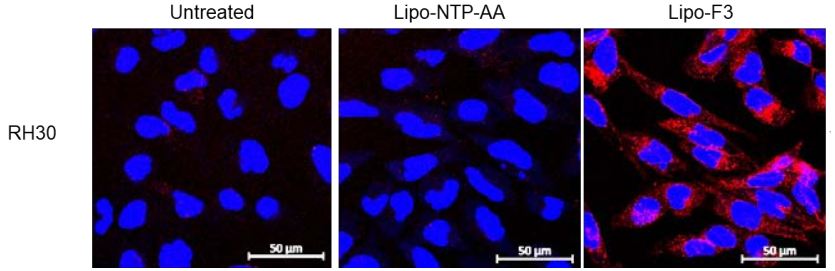
Lipo-NC

Untreated

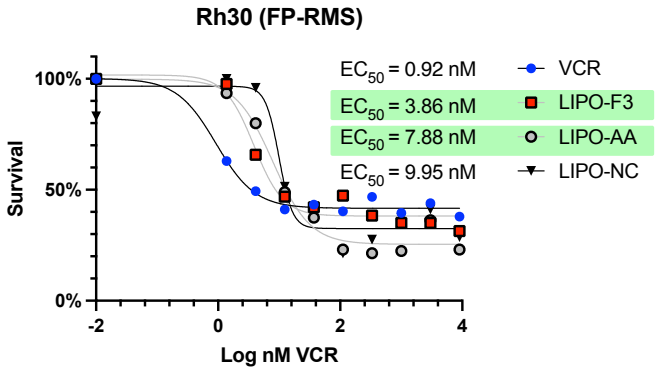


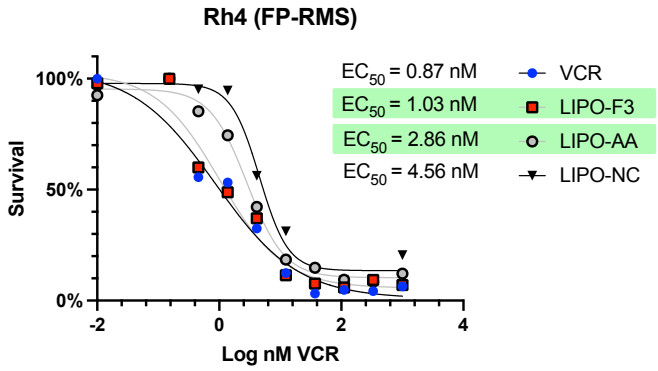
LIPO-F3 / LIPO-AA
Rh30: 170-fold
Rh4: 63-fold
RD: 37-fold

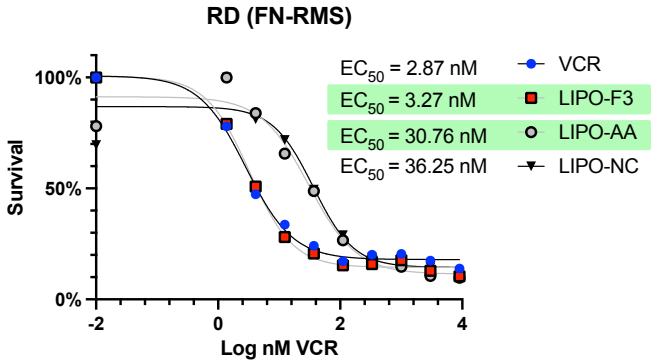




F3-Liposomes – In Vitro Cytotoxicity VCR







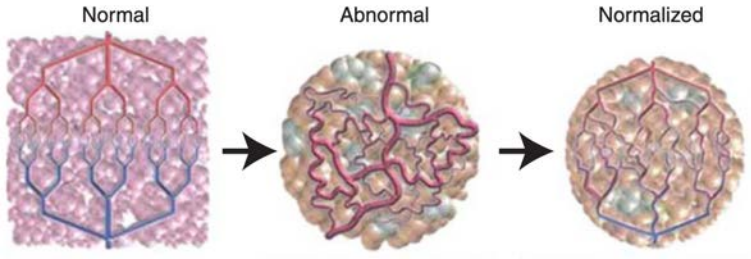
- 2h Incubation before wash and medium change
- 48h incubation at 37°C in 5% CO_2
- MTT assay

Conclusions and Outlook

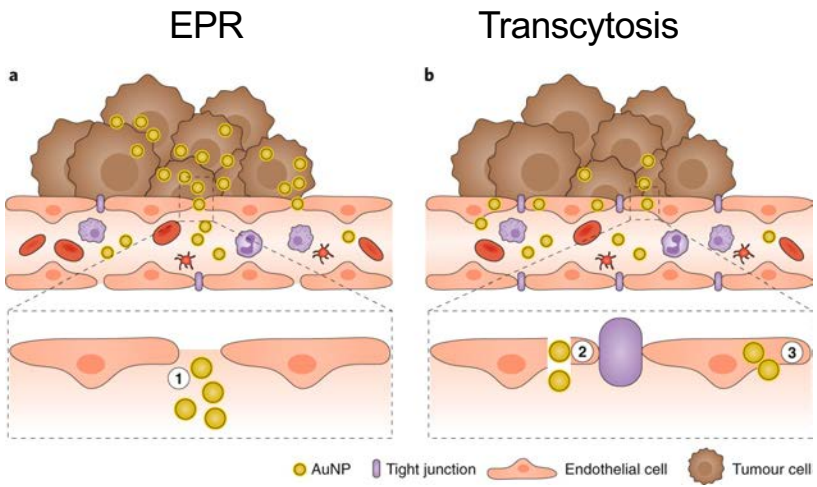
- Liposomal formulation of VCR can increase circulation $t_{1/2}$ and accumulation in RMS tumors in mice
- The RMS-targeting peptide TmR or FGFR4-targeting nanobodies do not further increase accumulation *in vivo*
- F3-liposomes bind to RMS cells **5-10**-fold better than TmR-liposomes *in vitro*

- EPR effect might be dominant

Conclusions and Outlook



EPR effect (?)



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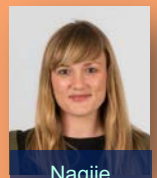
- **Vascular normalization is being studied to investigate effect on permeability**
- **Alternative strategies to promote vascular transcytosis might be beneficial**

Acknowledgements

pHOncoLab

Alumni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