"Drug delivery challenges and tumor resistance"

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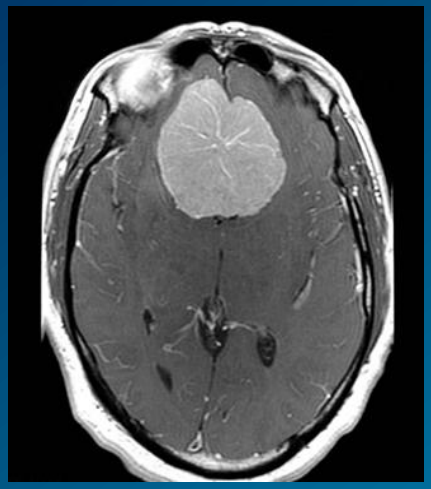
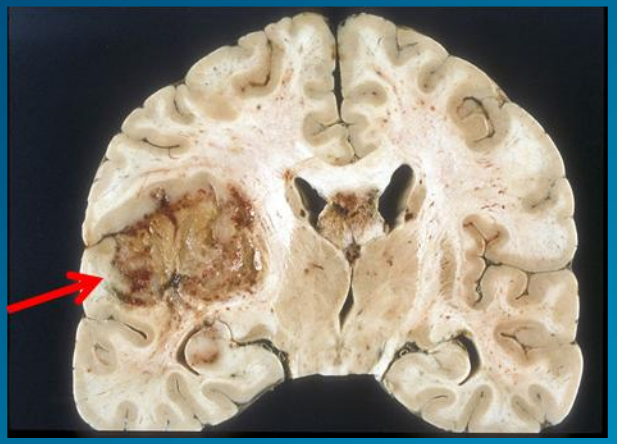
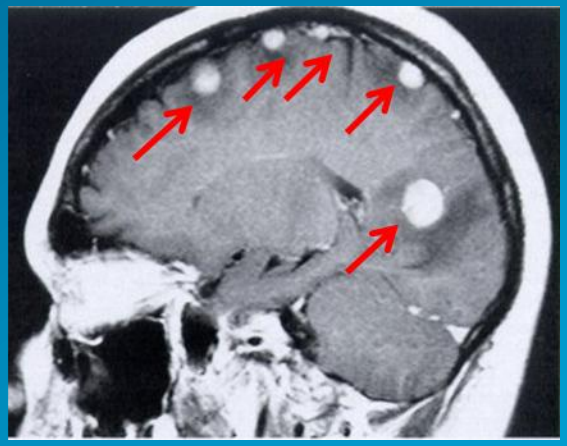
Primary and secondary brain tumors represent a significant public health challenge.







At any time in the U.S. there are approximately 360,000 primary and secondary brain cancer patients.

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Glioblastomas (GBM) are the most common brain cancers in adults with an extremely poor prognosis

WHO Grade	Designation	Frequency (% of gliomas)	5-year Survival (%)	Median Survival
II	Diffuse astrocytoma Oligodendroglioma	1.7 9.2	46.9 70.5	3-8 yrs 7-10 yrs
III	Anaplastic astrocytoma Anaplastic oligo	7.9 5.1	29.4 40.1	2-3 yrs 3-5 yrs
IV	Glioblastoma (GBM) (13,000 new GBM patients per year in U.S.)	50.7	3.3	9-15 mos

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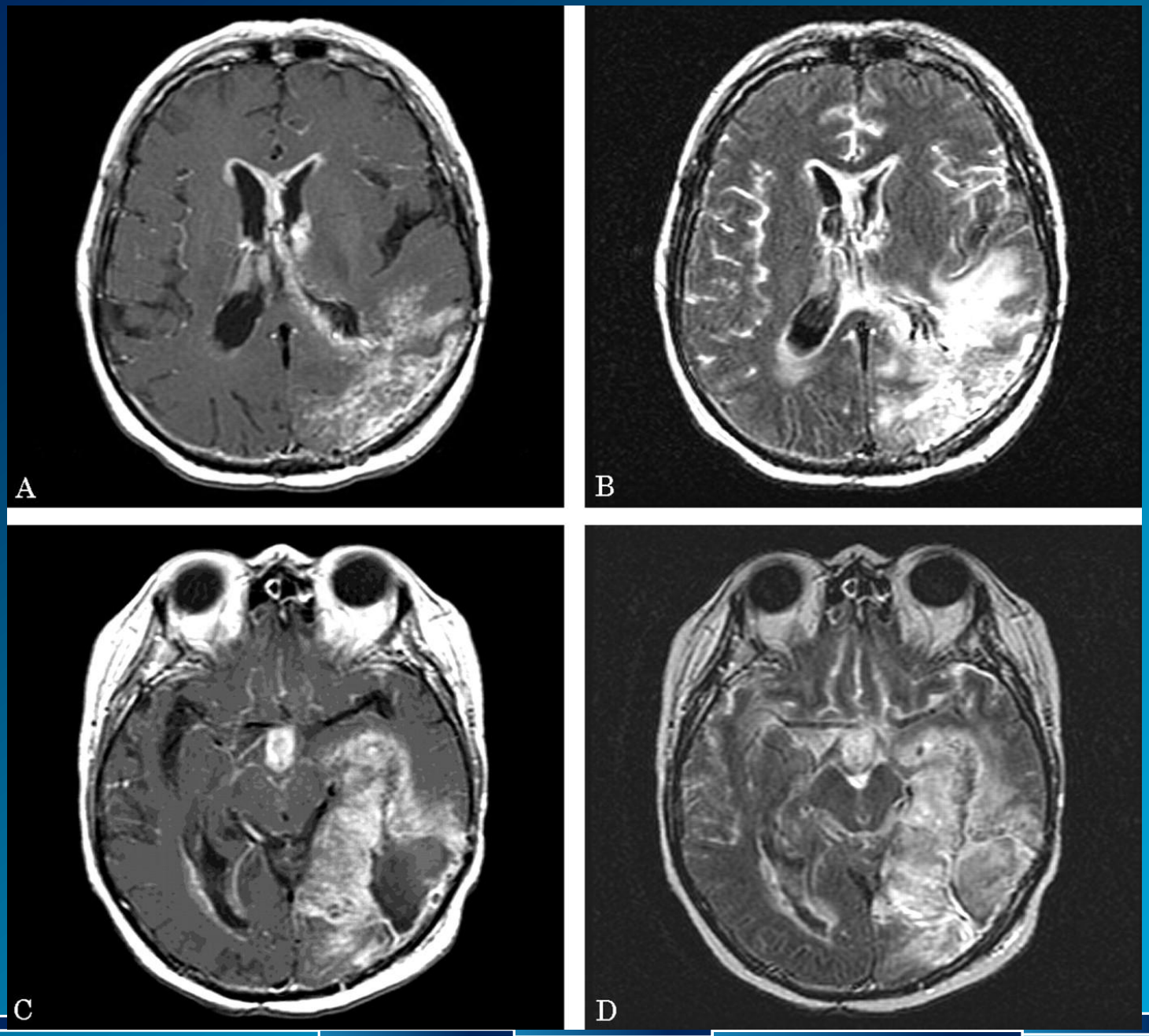
- Causes of GBM drug resistance multifactorial
- Stem-like compartment drives these tumors and needs to be addressed by agents that can cross BBB
- Physical distribution of agents can in effect be a limiting factor and cause of resistance

The cells that drive GBM are resistant and they invade



Begemann M et al.
Neurology 2004;63:E8

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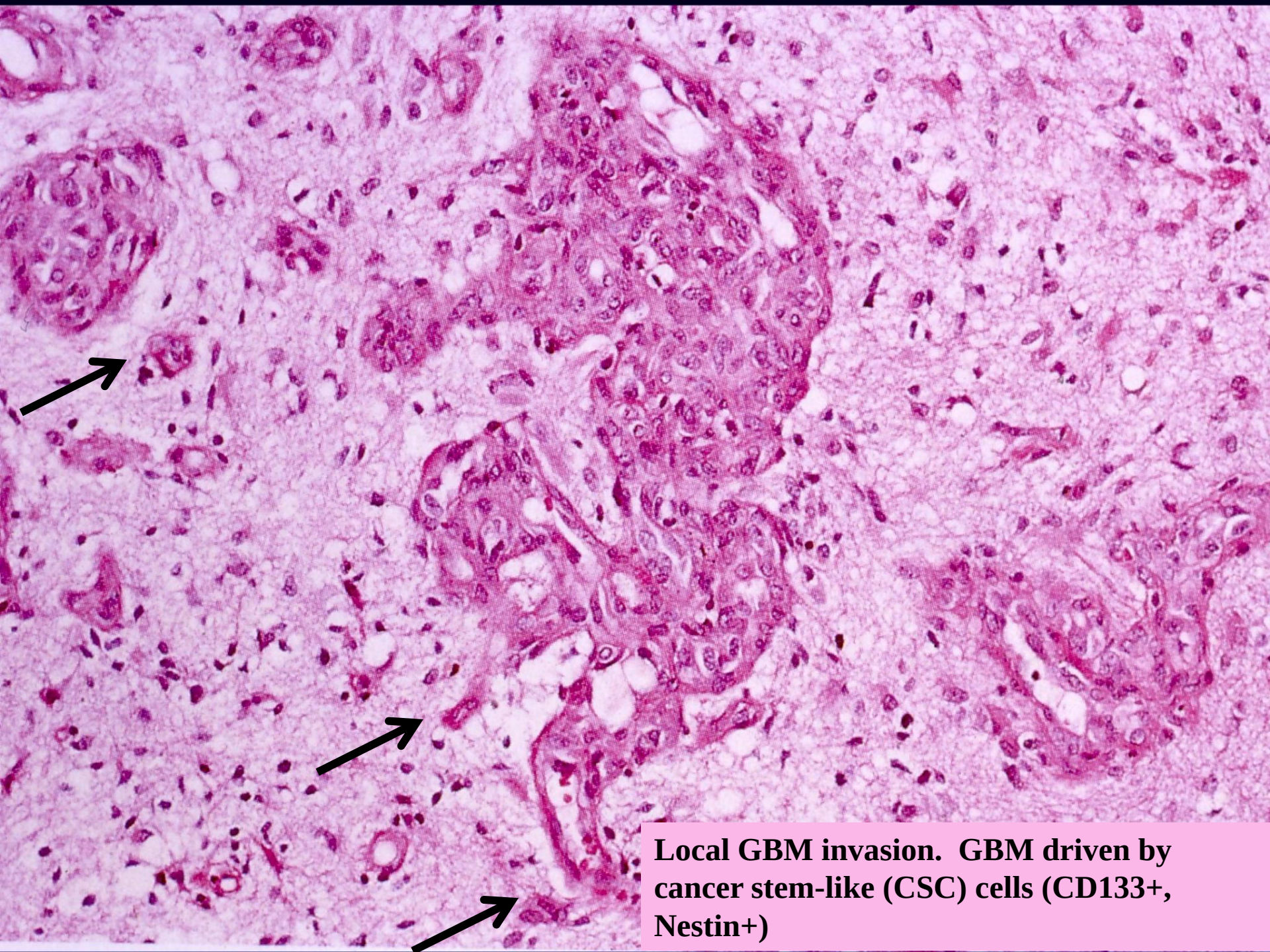
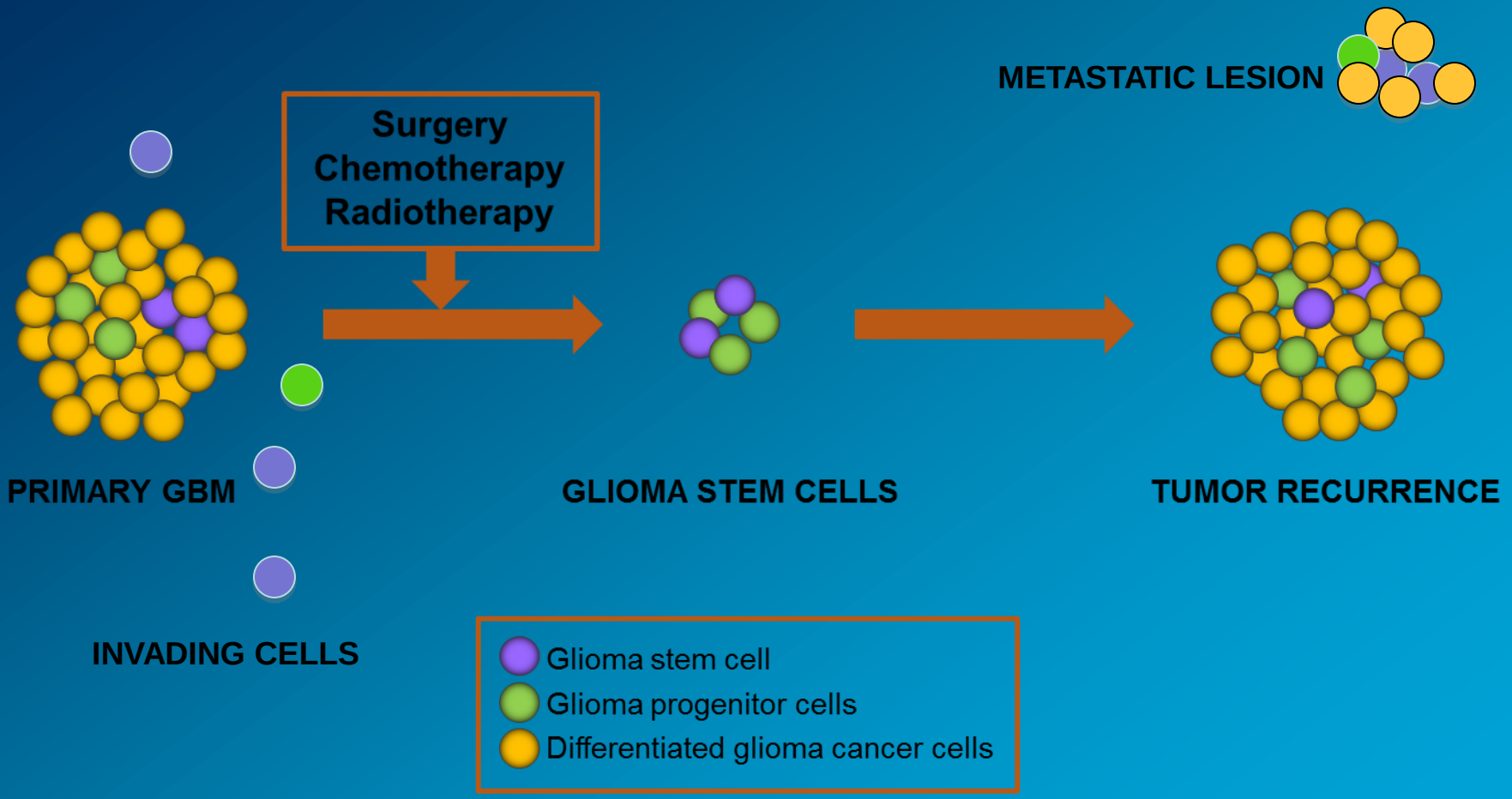


Local GBM invasion. GBM driven by cancer stem-like (CSC) cells (CD133+, Nestin+)

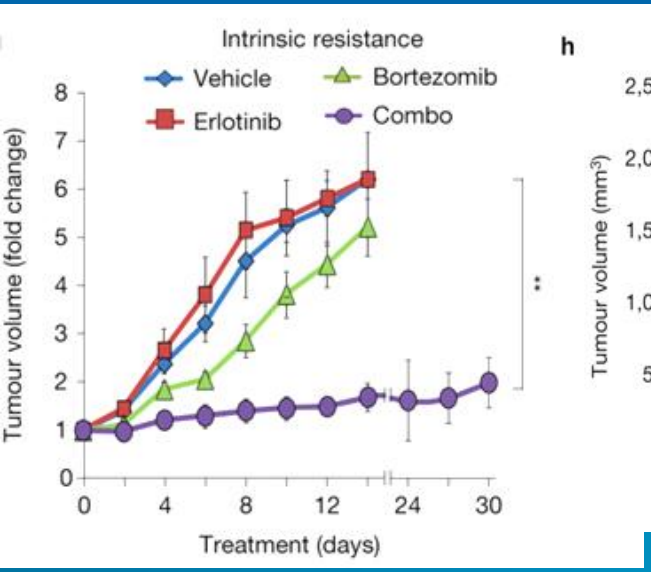
Conventional Therapies Target The Tumor Bulk, But Display Limited To No Efficacy Toward GSCs

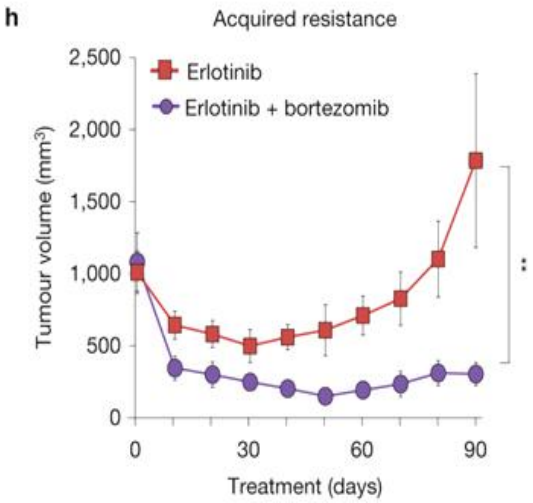


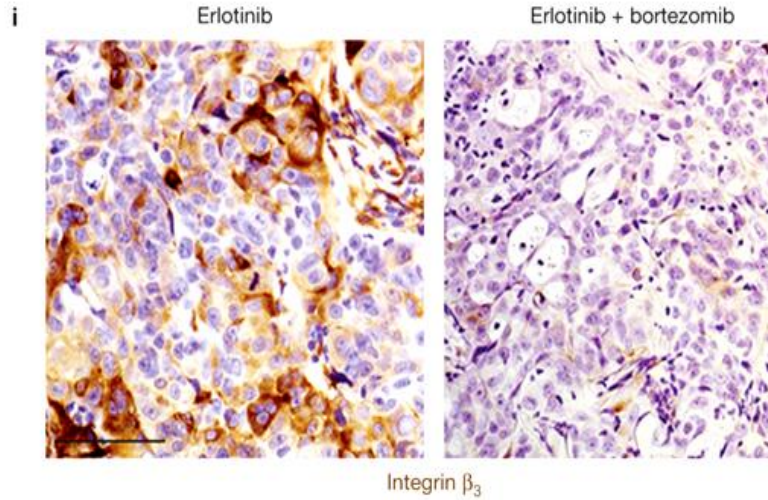
Targeting Glioma Stem Cells will be Critical for Long-term Remission

A β3 integrin-KRAS-RalB complex drives tumor stemness and resistance to EGFR inhibition. *Nat Cell Biol. 2014 May ; 16(5): 457–468.*

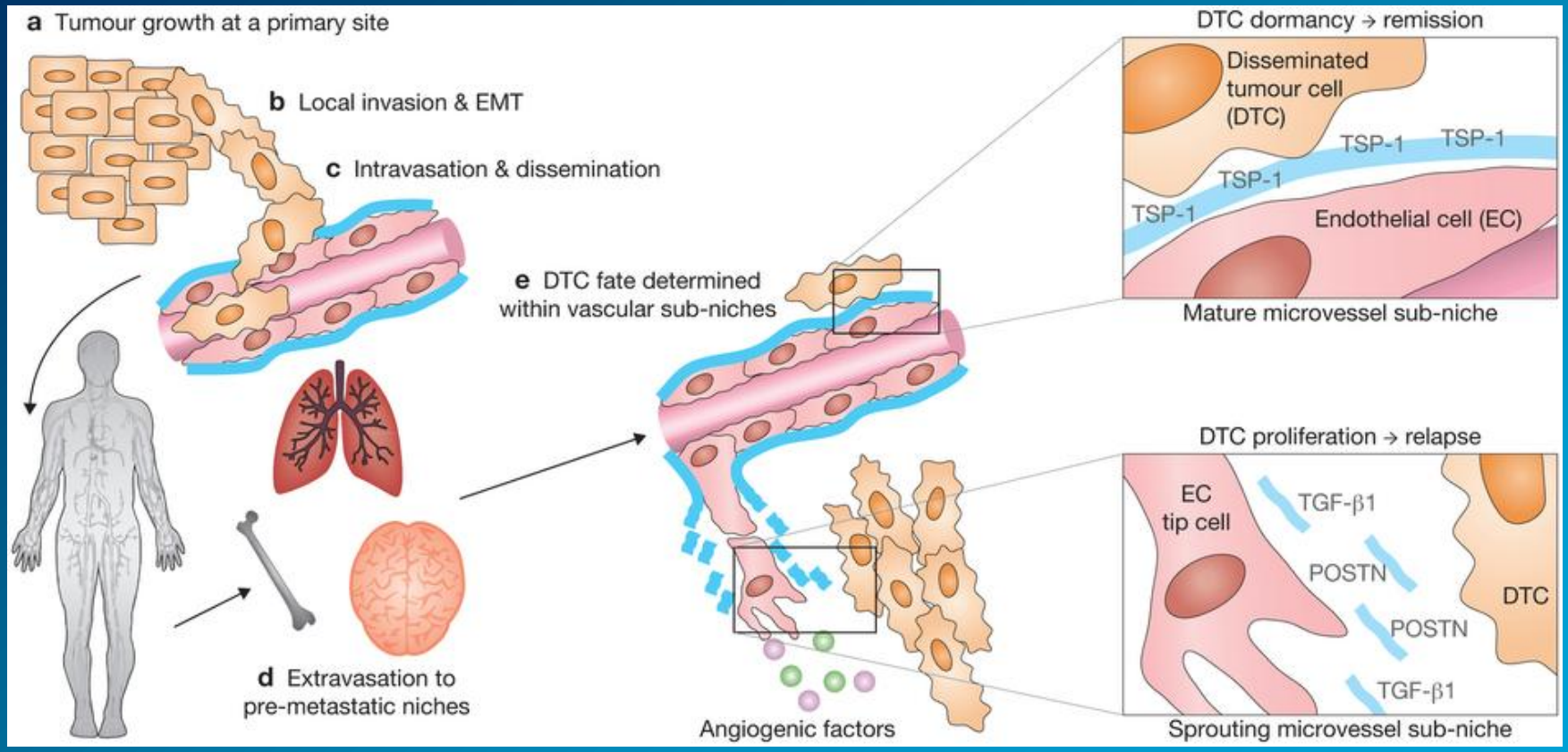
Erlotinib – inhibits EGFR, Bortezomib – proteosome inhibitor







Do tumors model niches for stem like cells? Role of extracelleluar matrix? Hyaluronan and CD44 in GBM?



Nature Cell Biology ***15, 721–723 (2013)***

Reconstructing and Reprogramming the Tumor-Propagating Potential of Glioblastoma Stem-like Cells

Mario L. Suva et al,

Cell 157, 580–594, April 24, 2014

Identified a core set of neurodevelopmental TFs (POU3F2, SOX2, SALL2, and OLIG2) essential for GBM propagation in preclinical models.

These TFs coordinately bind and activate GSC-specific regulatory elements and are sufficient to fully reprogram differentiated GBM cells to “induced” GSCs, recapitulating the epigenetic landscape and phenotype of native GSCs.

Oligodendrocyte factor – 2 (OLIG2) is a basic helix-loop-helix (bHLH) transcription factor

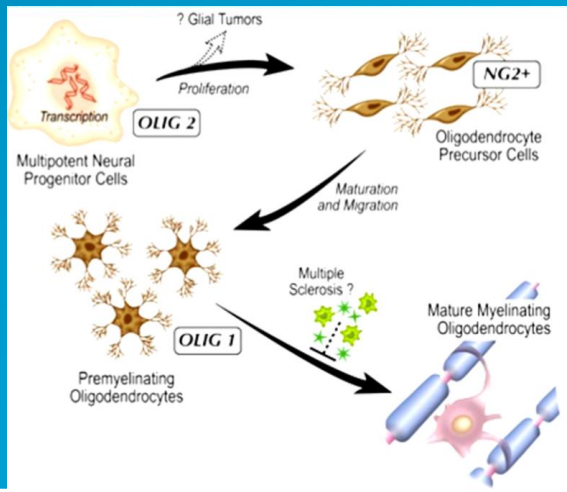
OLIG2 is expressed early by brain stem/progenitor cells and during regeneration by progenitor cells

Expressed by 100% of diffuse gliomas – mainly by cancer stem-like cell compartment – ONLY IN BRAIN

OLIG2 represses both p53 and p21 which are tumor suppressors – and OLIG2 is thought to be responsible for radiation resistance

Mehta et al, Cancer Cell. 2011 March 8; 19(3): 359–371.

80% of high grade gliomas have intact p53.

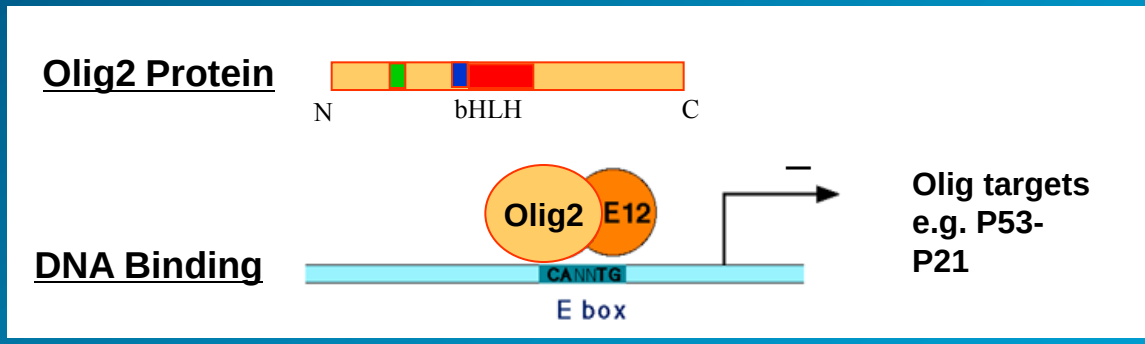


Rowitch and co-workers

Olig2 is a Critical Regulator of Neural Stem Cell Biology

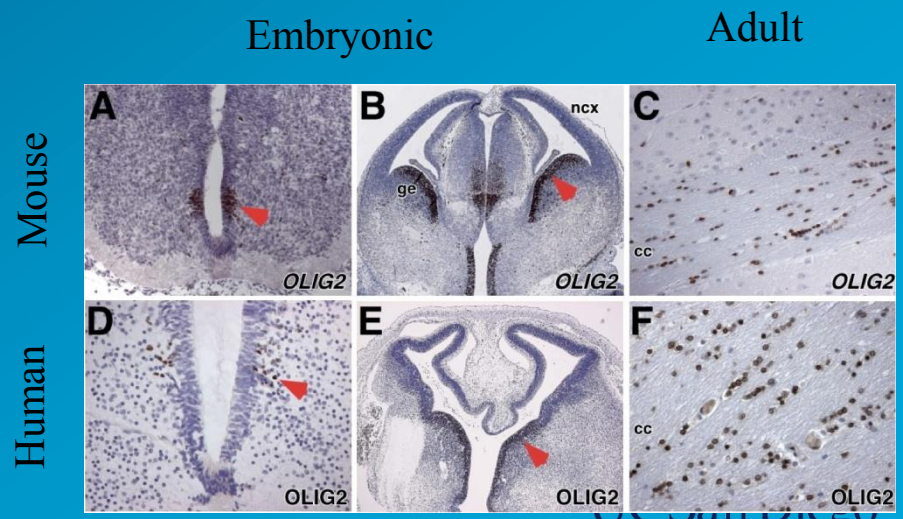
Gene

- bHLH transcription factors
- Co-localized (within 40 KB) on chromosome 21
- Binds DNA as heterodimer and is a transcriptional repressor (bind E-box elements (CANNTG))



Biology

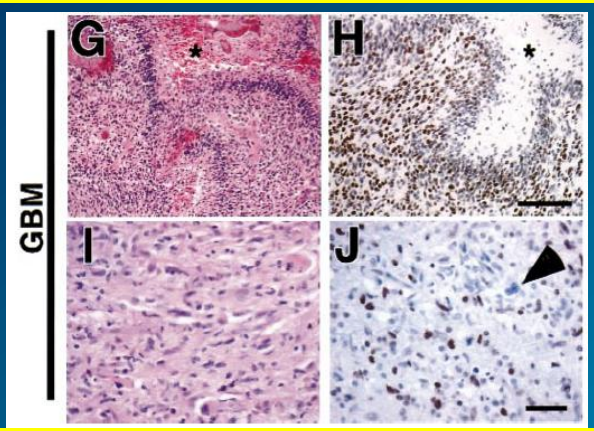
- Regulates the formation of progenitor cells that give rise to neurons and glia
- **Olig2 plays a role in maintaining the replication-competence of neural progenitor cells via P53-P21 axis**
- Expressed only in CNS: stem/progenitor cells and oligodendrocytes (active).
- **Activates repair pathways**

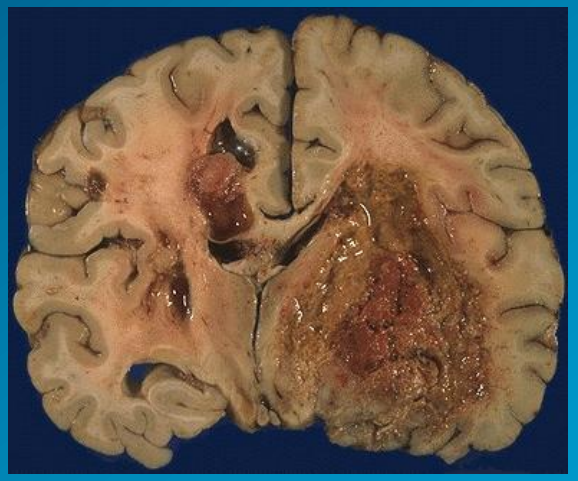


Reviewed in Meijer, et al Nat Rev Neurosci 2012

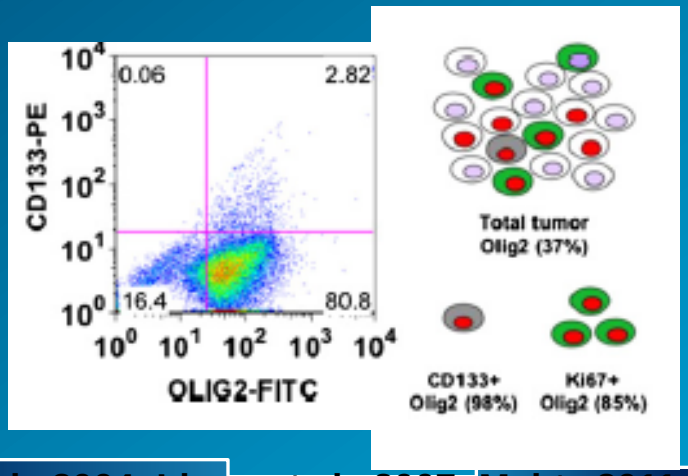
Role of Olig2 in Glioma Formation

A. Olig2 is universally expressed in diffuse gliomas

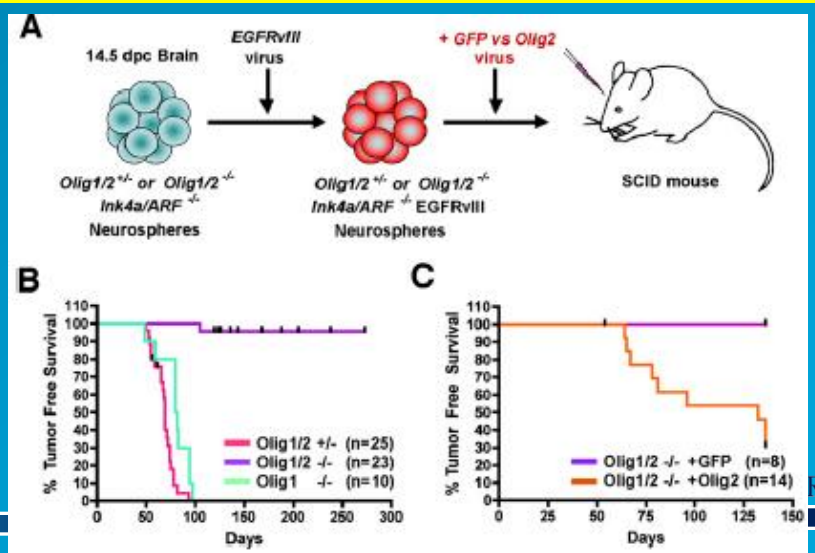




B. Almost all of CD133+ glioma stem cells are Olig2+

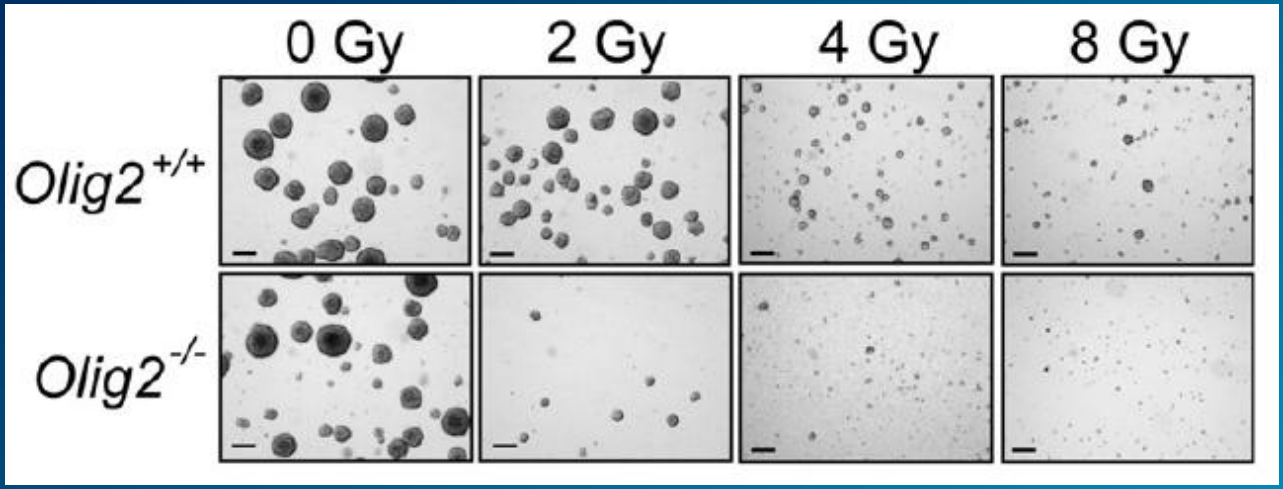


C. Olig2 function is required for glioma formation

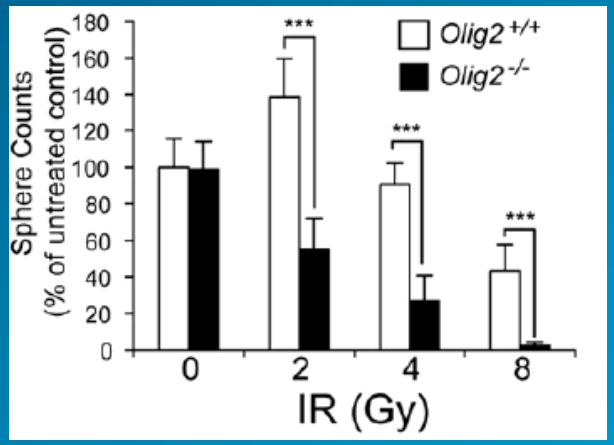


Ligon et al., 2004; Ligon et al., 2007; Mehta 2011

OLIG2 Promotes Radiation Resistance



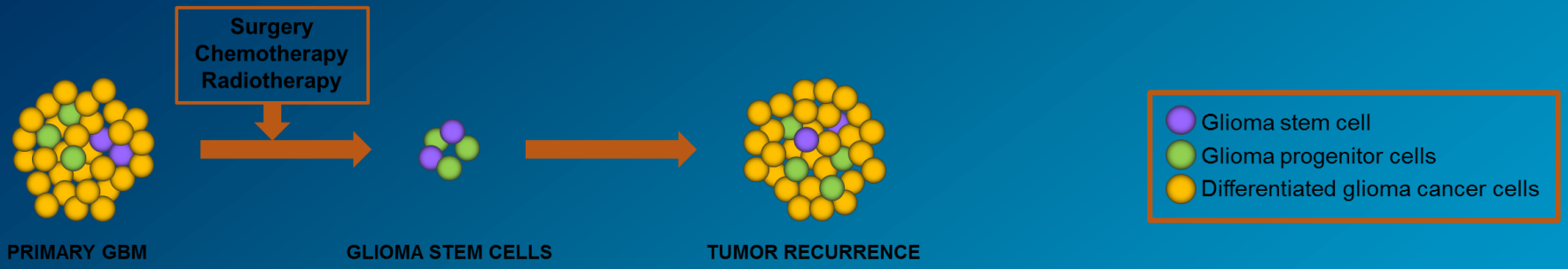
Differential response of Olig2+/+ and Olig2-/- neural progenitors to ionizing radiation. Secondary neurosphere assays were counted at day 5 following treatment of Olig2+/+ or Olig2-/- cells with 2, 4, or 8 Gy of ionizing radiation. Scale bars = 100 mm.



Quantitation of data above. The bars in the histogram represent the percentage of secondary neurospheres formed in irradiated samples relative to the untreated control samples.

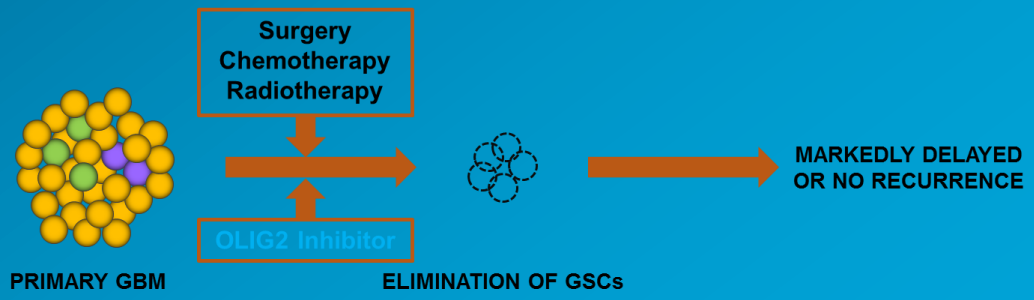
Mehta, S. et al. The central nervous system-restricted transcription factor Olig2 opposes p53 responses to genotoxic damage in neural progenitors and malignant glioma. Cancer cell 19, 359–71 (2011).

Olig2 Inhibition of GSCs to Overcome Radiation Resistance



Rationale for targeting OLIG2

- Expression of OLIG2 drives tumorigenesis and promotes resistance to chemotherapy and radiation therapy
- Highly expressed in all diffuse gliomas and nearly 100% of GSC
- Typically not active in normal brain tissue
- Not found in normal tissues outside the CNS



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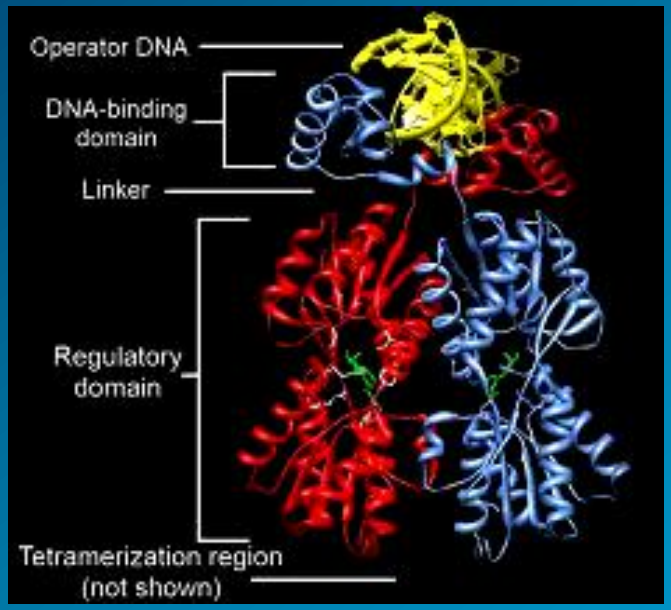
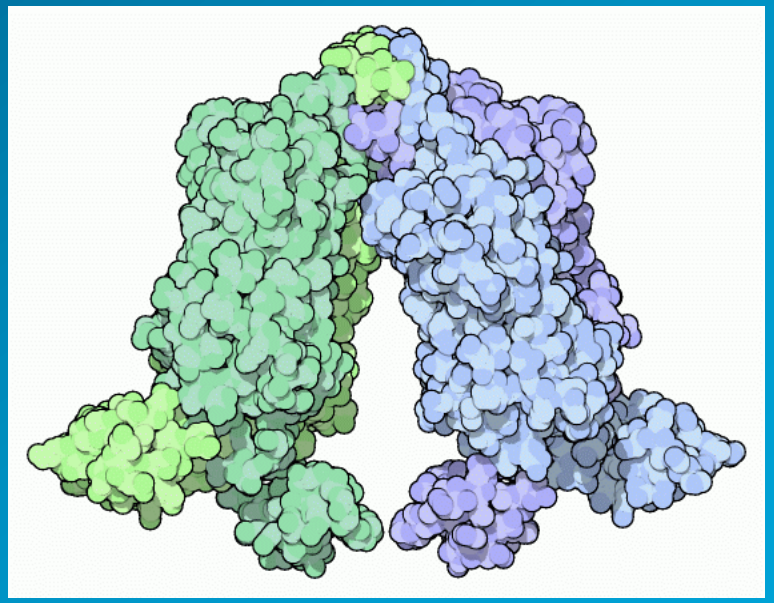
OLIG2 inhibitor may be effective as single agent or with radiation

However,

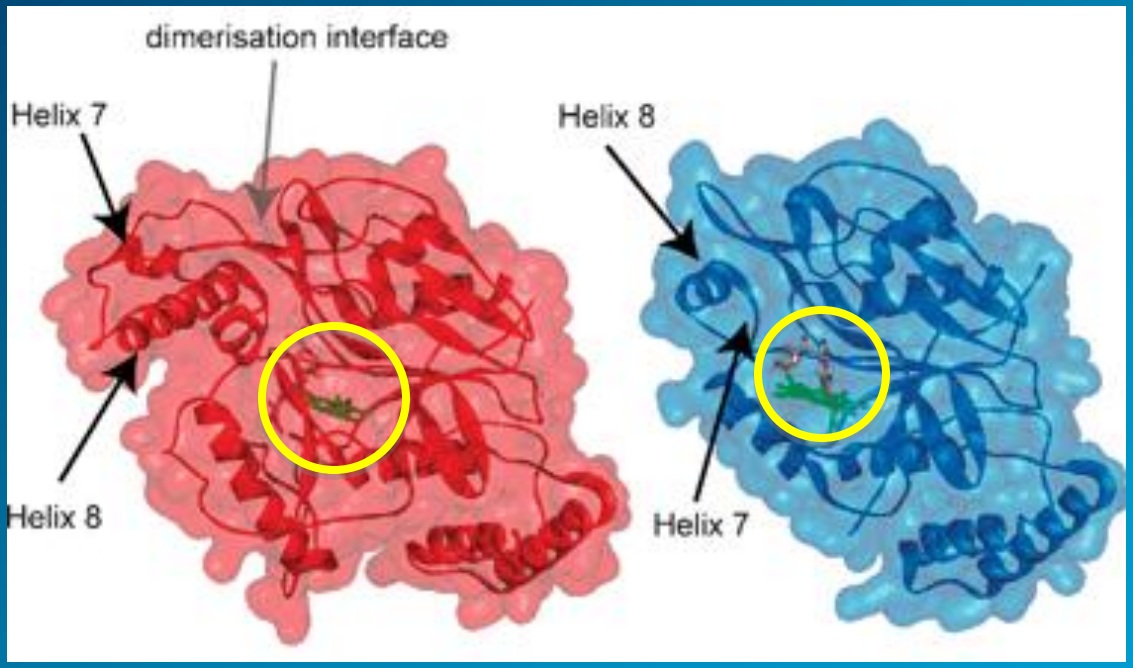
…it is challenging to develop transcription factor inhibitors and success has been quite limited…

… expansive protein-protein interfaces and lack of hydrophobic pockets





Previous TF inhibitor design efforts have focused on small, often single binding ‘hotspots’



Our team had to develop a different targeting approach….

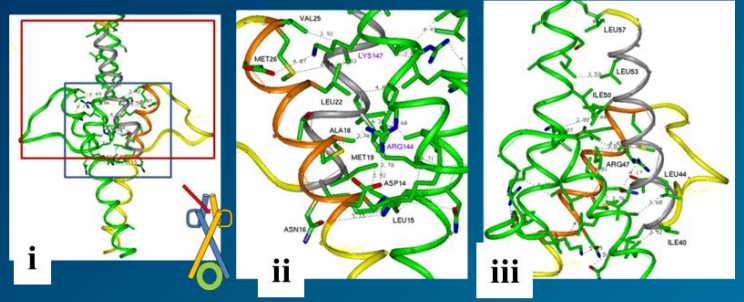
Started with an innovative rational design algorithm .
The resultant structure scaffold solutions guided in silico searches of the NIH and other databases – over 2 million structures.



Dr. Tsigelny (Russia- left), Dr. Wrasidlo (Germany-middle), Dr. Mukthavaram (India-right)

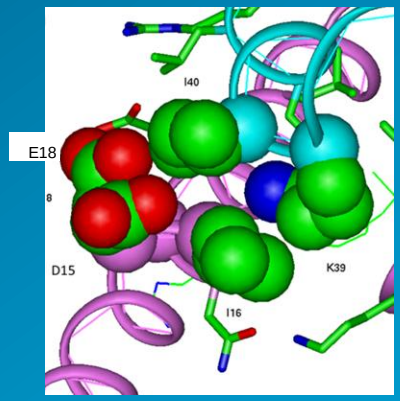
Olig2 Inhibitor Screening: Multiple Pharmacophore Model Developed From Homology-Based *In Silico* Structure

OLIG2 Homology Model

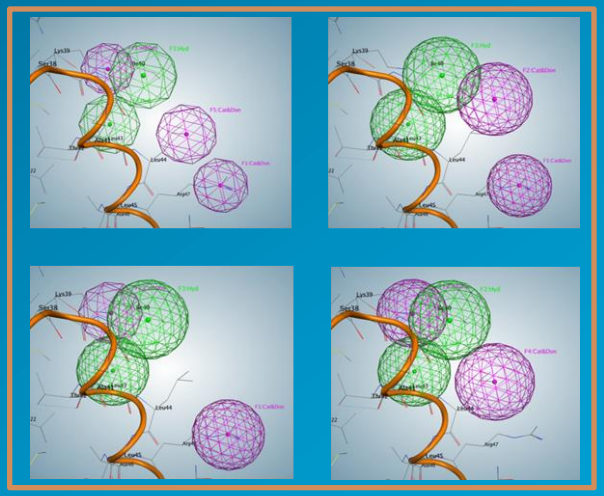


(i) general structure
(ii) enlarged region (middle interface)
(iii) enlarged region (upper interface)

Identification of specific sites of interaction



Development of pharmacophore hypotheses for compound binding sites



Iterative modeling and the underlying methodology

In collaboration with the UCSD Supercomputer and Medicinal Chemistry

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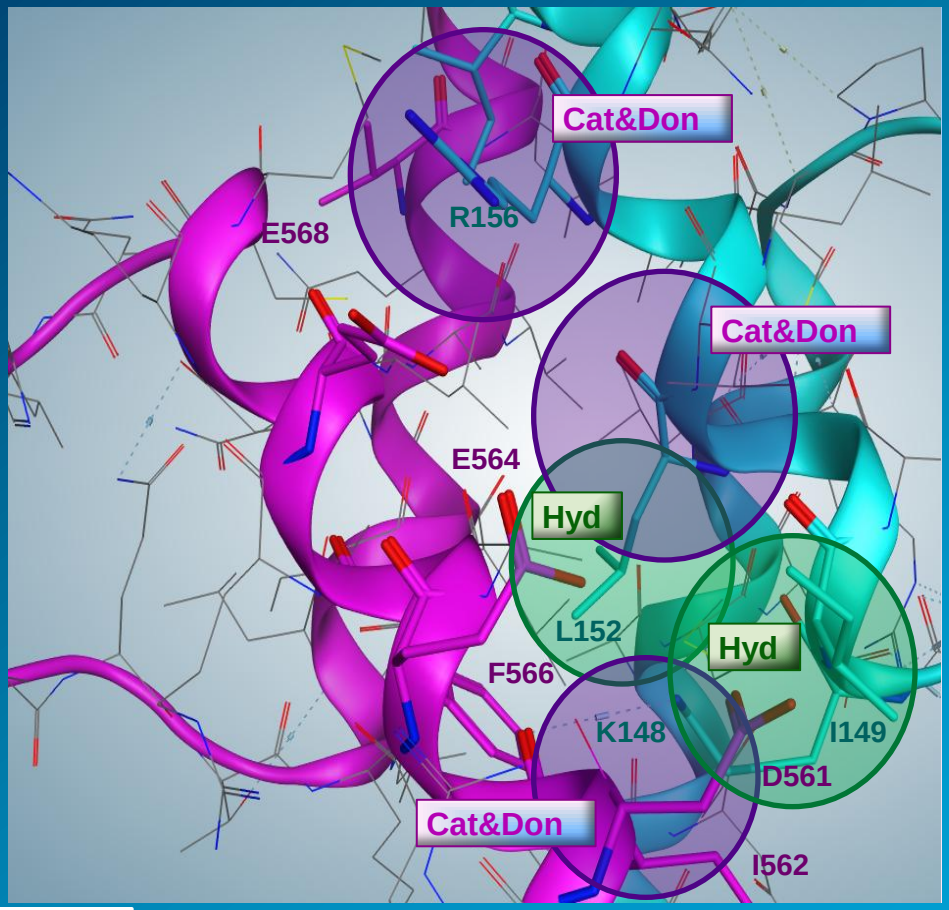
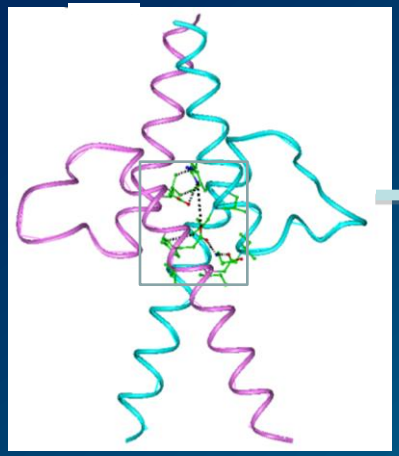
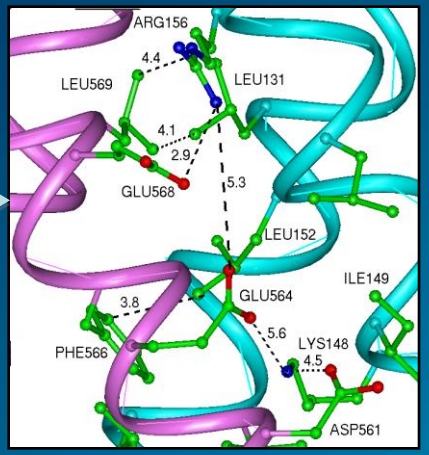
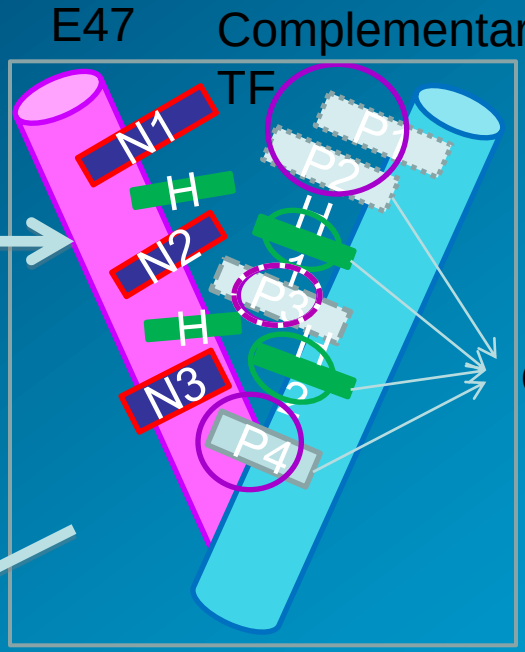
Modeled the entire dimerization surface as an expansive parental binding site (pharmacophore) comprised of multiple daughter sites



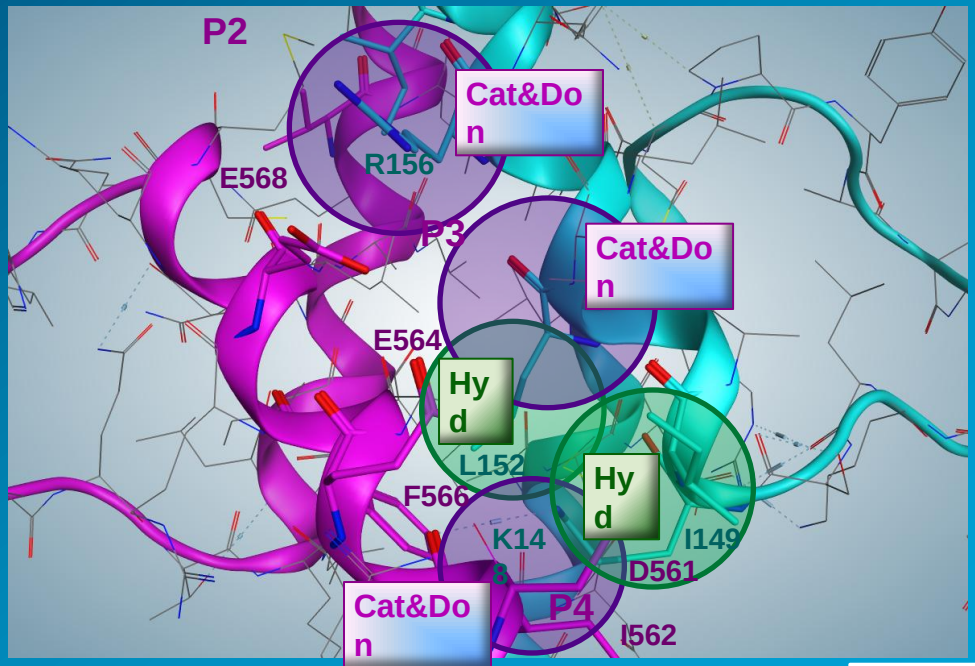
Parental Pharmacophore







Homology modeling. Used neuroD1-E47 dimer as a template. NeuroD1 has very similar sequence to OLIG2.



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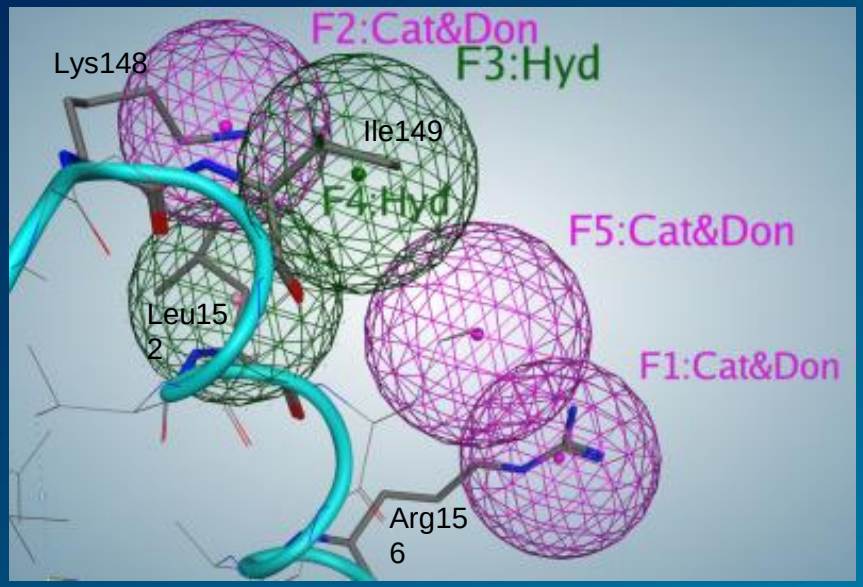
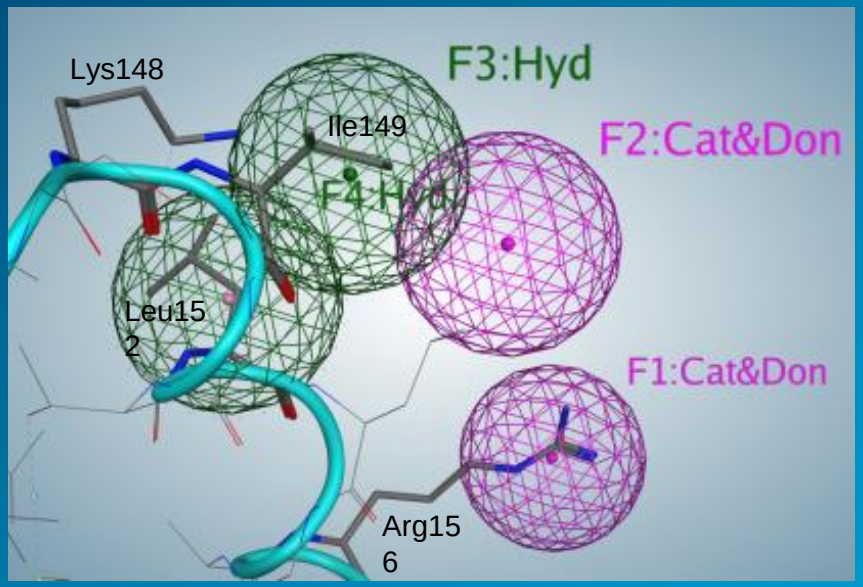
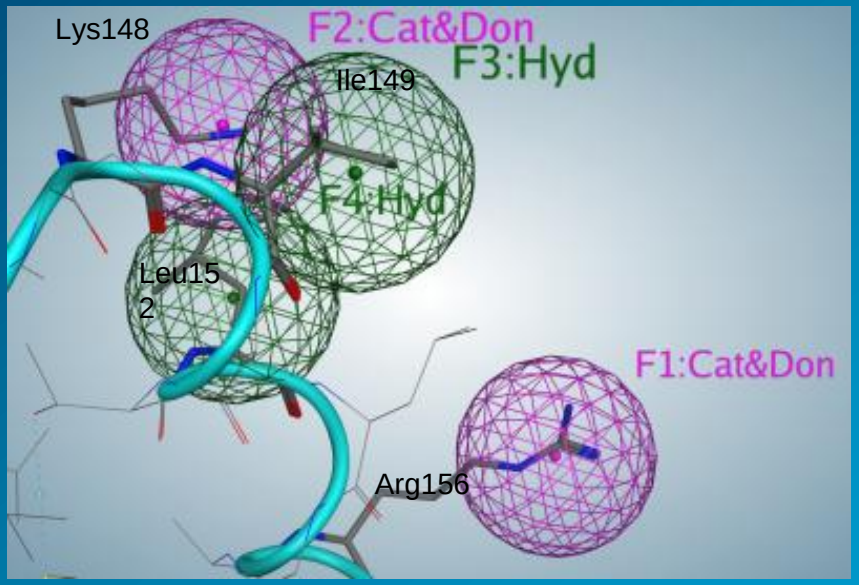
parental pharmacophore



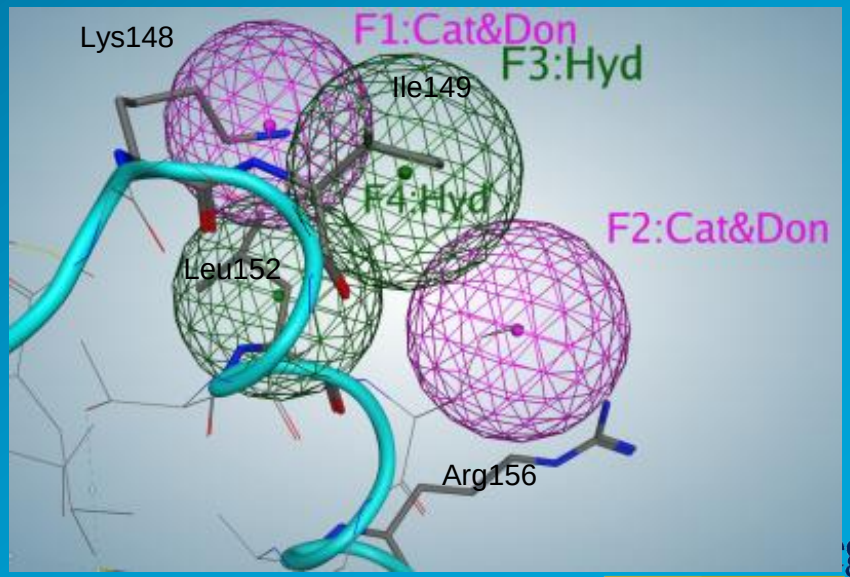
group-1 pharmacophore



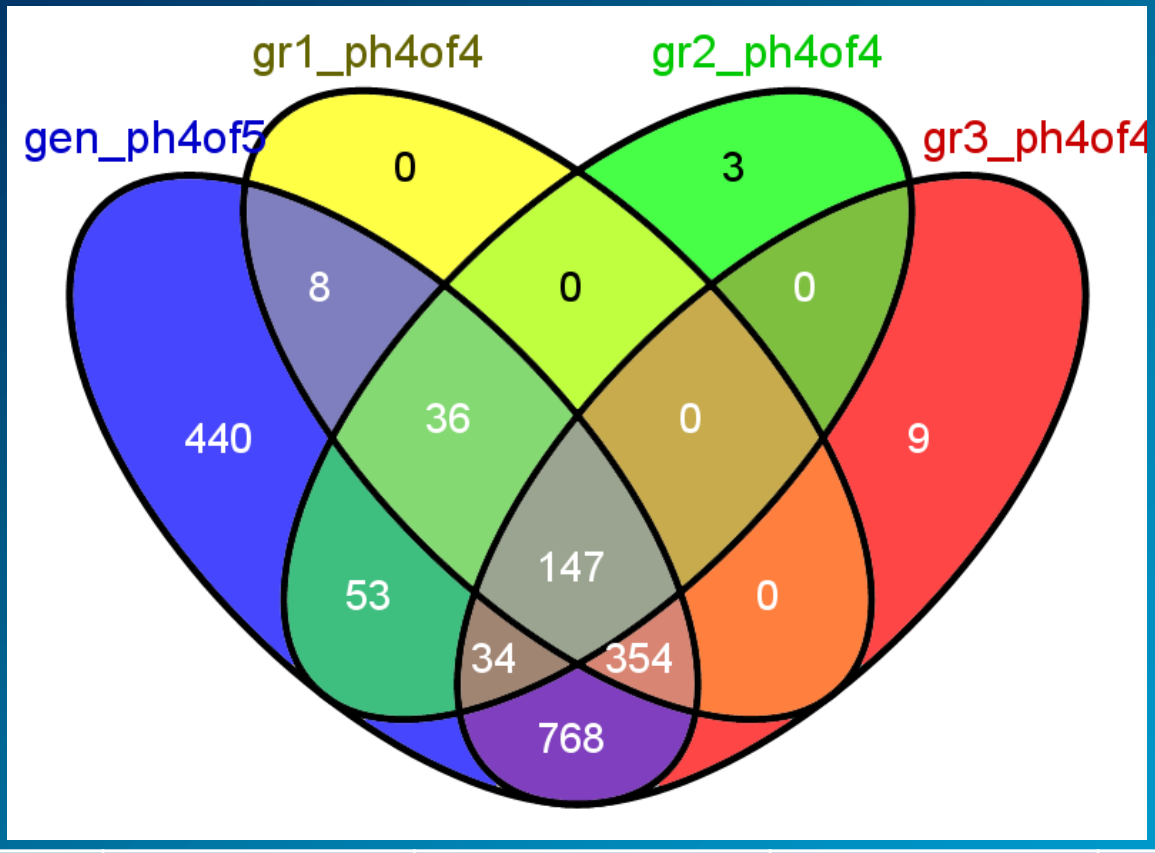
group-2 pharmacophore



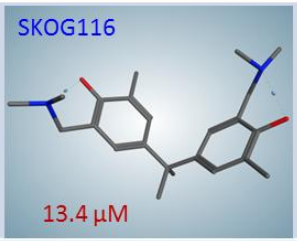
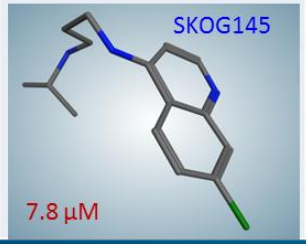
group-3 pharmacophore

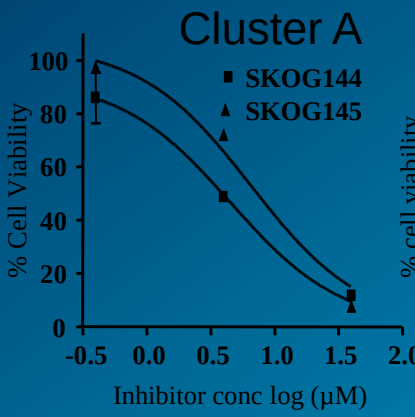


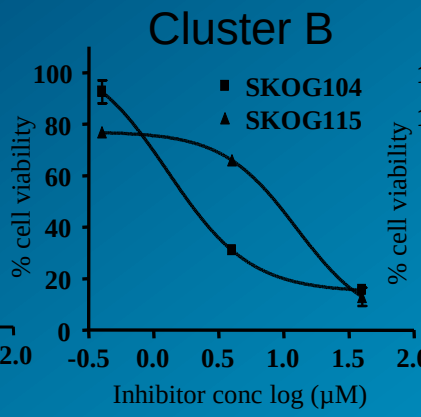
Venn diagram indicating number of structures binding pharmacophores

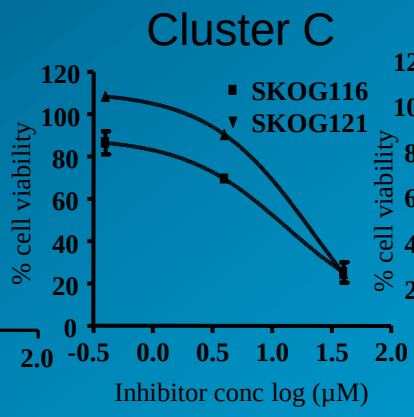


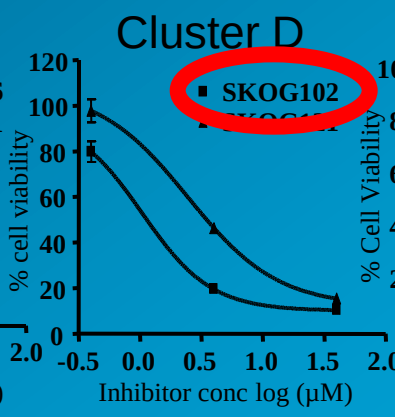
Pharmacophore	gen_pha4of5	gr1_pha4of4	gr2_pha4of4	gr3_pha4of4
Compounds	1840	545	273	1312

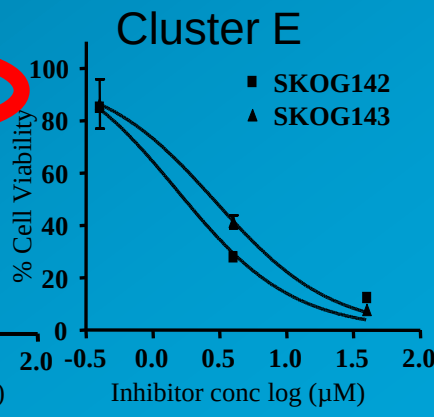
Cluster A (code1) 23 compounds	Cluster B (code2) 26 compounds	Cluster C (code5) 5 compounds	Cluster D (code8) 16 compounds	Cluster E (code72) 6 compounds
				
				











Comp No.	IC_{50}	Comp No.	IC_{50}	Comp No.	IC_{50}	Comp No.	IC_{50}	Comp No.	IC_{50}
SKOG144	4.5	SKOG104	1.74	SKOG116	13.4	SKOG102	1.04	SKOG142	1.24
SKOG145	7.8	SKOG115	12.4	SKOG121	22.2	SKOG141	2.56	SKOG143	2.87

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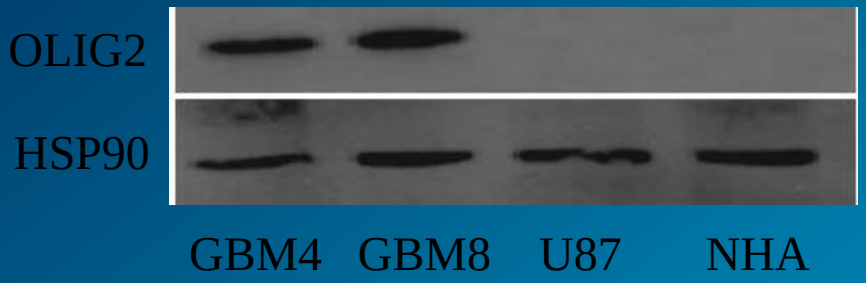
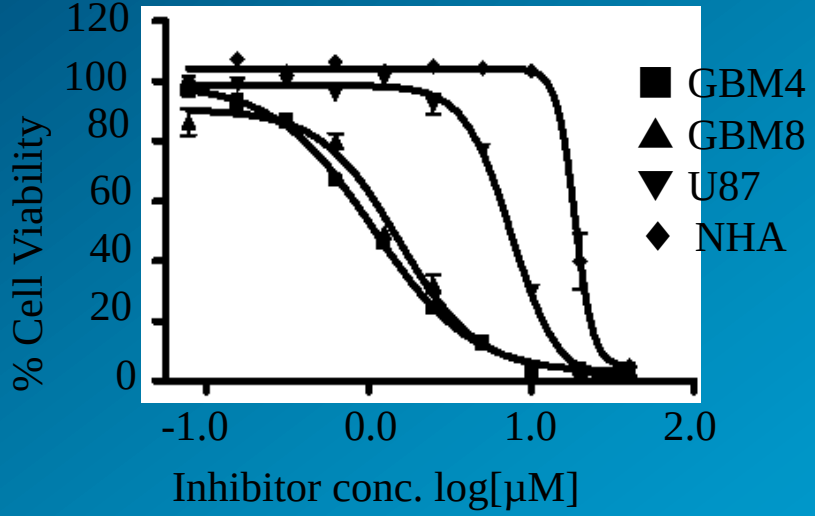
Selectivity: Only OLIG2 expressing cells are sensitive to OLIG2 inhibitor – SKOG102





	GBM4	GBM8	U87	NHA
IC_{50}	1.066	1.536	7.519	18.50

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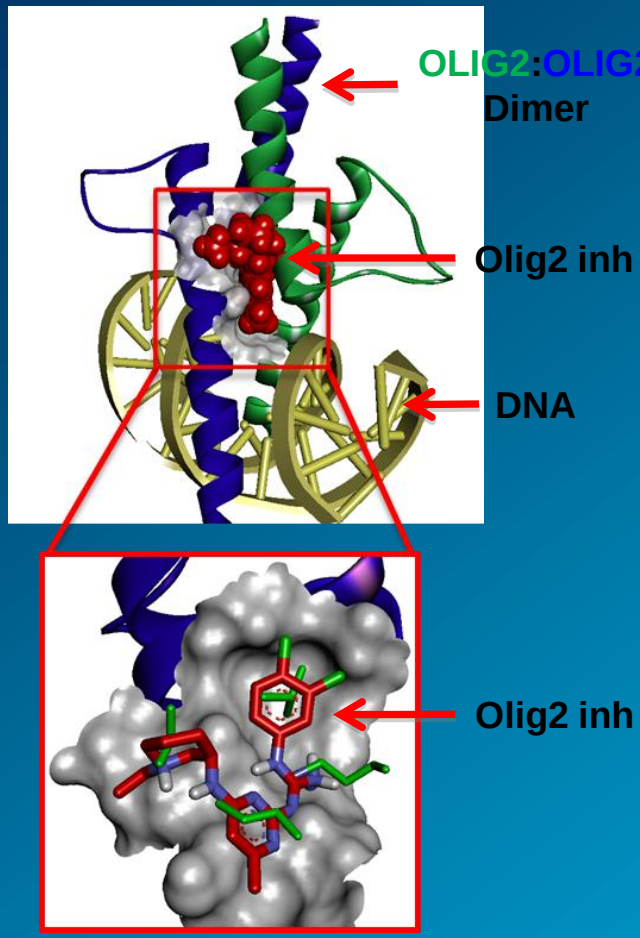
SKOG102:
Proposed Mode of Target Engagement

OLIG2 bHLH Model



Target engagement hypotheses:

1. Disrupt OLIG2 dimerization – **lead hypothesis**
2. Prevent DNA binding
3. Alter OLIG2 transcriptional complex function

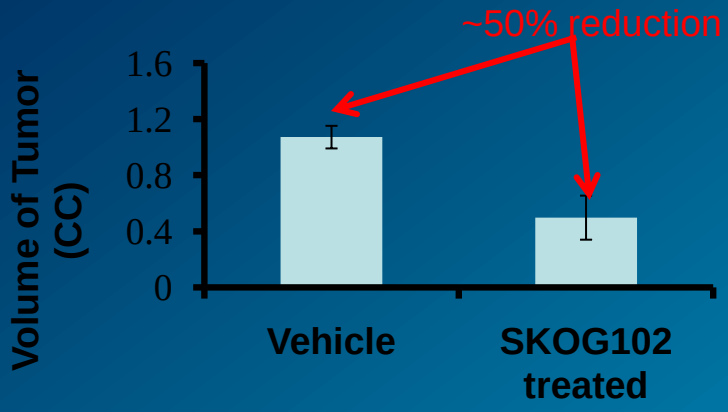
Target engagement studies in process:

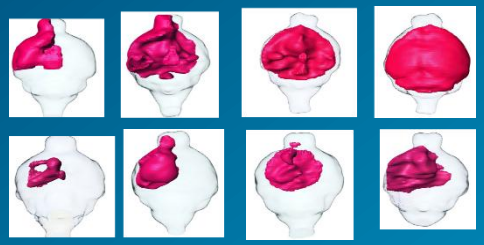
1. BIAcore (dimerization / DNA binding)
2. EMSA (DNA binding)
3. Protein Thermal Stability (direct cmpd binding)

Independent of the actual mode of compound effect, there is a specific mechanistic effect on OLIG2-mediated transcription.

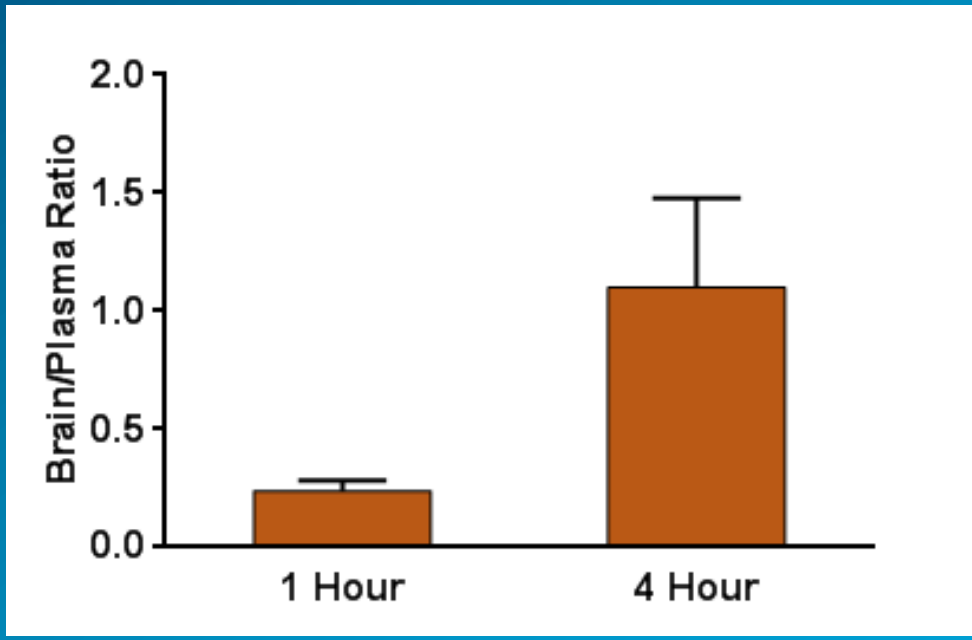
SKOG102 Reduces Tumor Growth In Vivo and Penetrates the BBB

Ex vivo Treated Orthotopic Study





Brain/Plasma Ratio at 1 & 4 Hours



- 1 hour B/P ratio is 0.24
- 4 hour B/P ratio is 1.10
- By comparison, reported temozolomide B/P ratio is 0.07 to 0.29
- Can further improve blood-brain barrier penetration

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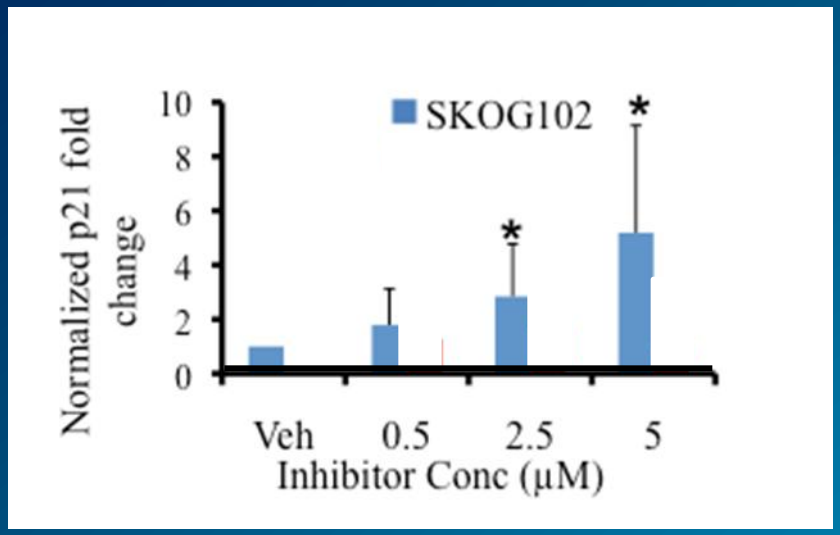
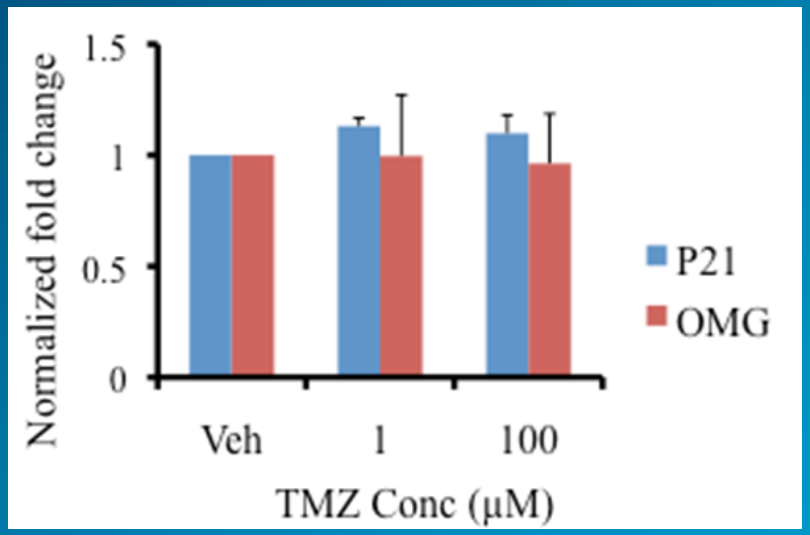
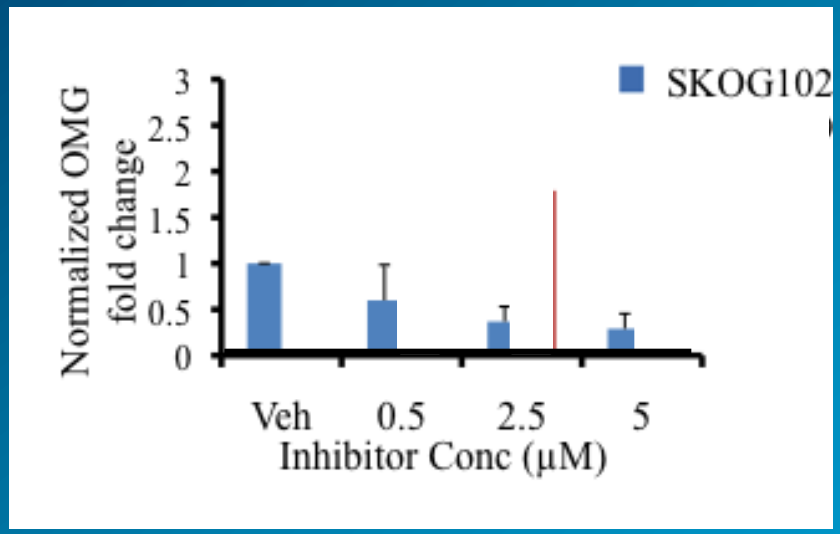
Downstream OLIG2 targets are specifically modulated by SKOG102







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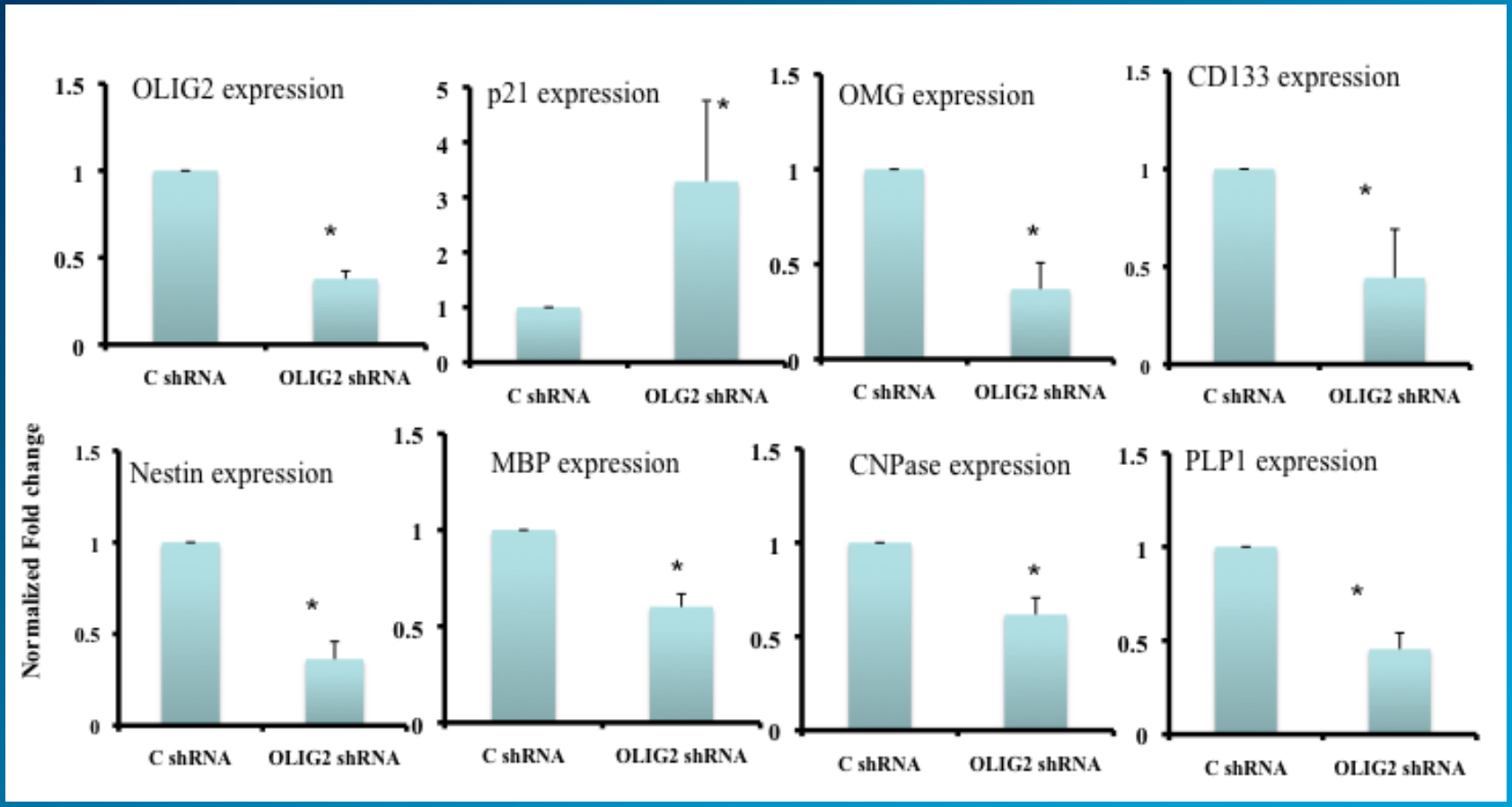
OLIG2 shRNA suppresses direct targets and cancer stem cell markers



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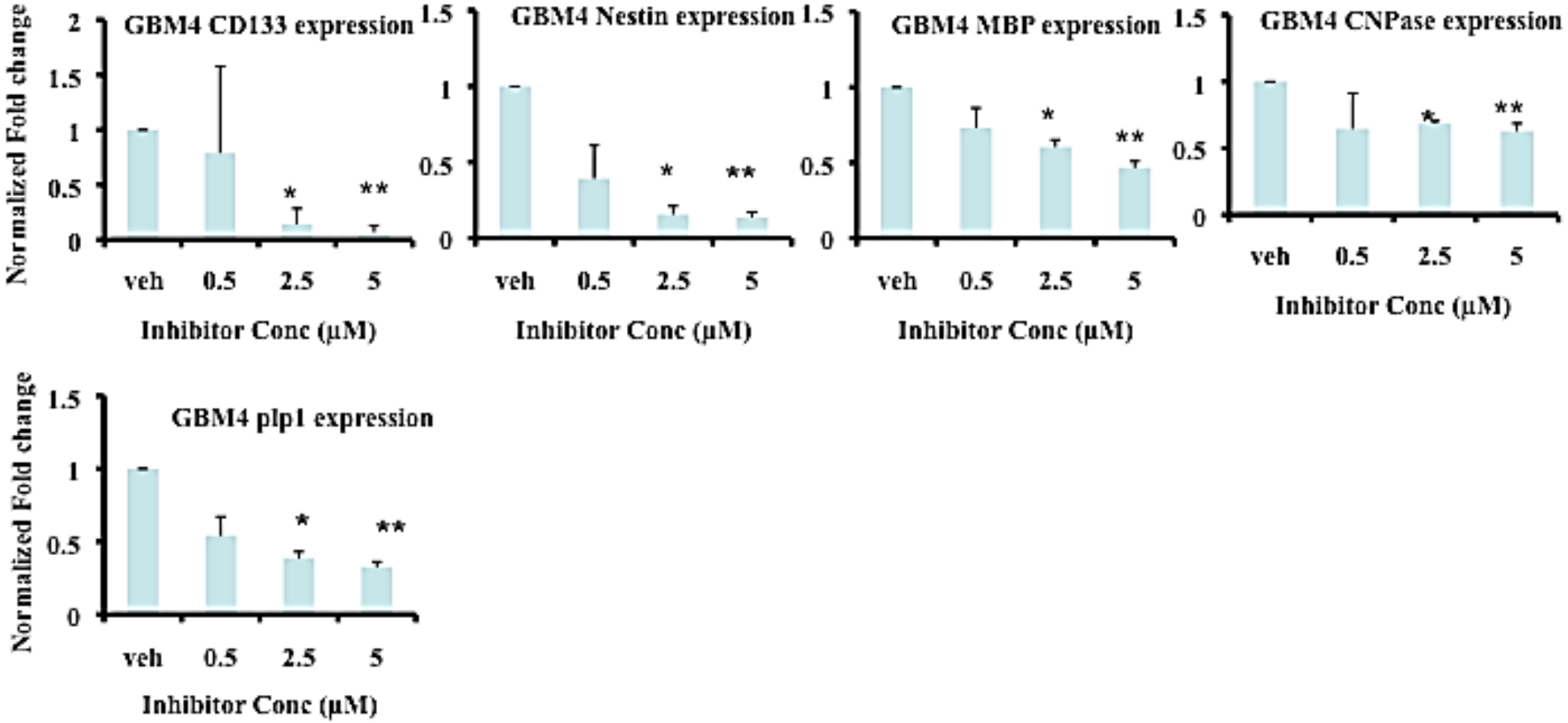
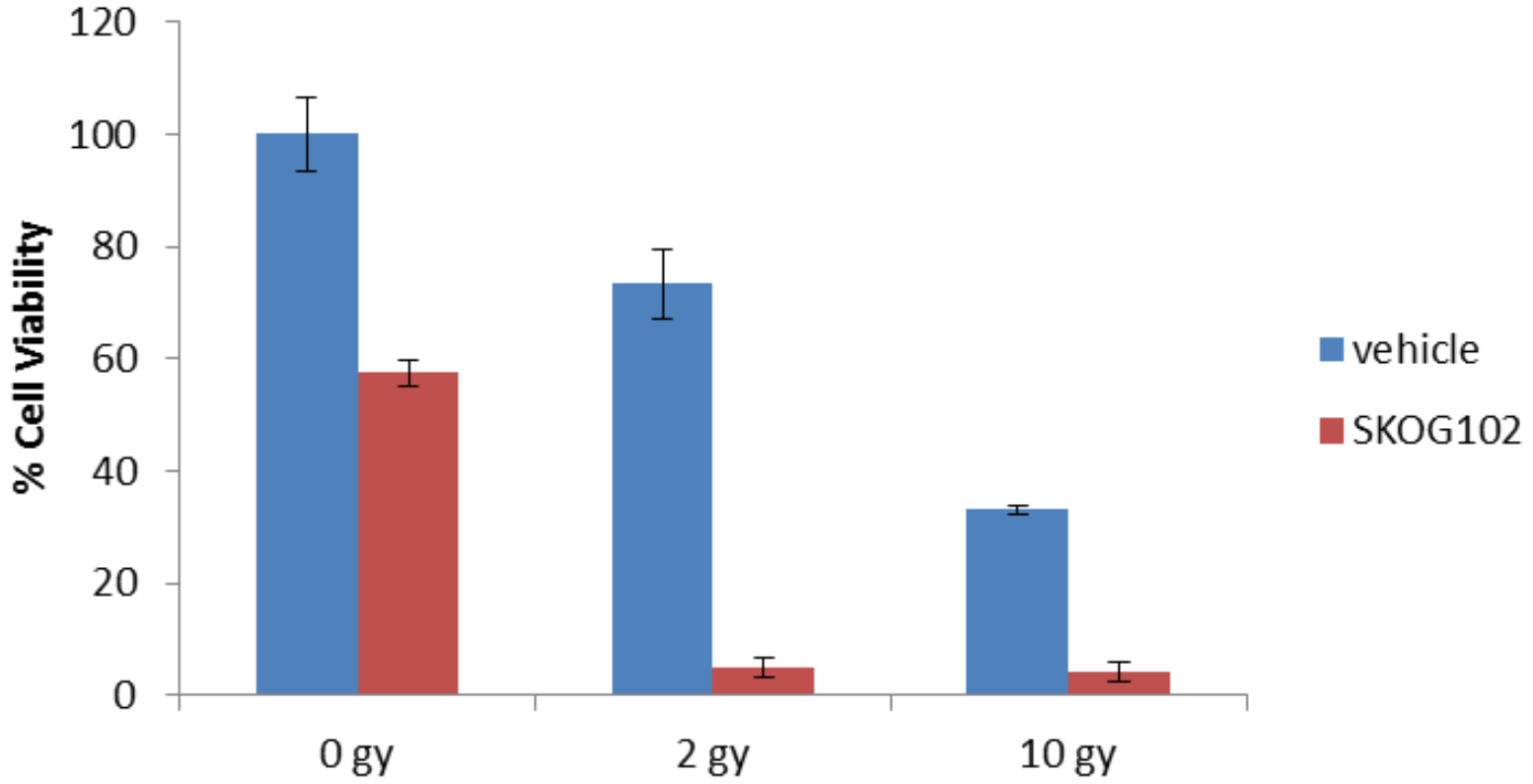
OLIG2 inhibitor suppresses cancer stem cell markers



OLIG2 inhibitor SKOG102 at 1μM (IC_{50}) *in vitro* sensitizes human cancer stem cell derived GBM (GBM8) to radiation



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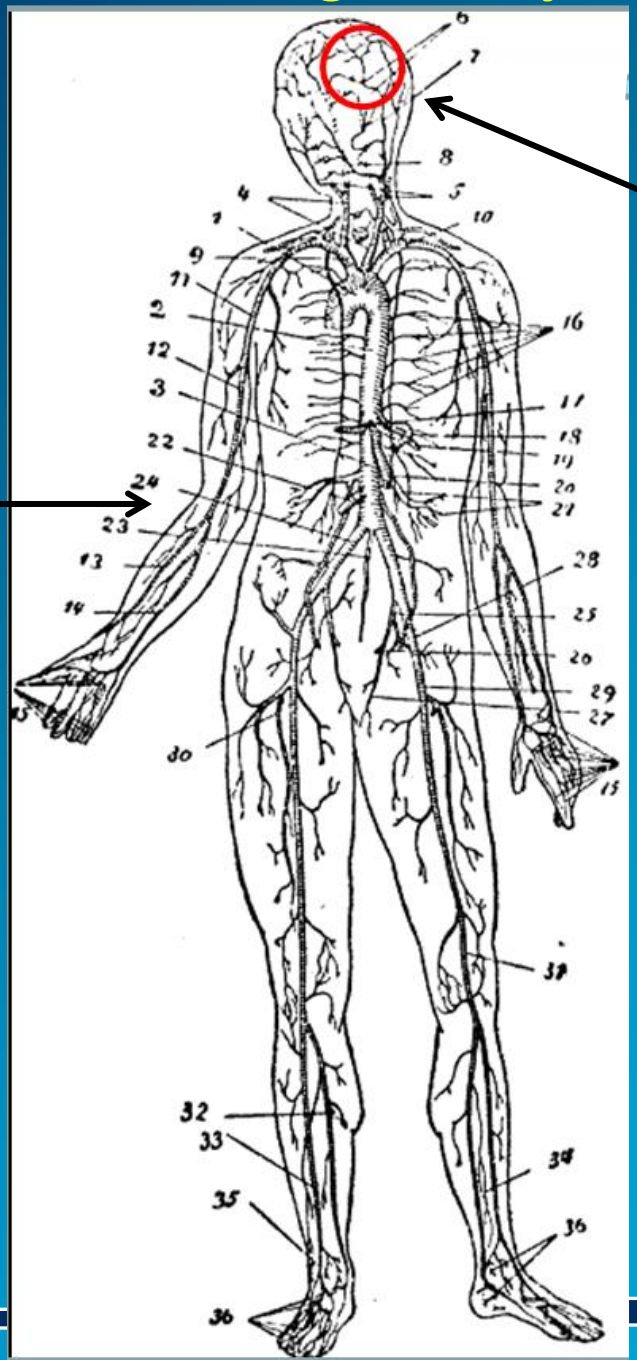
Drug delivery – A key obstacle in GBM

Drug delivery challenge 1: Conventional drug delivery is toxic to the whole body and limits dose



Brain tumor

Inject drug IV

TOXICITY TO:

Blood forming organs

Liver

Kidney

Intestine

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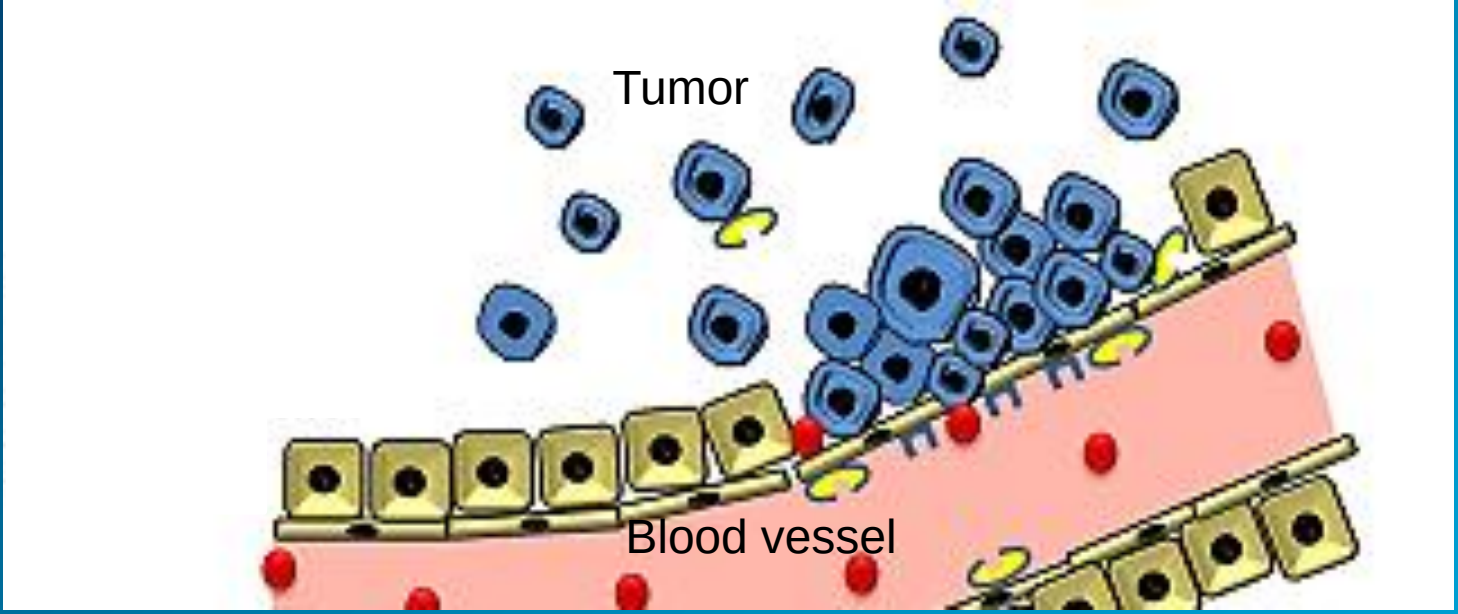
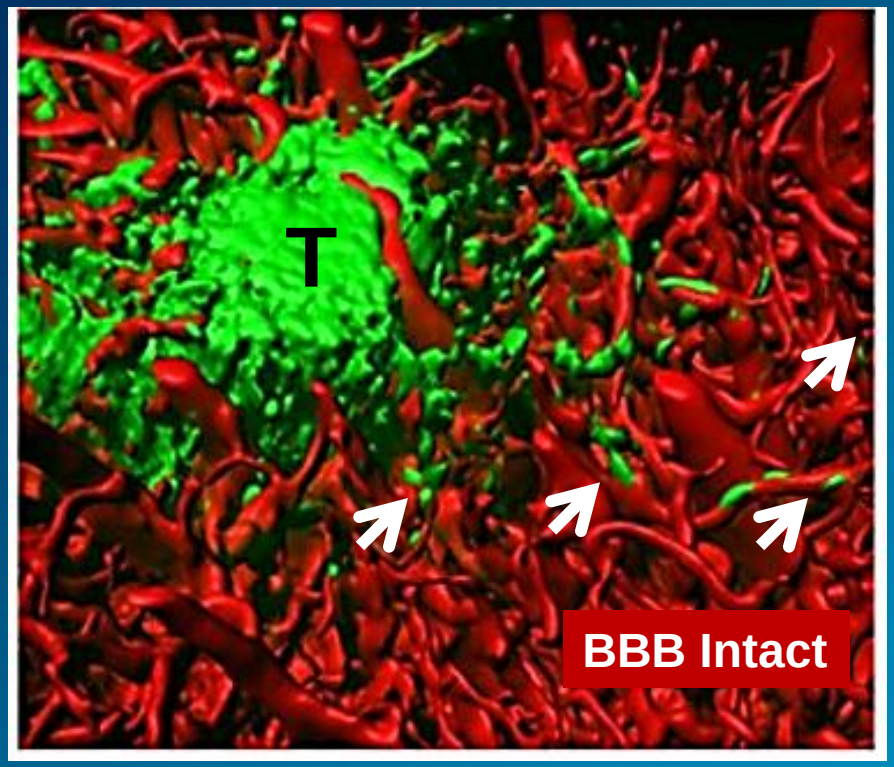
Drug delivery challenge 2: Drugs cannot penetrate multiple layers of packed tumor and normal cells which constitute a physical barrier

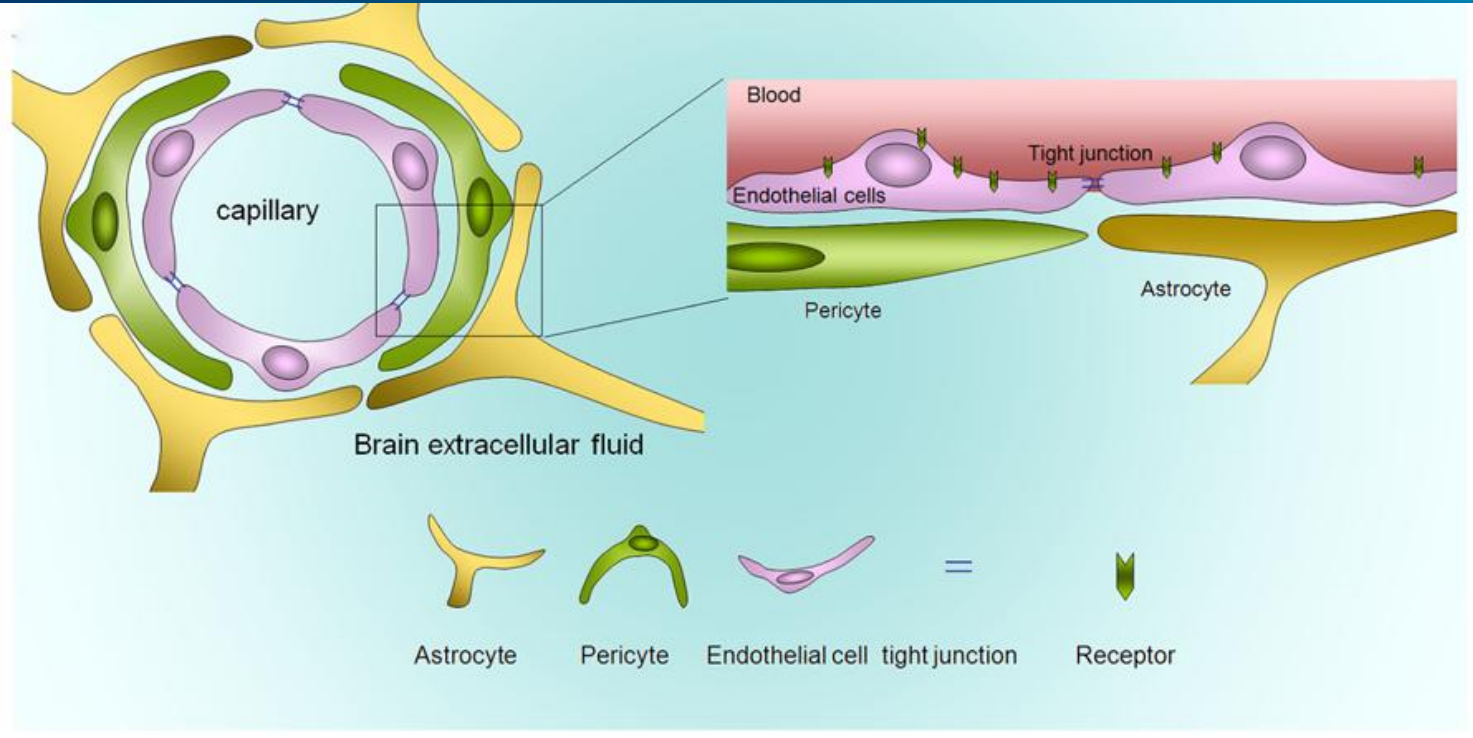


Drug delivery challenge 3: Drugs cannot enter brain to engage invading cells because of blood brain barrier (BBB) which pumps out drugs

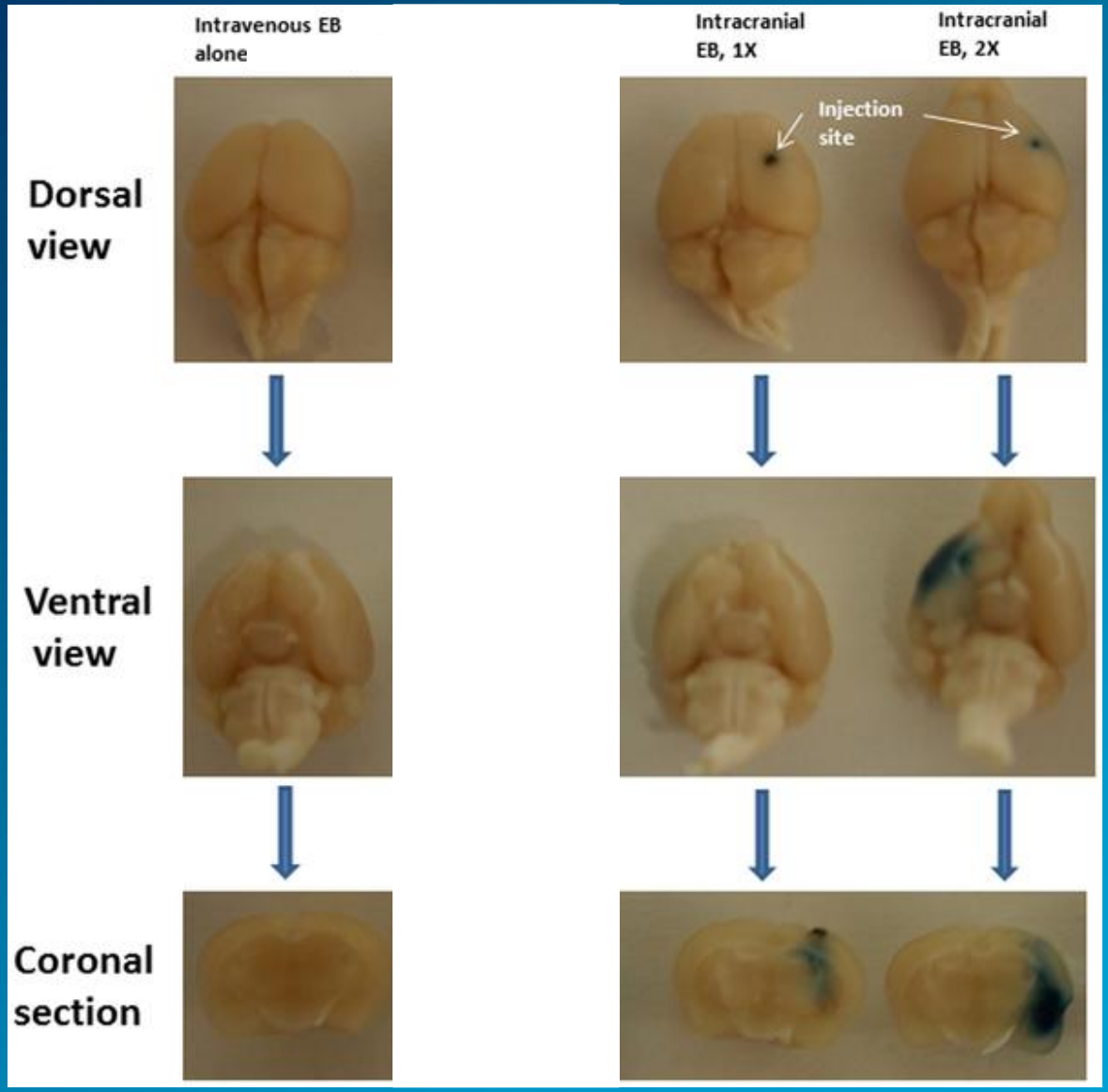


Frank Winkler – German cancer research center
Glioblastoma in living mouse

BBB is designed to protect the brain from toxins but does block drugs



The Blood Brain Barrier: Qualitative comparison of brain uptake of Evans Blue (EB) via intravenous and direct intracranial injections.



Sarkar G, Curran GL, Sarkaria JN, Lowe VJ, et al. (2014) PLoS ONE 9(5): e97655.

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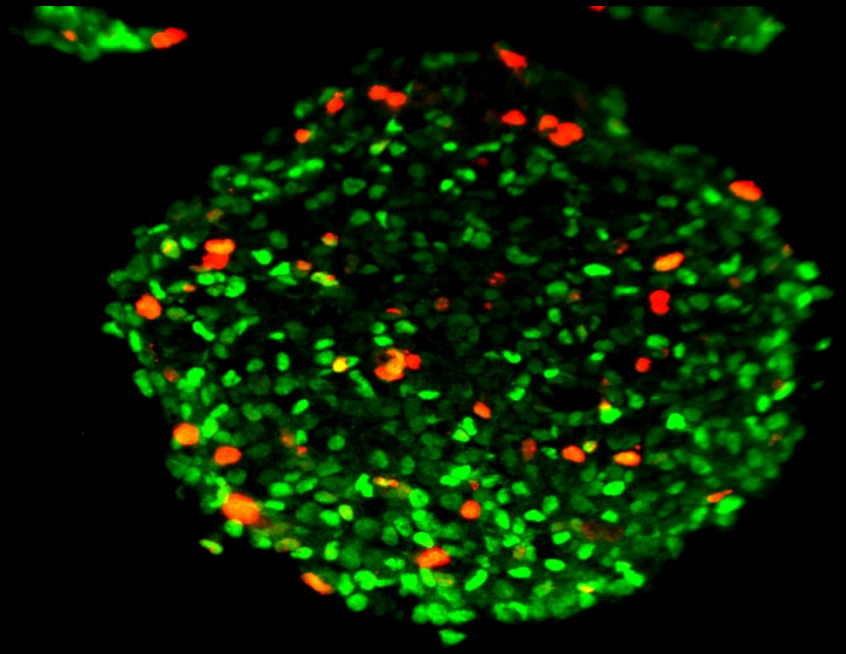


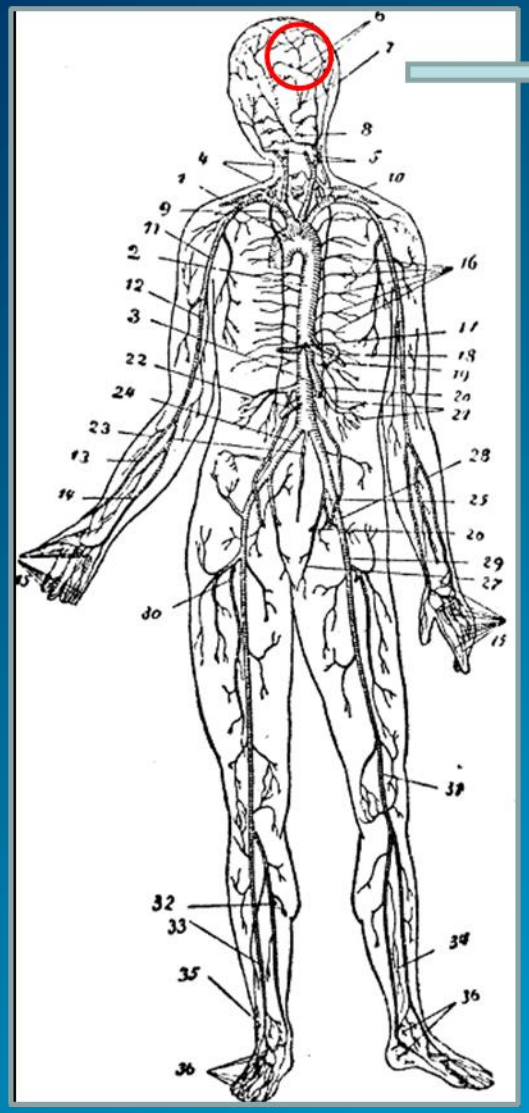
Our approach has 2 main parts:

1. Reformulate potent agents via a targeted nanoparticle delivery system.
   -to target just tumors and to bypass efflux pumps

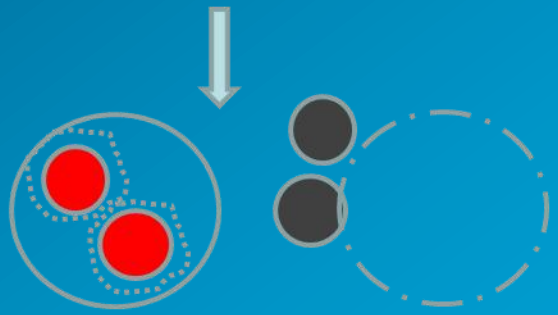
2. Develop new agents that can engage CSCs and enter brain.

Nanoparticle delivery specifically targets tumors

Inject nanoparticles



NPs accumulate at tumor.



Nanoparticles release drug at tumor

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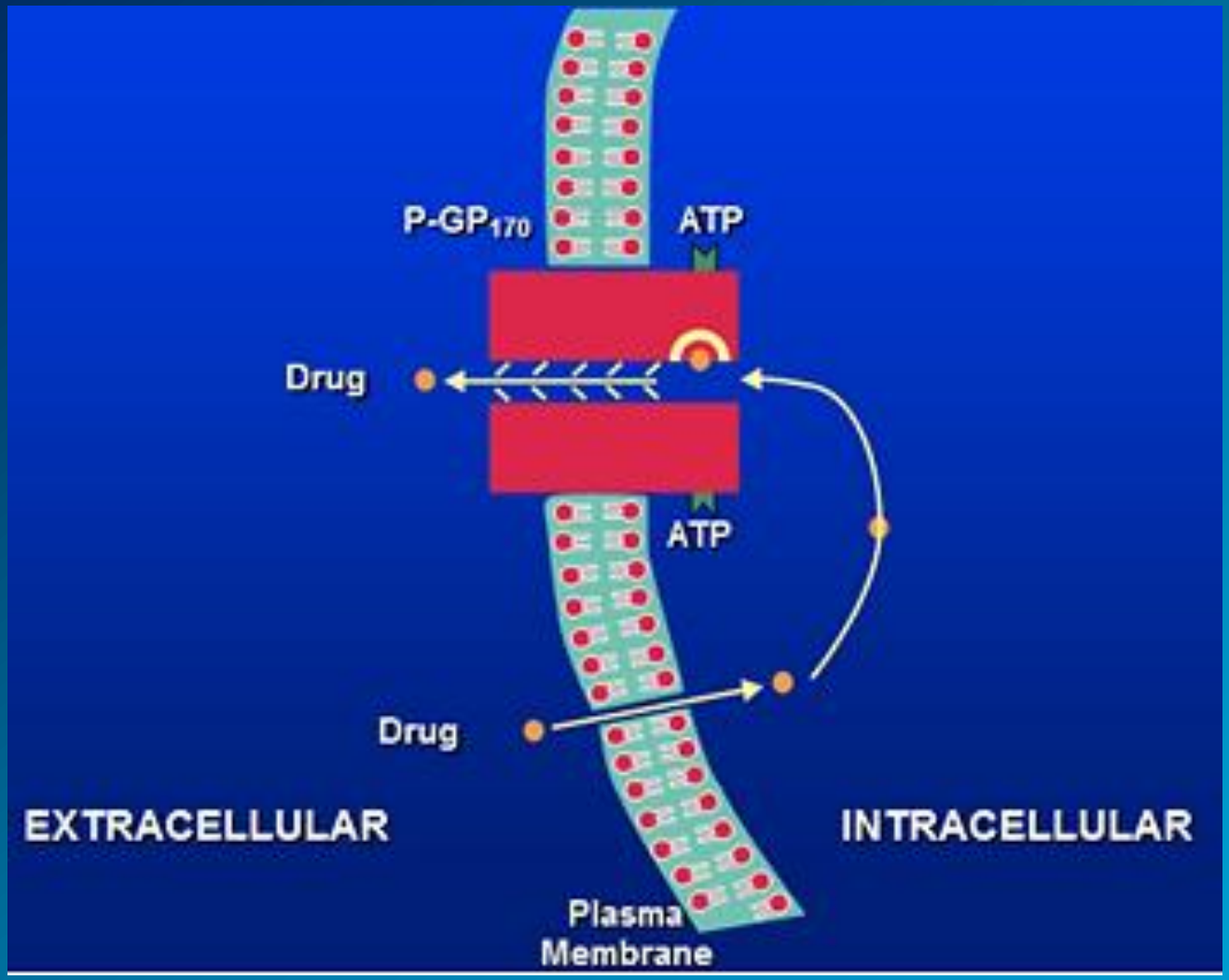
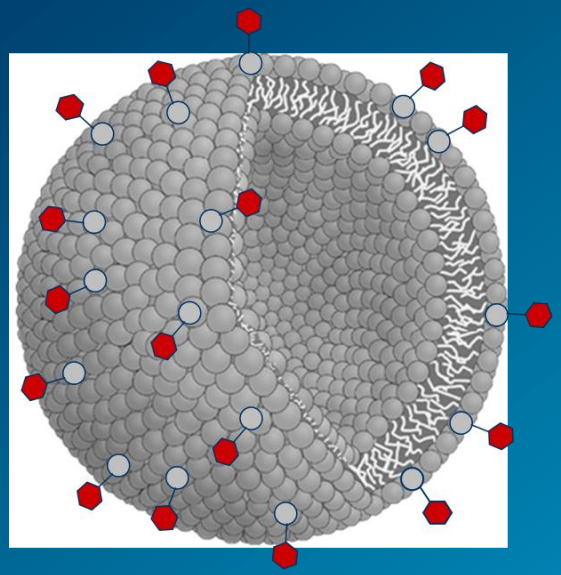
Drug delivery challenge 4: Tumor cells and BBB can pump drugs out



Pgp inhibitors so far have proved too toxic.

Nanoliposomes reformulate potent agents that failed because of systemic toxicity

Specific drug therapy challenges can be addressed by nanoliposomes



Optimal size is 50-200 nm.

A 100 nm liposome = 100,000 lipid molecules
10 mM lipid = 1 x 10^{-5} moles/ml = 6x10^{18} molecules/ml
1 ml of lipids from 10 mM stock = 6x10^{13}NP
Void volume/particle = 70% = 4 x 10^{-17} ml/particle
Thus 6 x 10^{13} particles = 2.5 uL = 3 mg drug/ml NP at 100 % packing

At 10% drug loading = or 300 ug drug /ml NP
At 200 uL inj/mouse = 60 ug / ml = 3 mg/kg mouse

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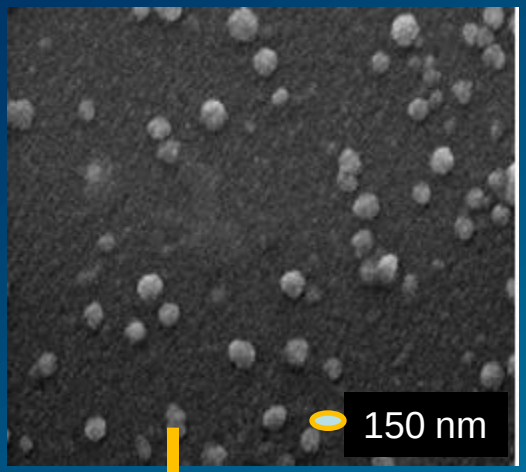
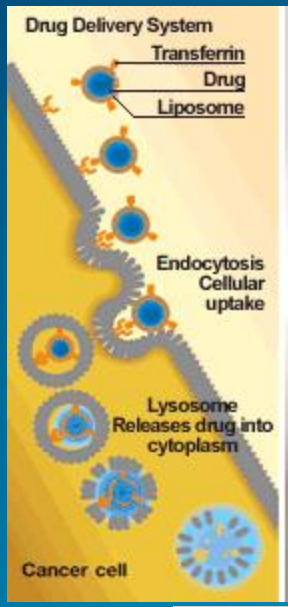
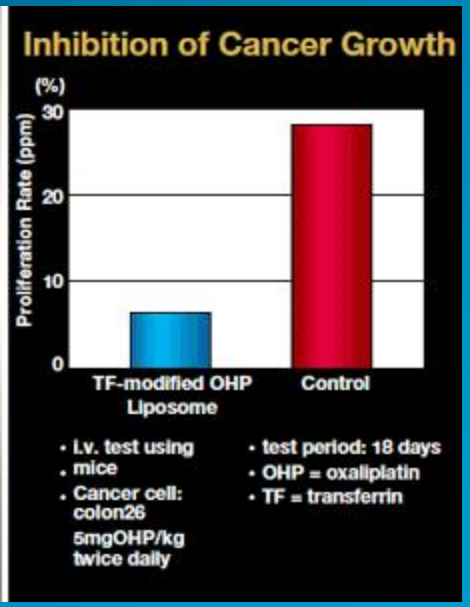
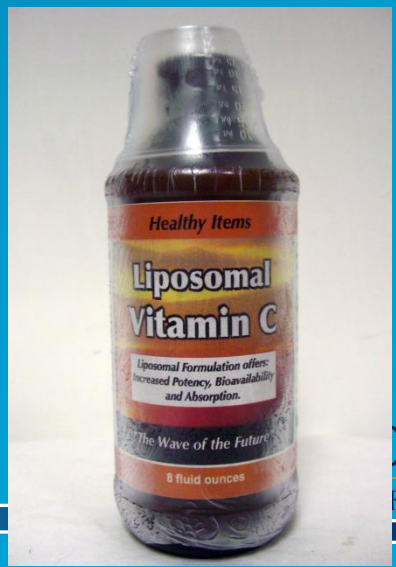
Recent escalation of liposomal nanoparticle formulations to enhance the therapeutic index of existing agents

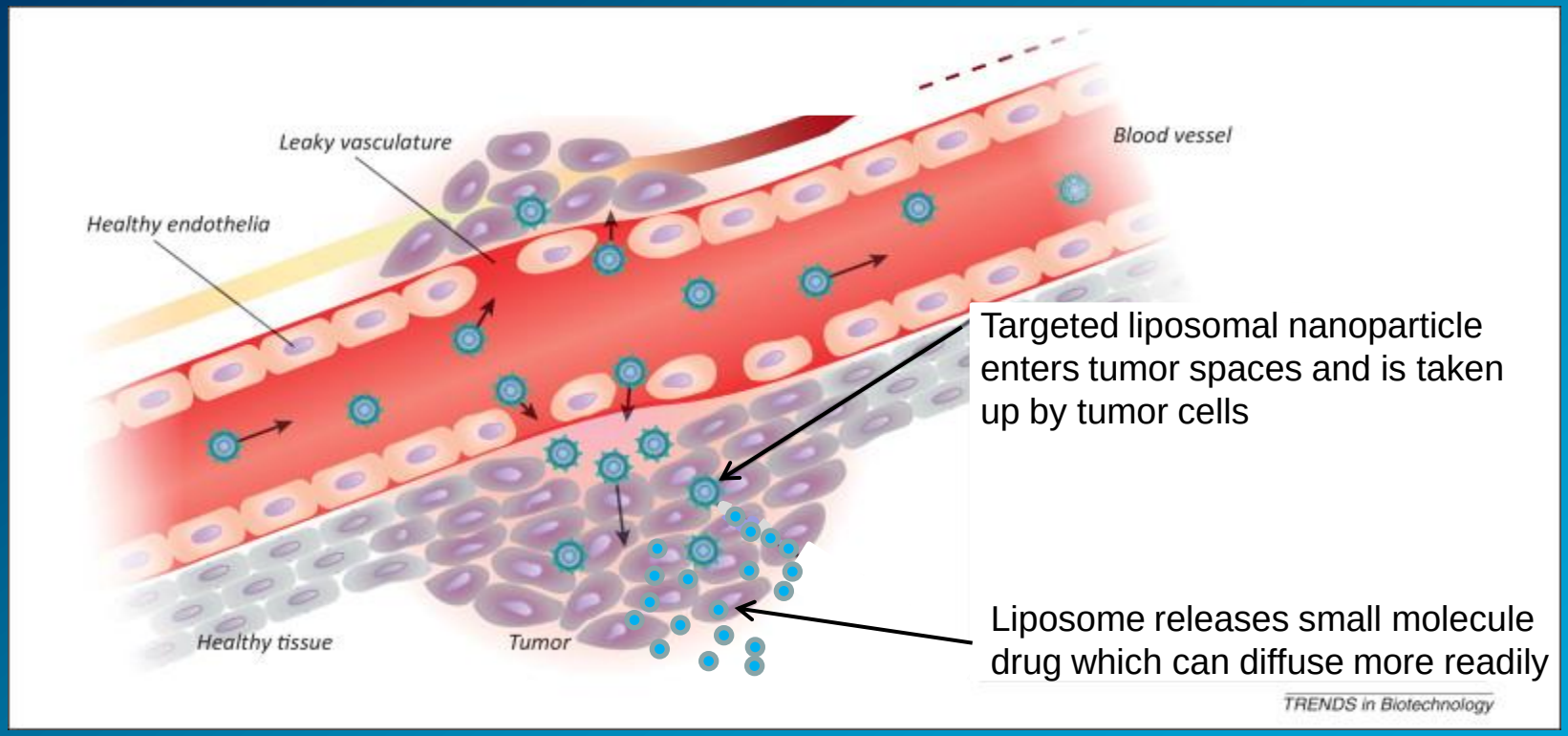








If nanoparticles release a small molecule payload at the tumor diffusion may be more effective – can trigger the particles to release at tumor



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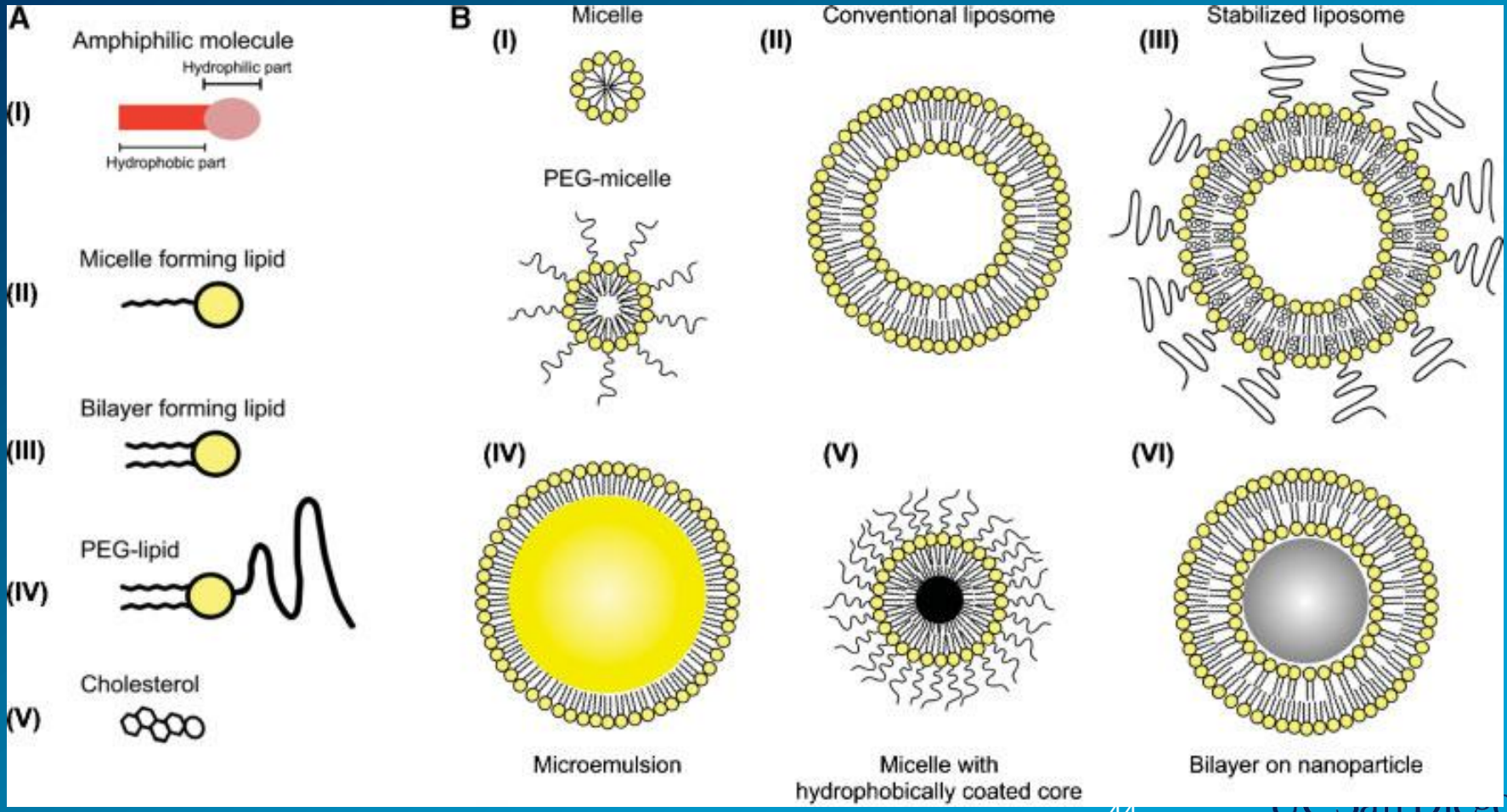
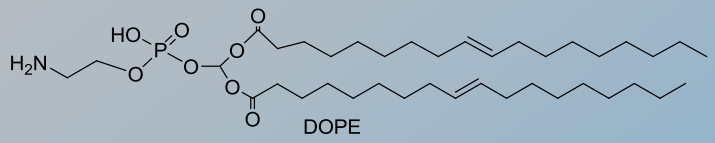
Types of encapsulating lipid based nanoparticles



Mulder W, Biomed., 2006

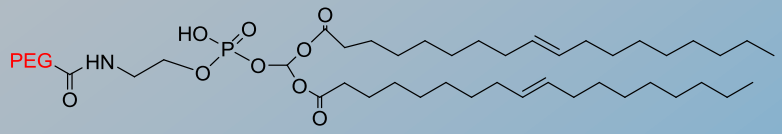
Components of encapsulating liposomes



DOPE

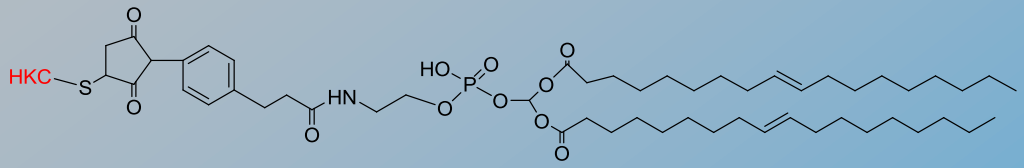
Function

Base lipid (40%)



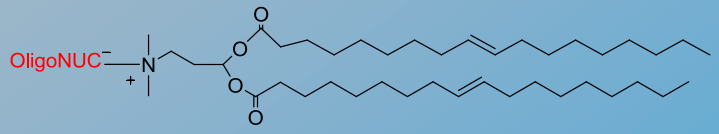
PEG-2000-DOPE

Stealth component (15%)



MPB-DOPE Hisoligolys

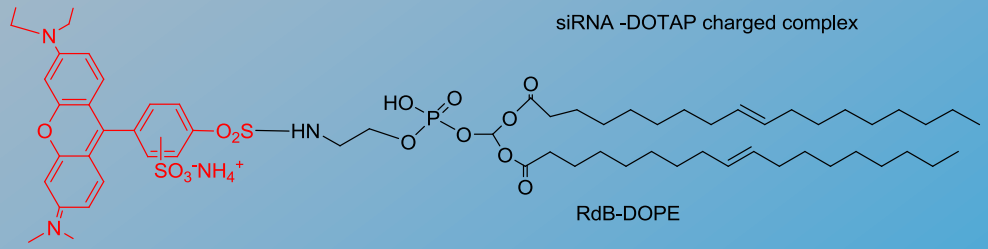
Proton sponge (4%)
Targeting peptides



siRNA -DOTAP charged complex

Oligo NT carrier (40%)

RdB



RdB-DOPE

Fluorescence probe (1%)

Structures in **red** indicate various lipid modifications (functionalization)

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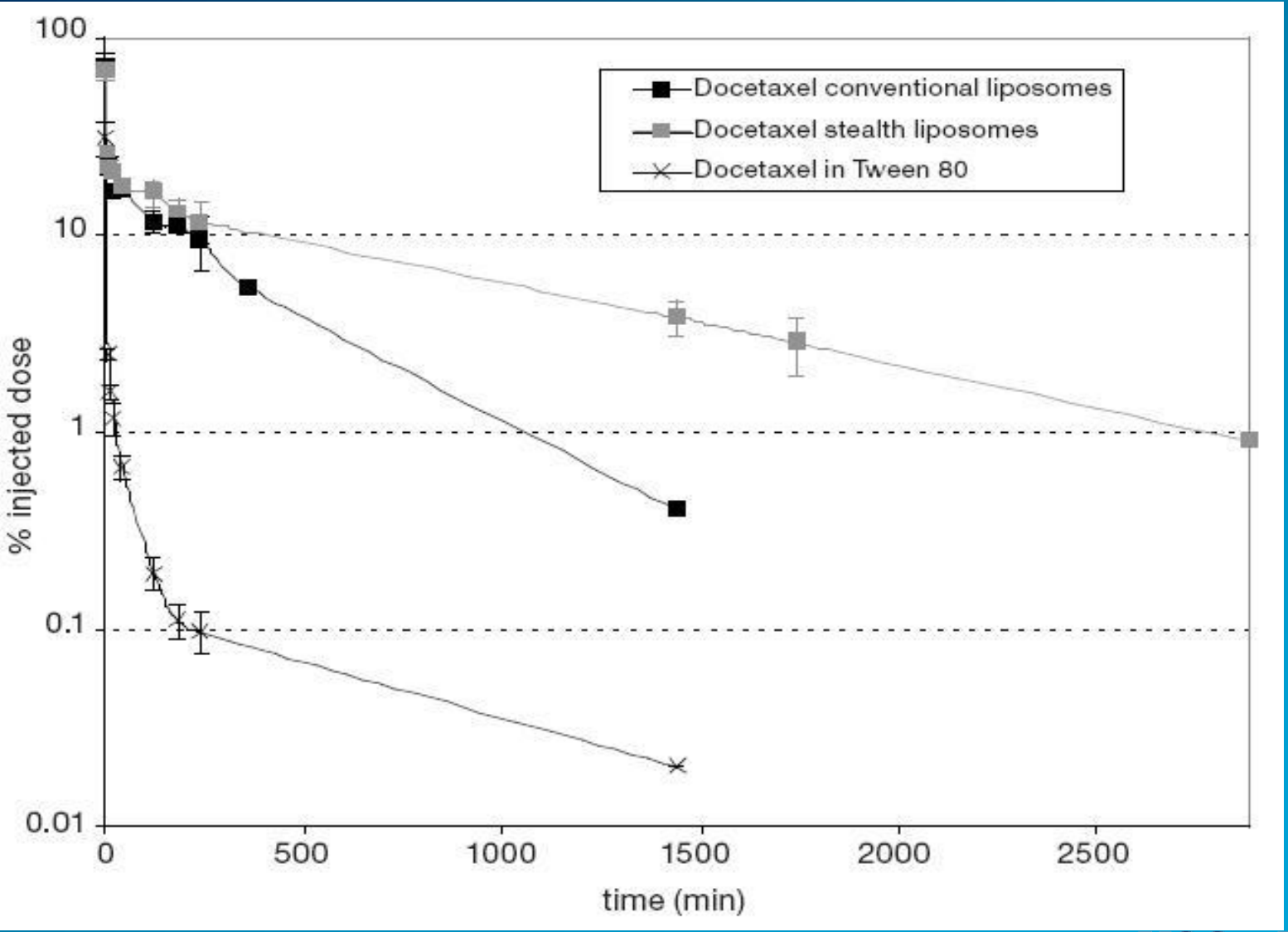
Effect of encapsulation and stealth on drug circulation time



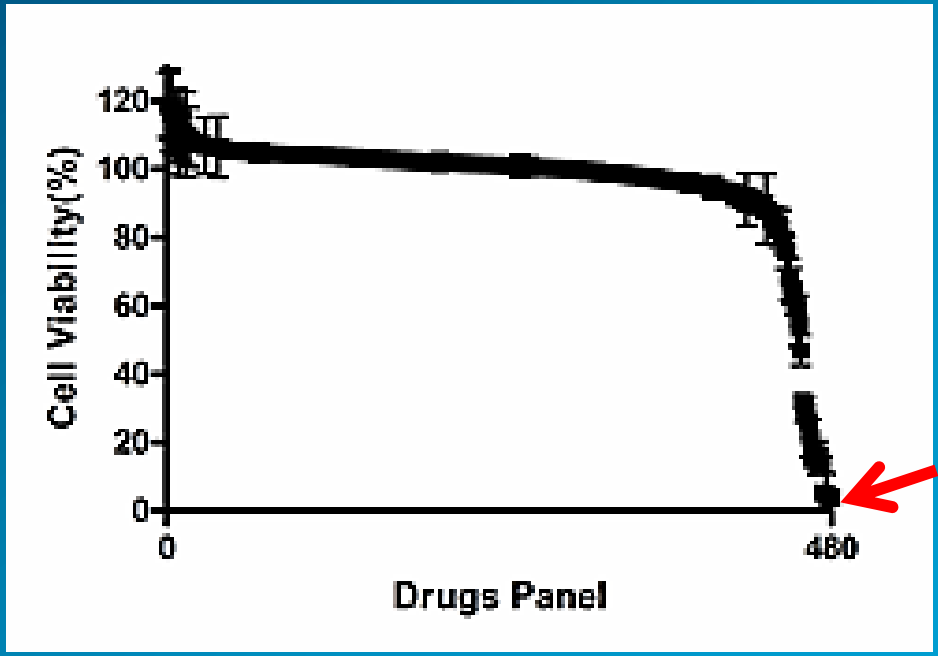
Primary advantages of using nanocarriers for therapy

Drug Encapsulation	- NP prevents contact with healthy tissue
EPR Effect	- NP specifically enters tumors through leaky vessels
Drug targeting	- NP targets cell surface receptors – transferrin/insulin facilitate BBB permeation and bypass BBB efflux
Cell uptake	- NP bypasses efflux pumps

Many promising agents have failed in GBM because of toxicity issues

e.g., Imatinib (panel of receptor tyrosine kinases), Desatinib (src kinase)

We screened 480 compounds (included 450 from NIH clinical collection) staurosporine was one of most potent agents against U87 GBM *in vitro*.



Staurosporine is a potent pan-kinase inhibitor that overcomes chemotherapy resistant cells.

Staurosporine in PI3K ATP binding pocket
Walker *et al*, *Mol Cell*, 6, 909–919, 2000

Stauro 7-hydroxy analogue showed promise in Phase II trials but dosing limited
NIH reluctantly abandoned due to toxicity and PK issues

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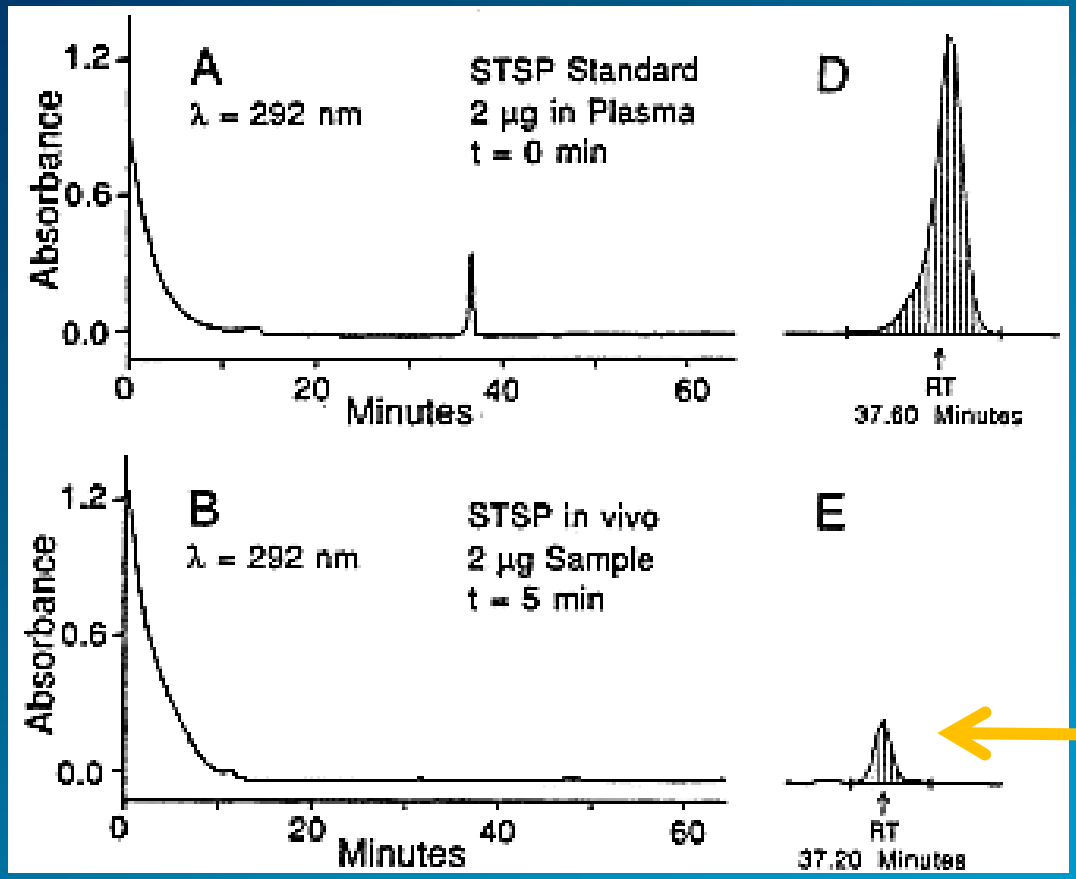
Staurosporine has poor PK due to rapid clearance



Only <0.7% left 5 min after IV bolus

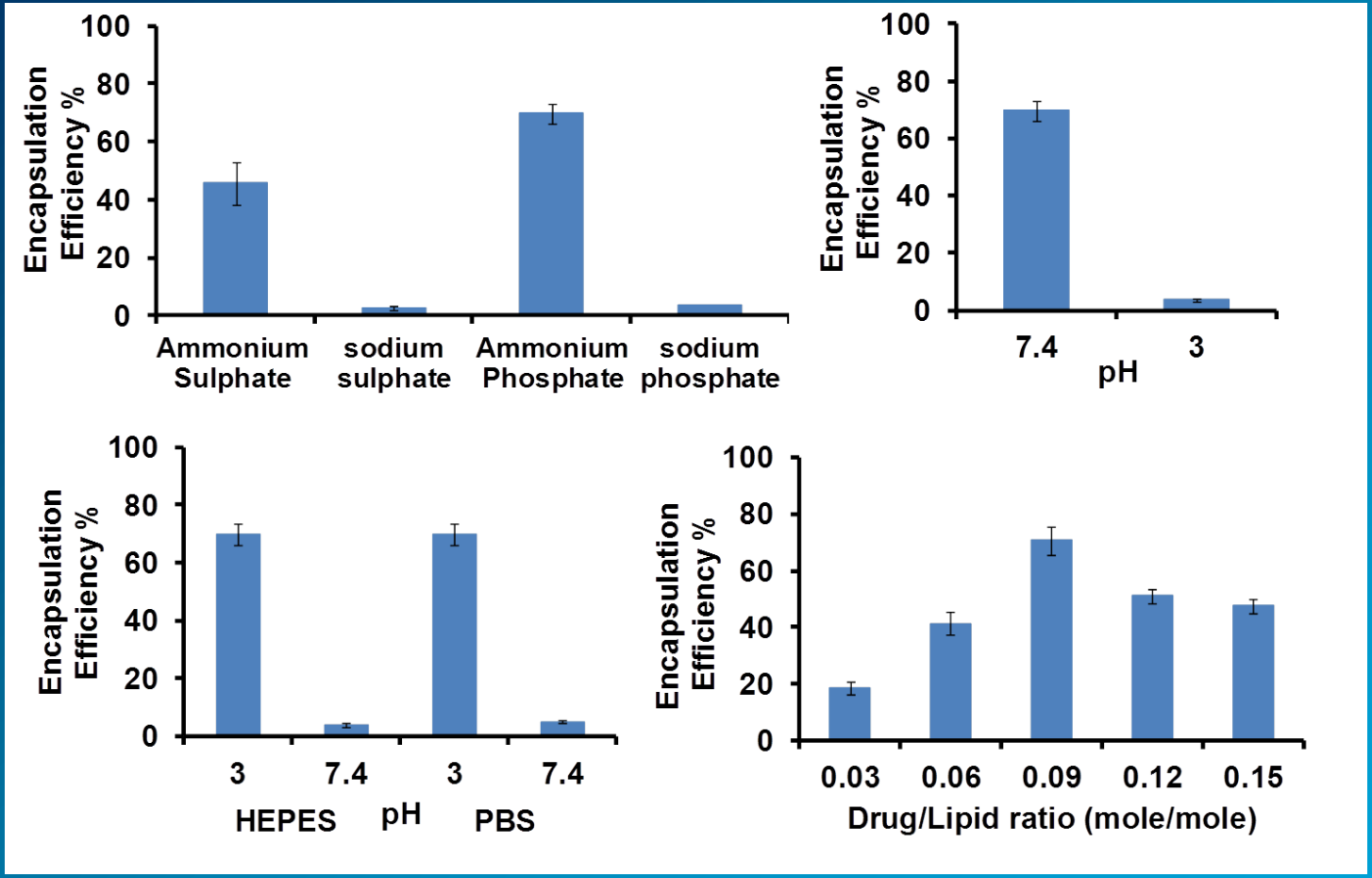
Gurley et al, J. Chromat Sci 1998

Obstacles to stauro therapy can theoretically be overcome by nanoliposomes

However loading of stauro has been reported to be very low <25%

The solution

- reverse pH gradient technology and buffer composition
- initially obtained 73% loading, now close to 100%

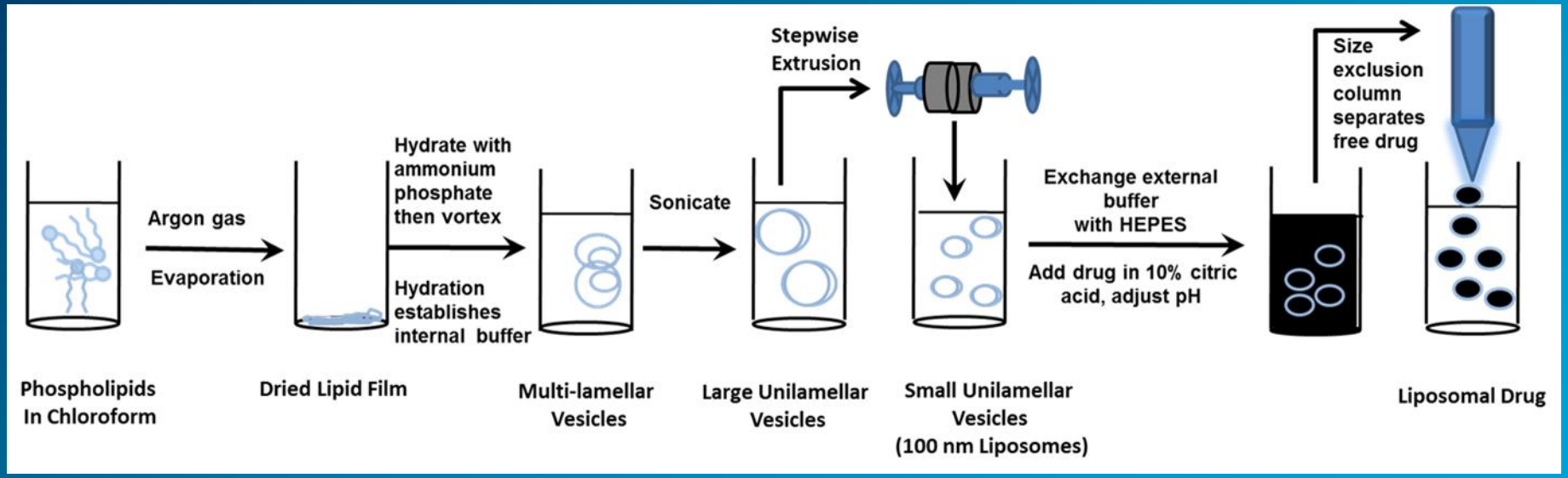


Mukathavaram et al, Int J. Nanomedicine

Synthesis of stauro loaded nanoliposomes

-straightforward method

-useful for a range of drug chemotypes



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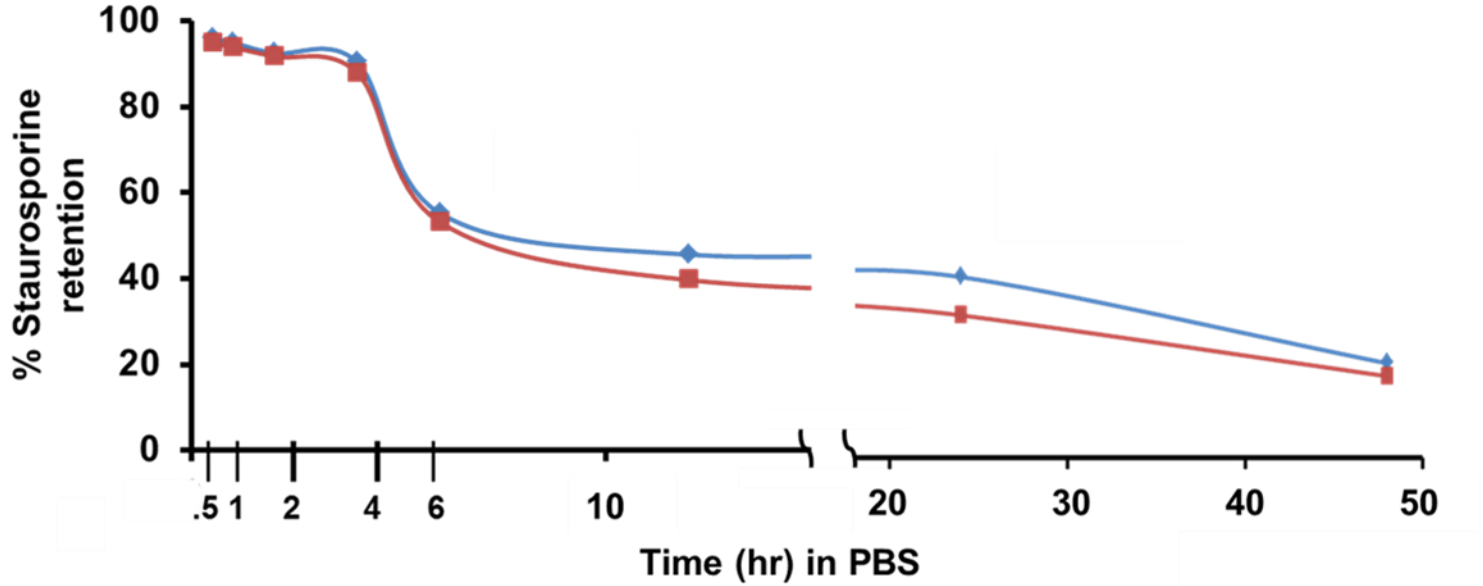
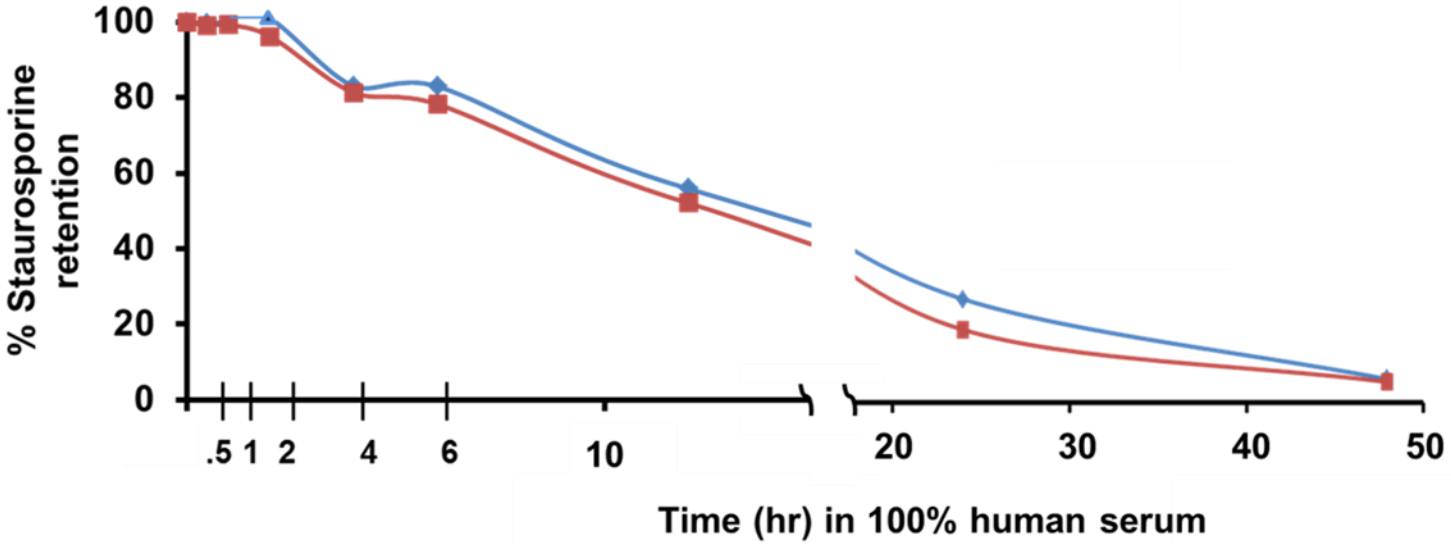
Liposomal retention of staurosporine





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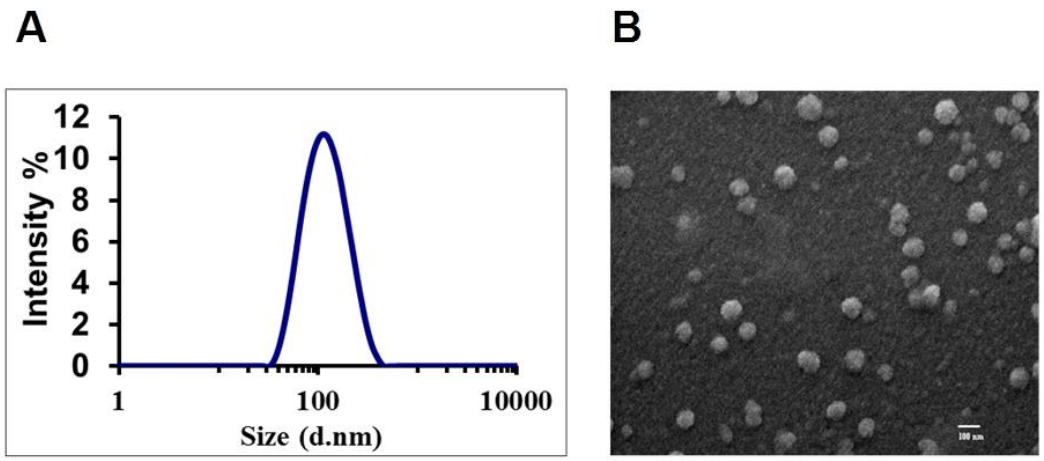
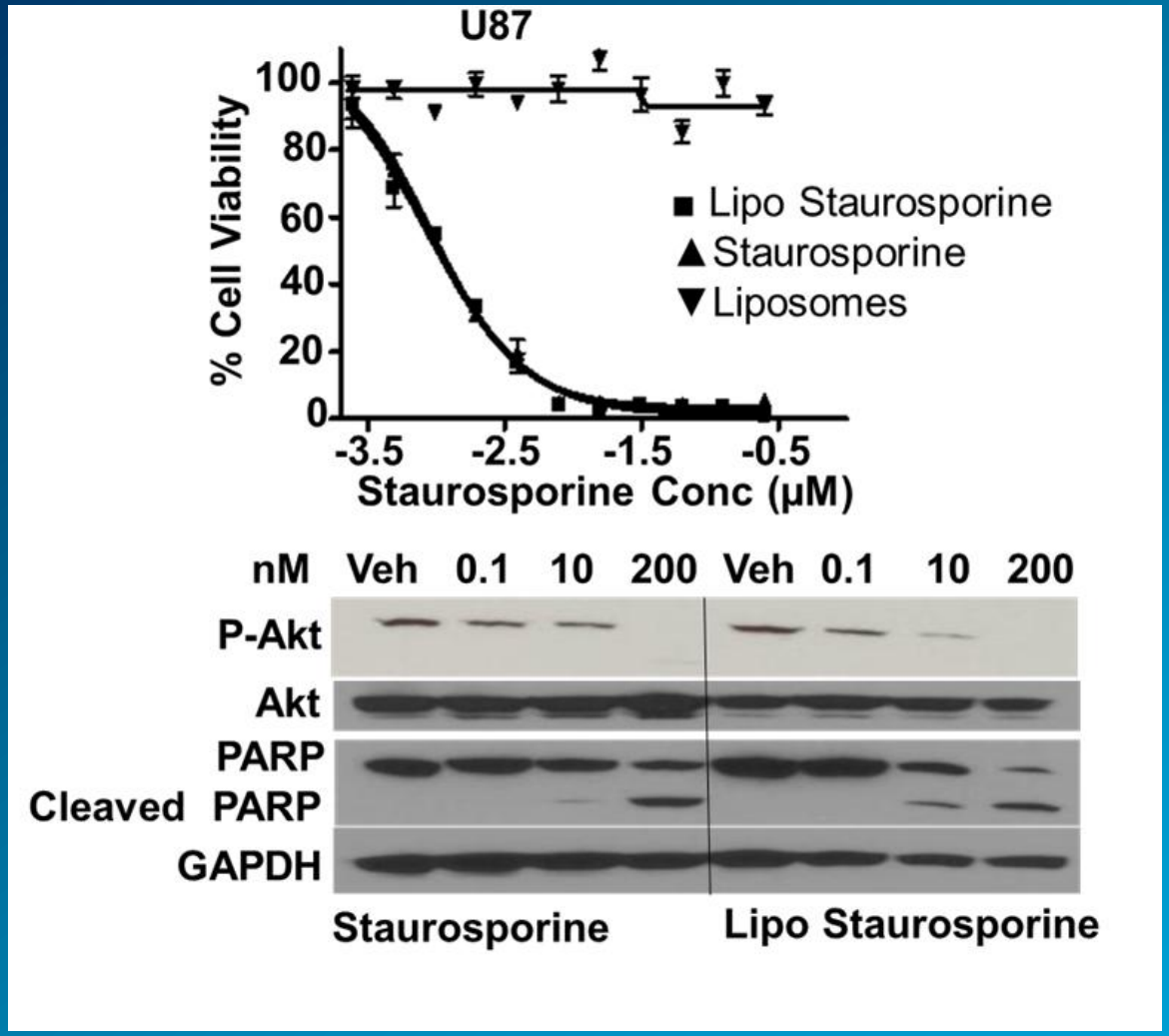
Confirmation of liposomal size and morphology



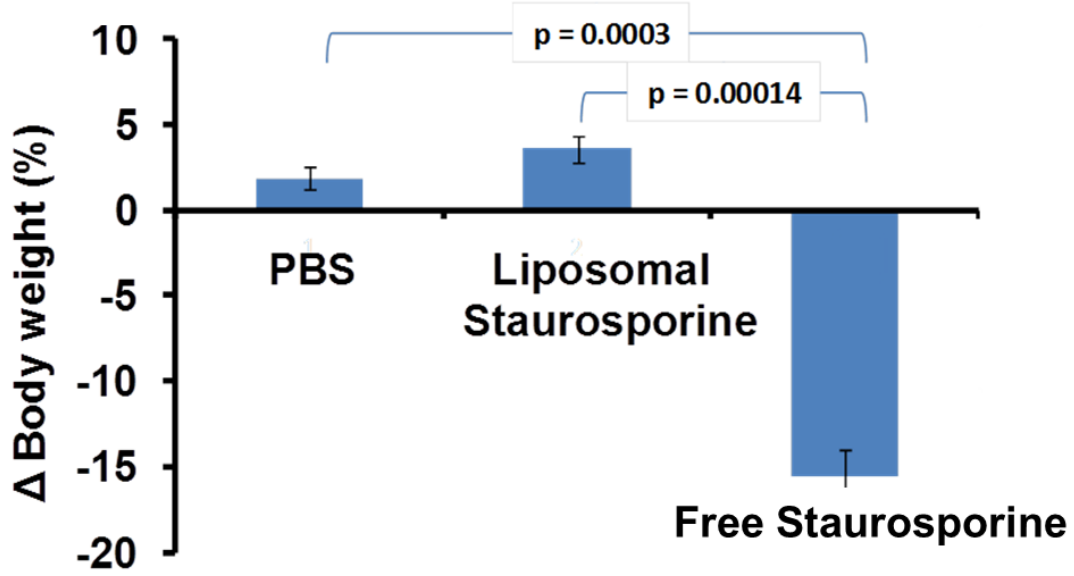
Characterization of staurosporine liposomes by DLS and SEM. **A:** Particle size distribution of staurosporine liposomes measured by differential light scattering (DLS). **B:** Representative scanning transmission electron microscope (SEM) images showing the general morphology of staurosporine liposomes.

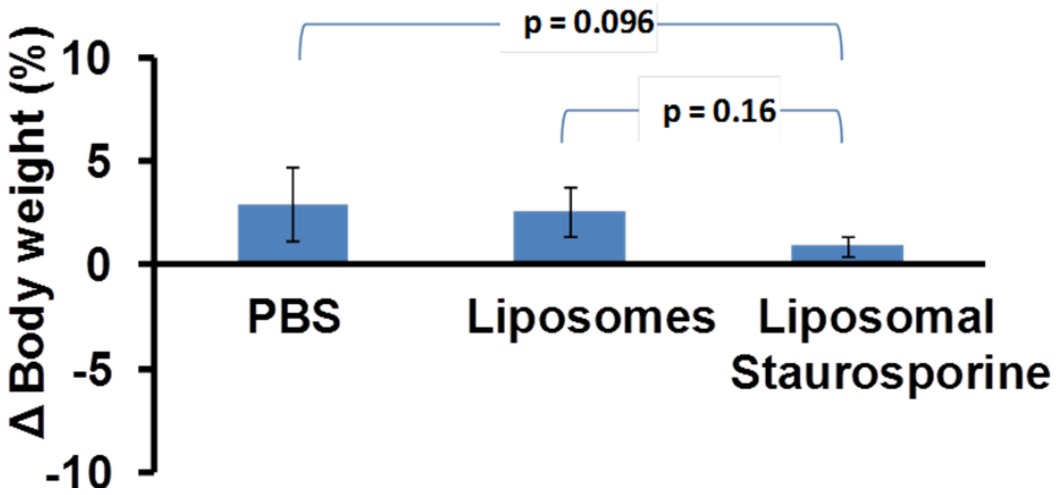
Mukathavaram et al, *Int J. Nanomedicine*, 2013.

Mode of stauro brain tumor cell killing not altered by encapsulation



Liposomal encapsulation prevents *In vivo* stauro toxicity





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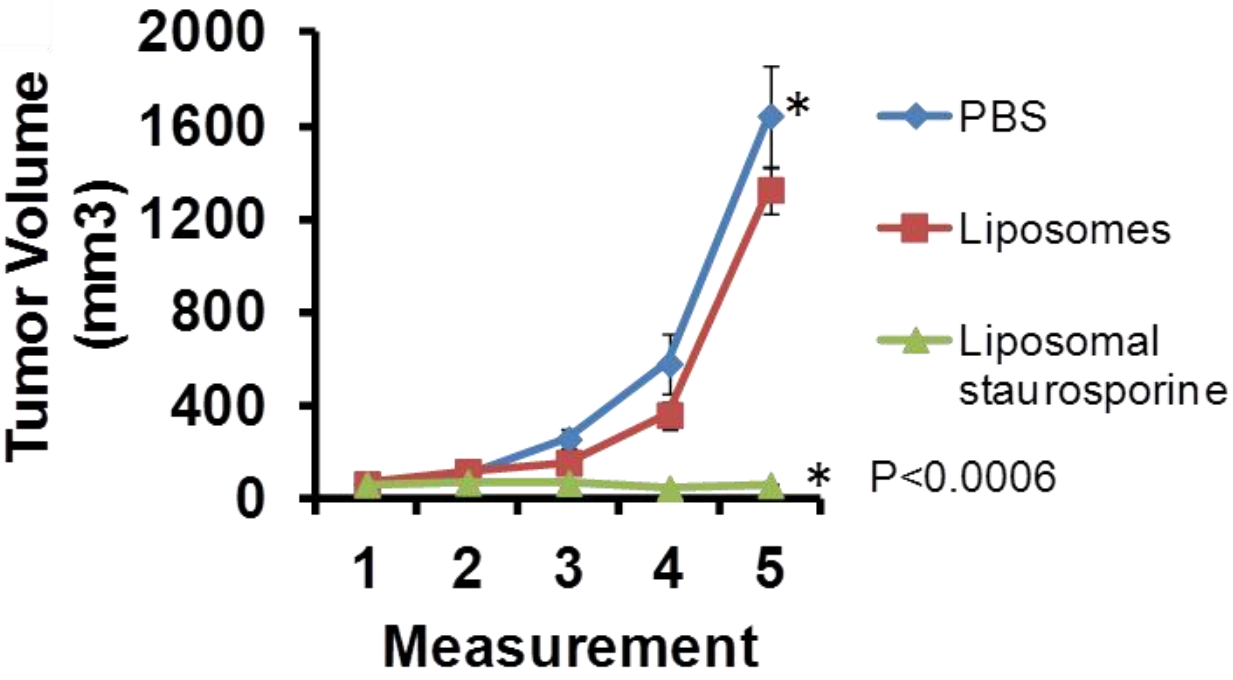
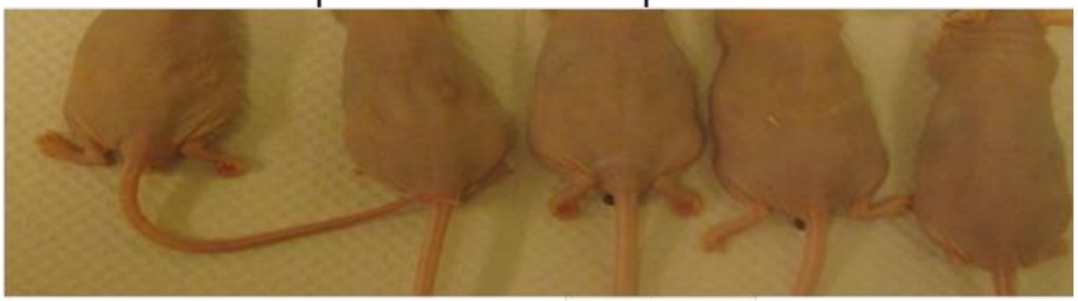
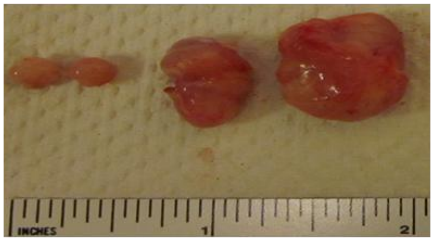
Low dose liposomal stauro is effective *in vivo* against established tumors



Liposomal Staurosporine



Excised



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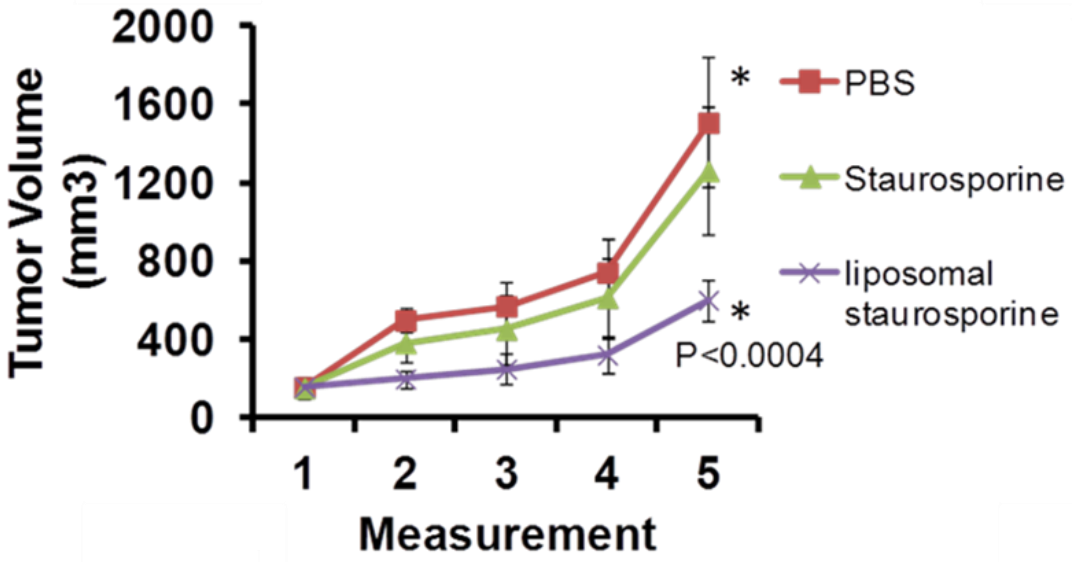
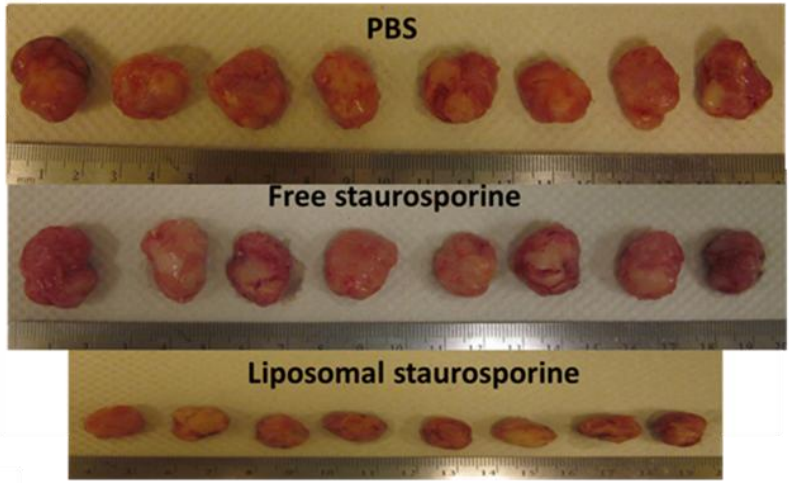
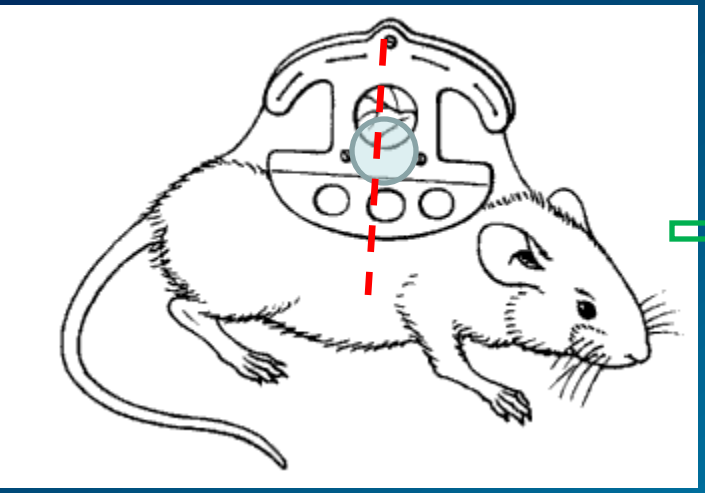
Very large (>300 mm^3) established tumors responded to liposomal stauro

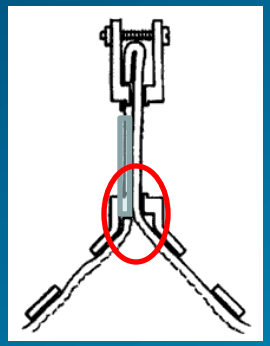


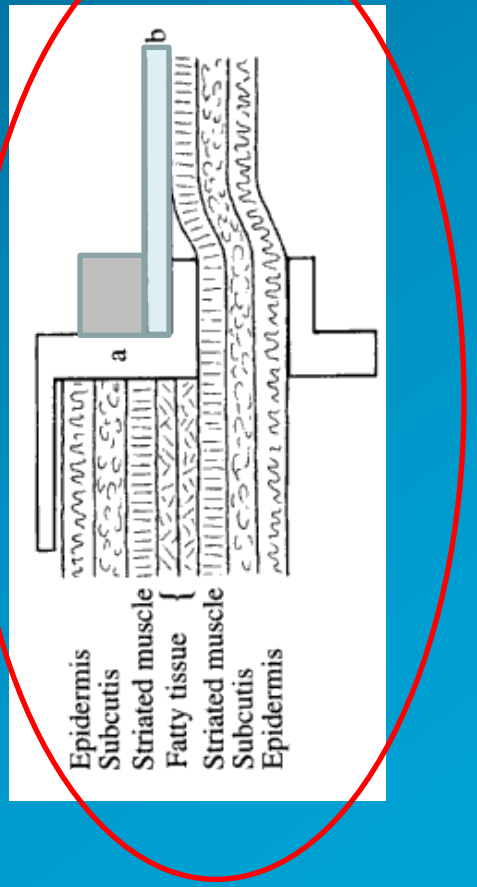


For orthotopic (brain implanted GBM) testing initially needed to determine whether our liposomal nanoparticles could accumulate at tumors

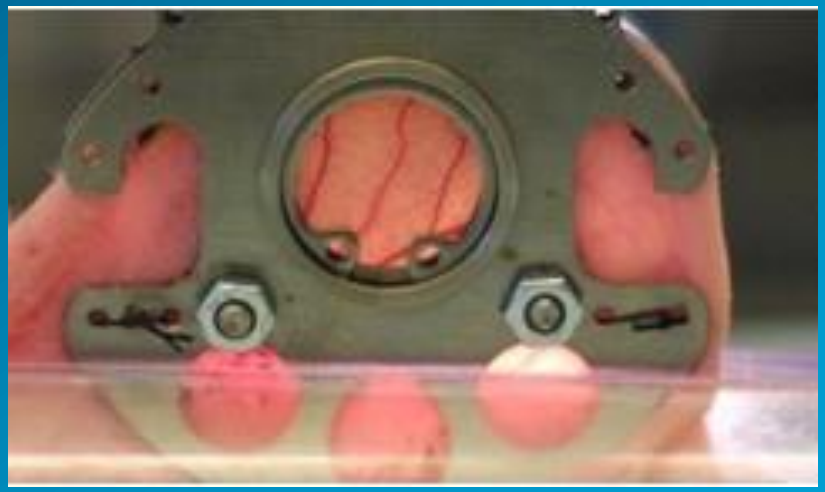
Platform for testing nanoparticle accumulation at tumors - rodent dorsal skinfold tissue window chamber







Two plates supporting dorsal skin within which a circular area of skin is removed, and the exposed tissue is covered by a thin layer of glass.



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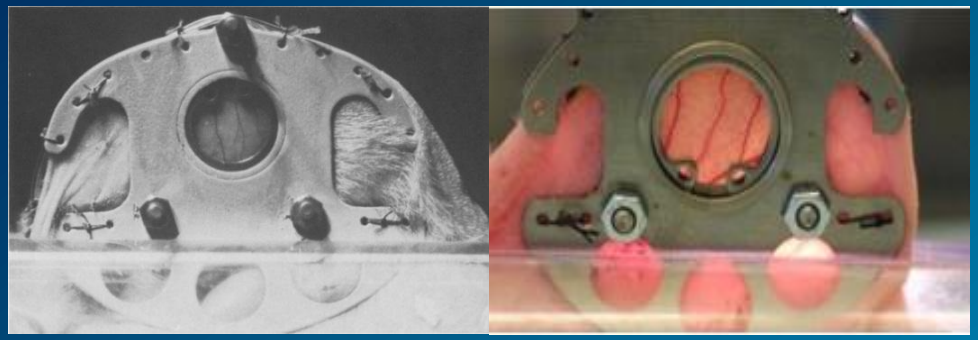
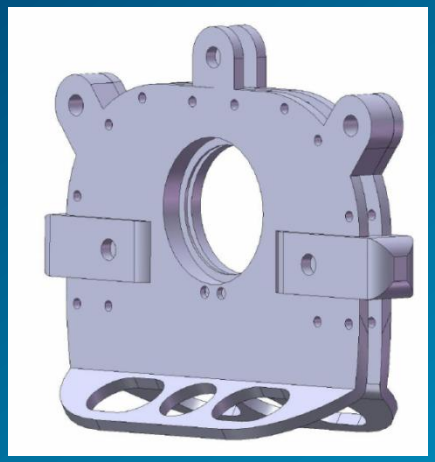
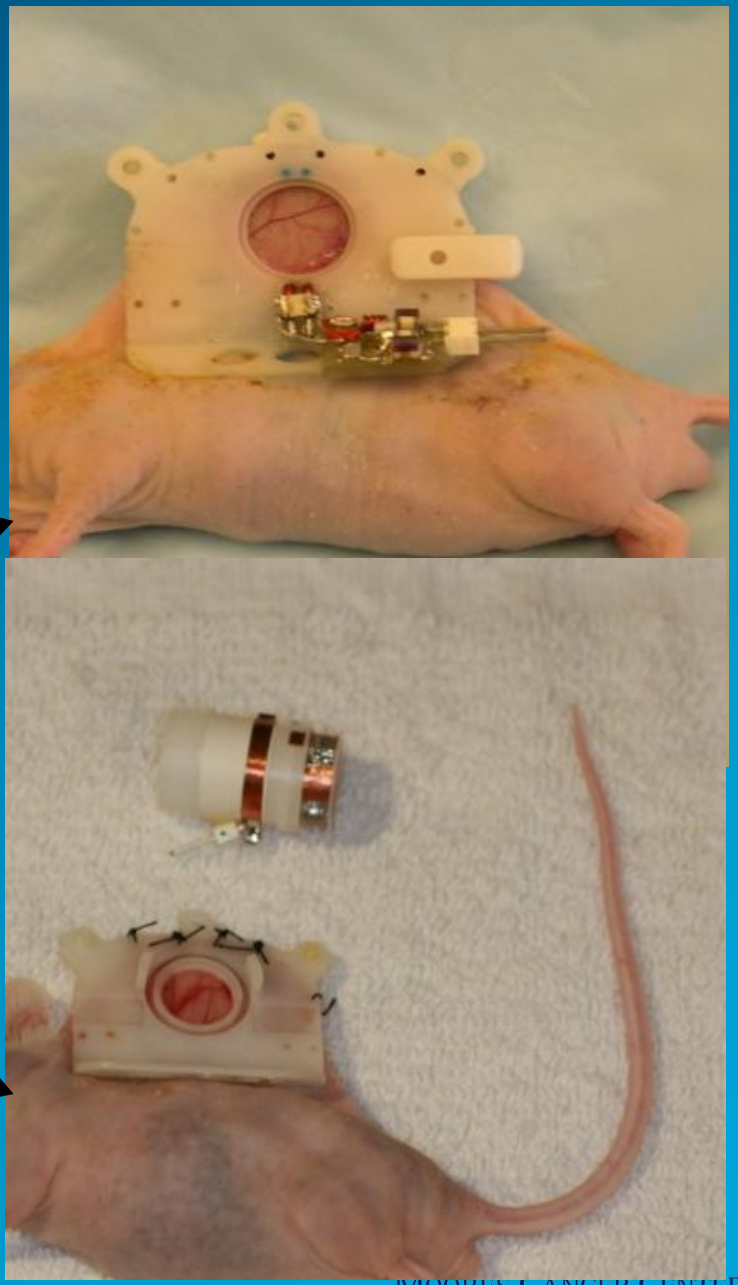
Adapted for combined modality nanoparticle imaging

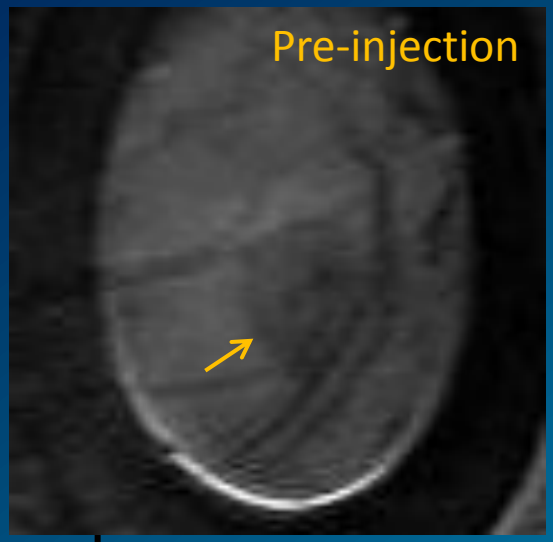


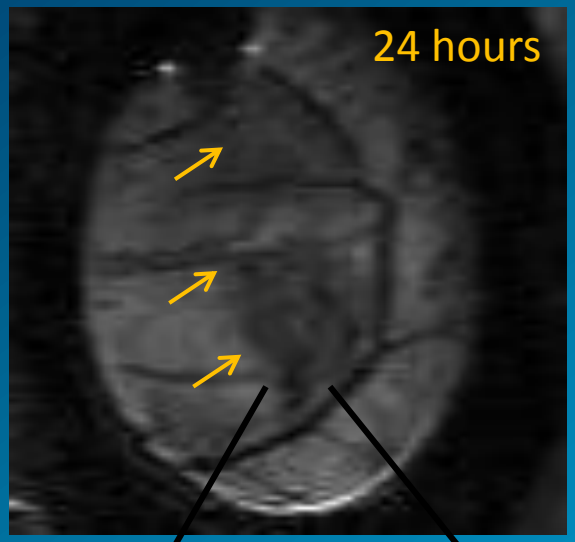
Aluminum/titanium frames (Messmer 1980/90)



Delrin® frame –
MR receive coil 3T

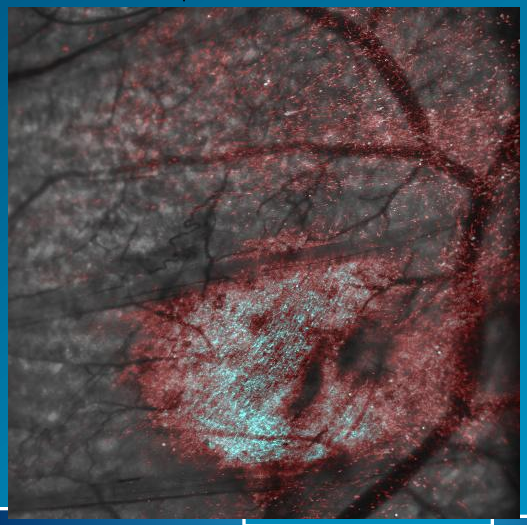




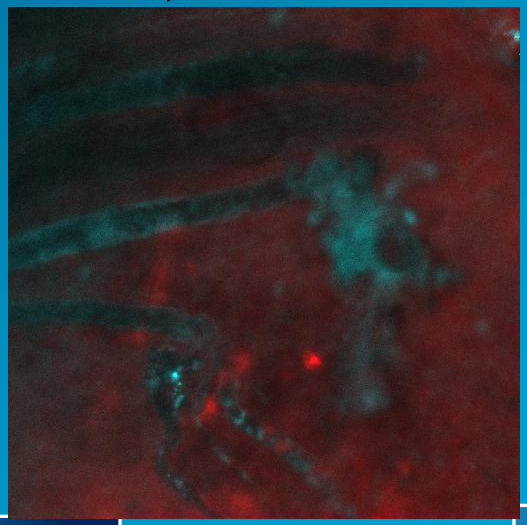


T2 weighted MRIs before and after injection with NPs.

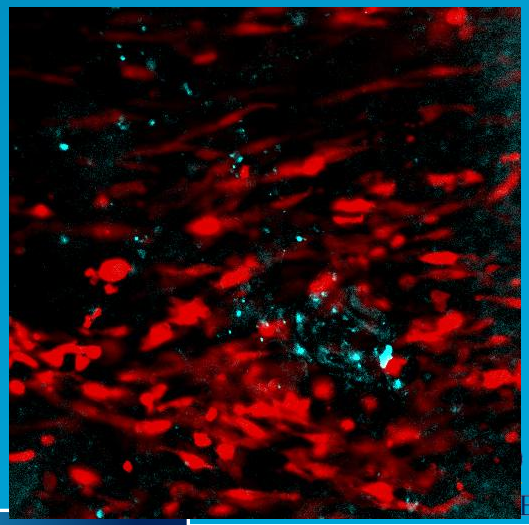
Fluorescence 4x



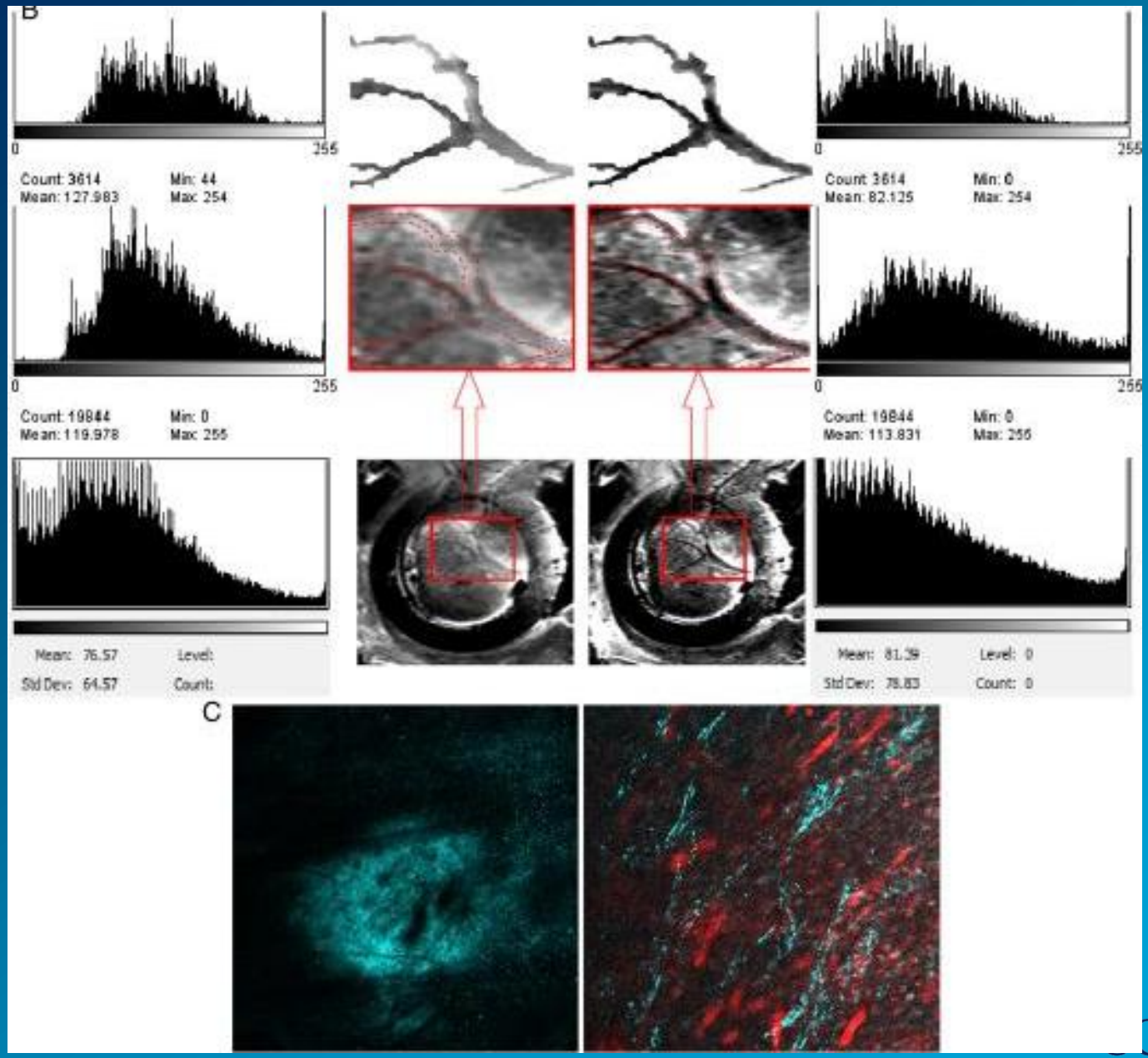
Confocal 20x



40x



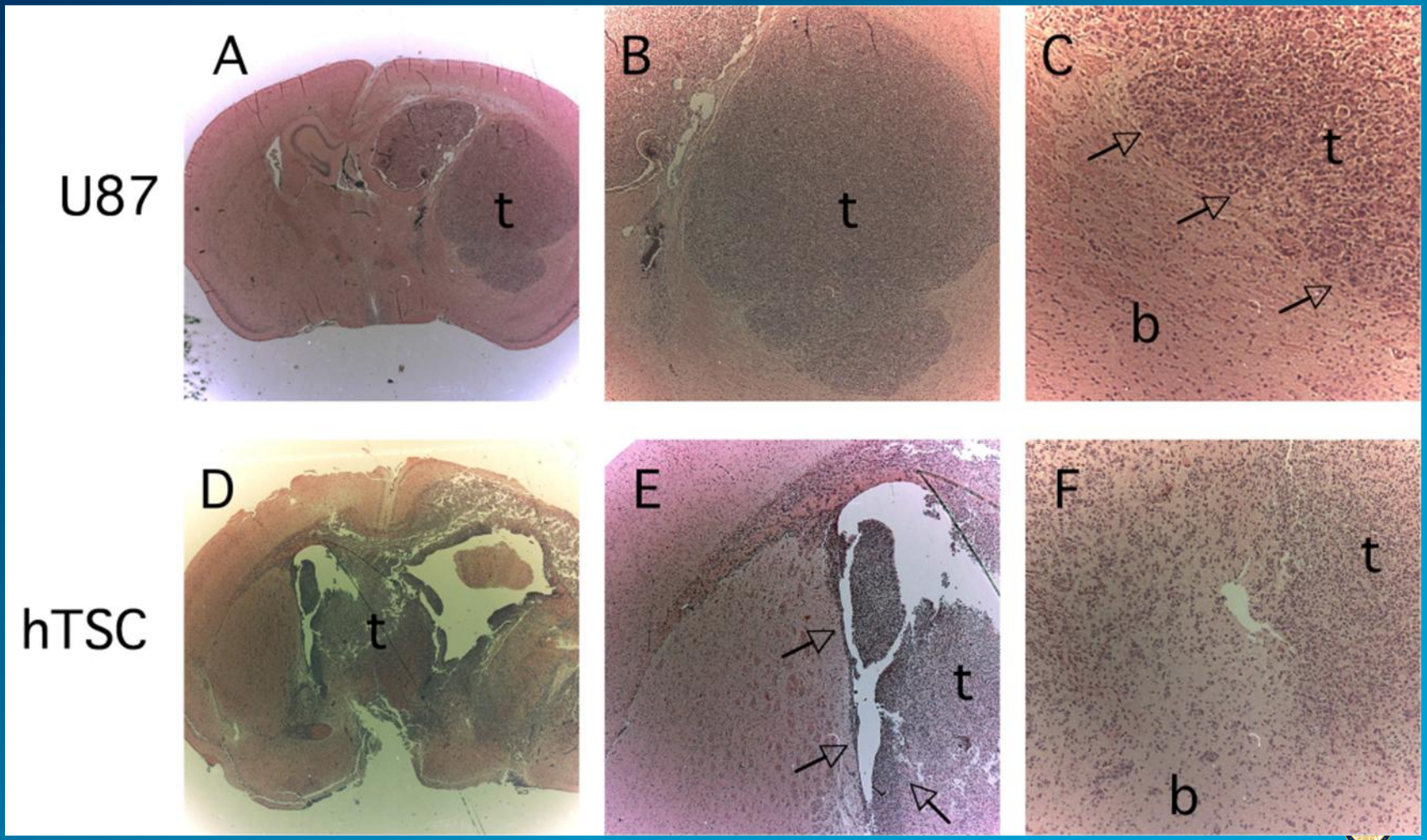
VERIFICATION OF TUMOR/VESSEL CONTRAST



Erten et al., *Nanomedicine*. 2010 Dec;6(6):797-807.

Orthotopic Screening

Human tumor stem cells vs. immortalized cell lines



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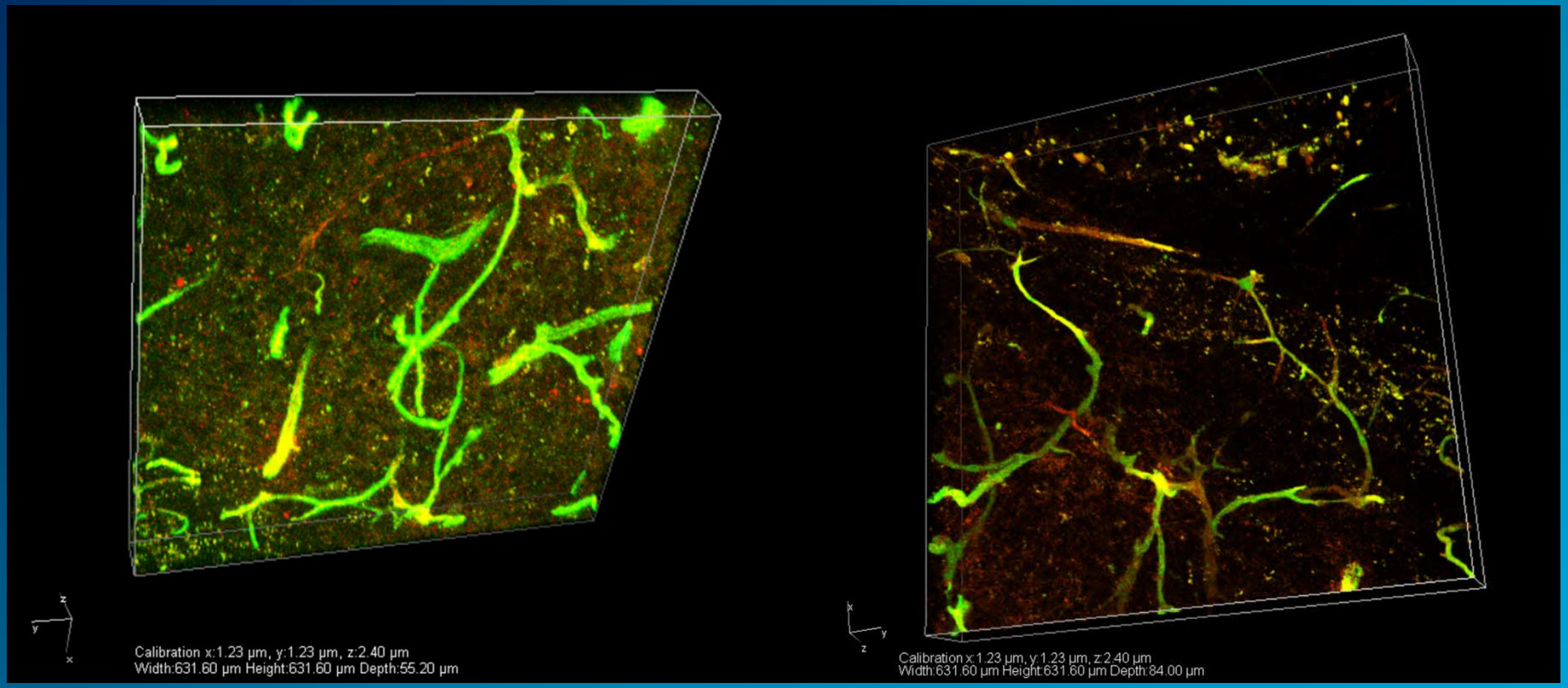
Orthotopic studies: liposomal nanoparticles BODIPY fluorescence labeled – **entered brain tumor**



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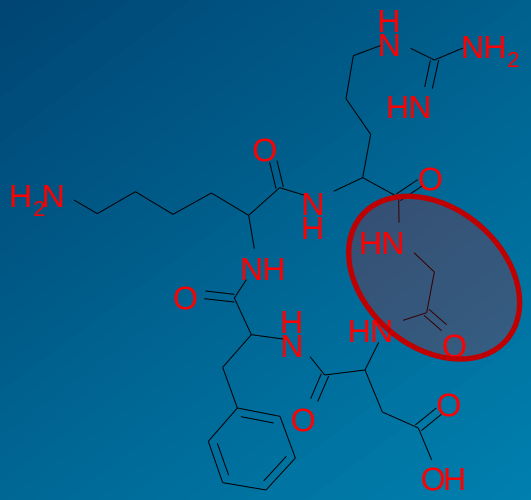
External liposomal ligands for targeting tumor cells and tumor vessels

Receptor	Ligand
avb3 integrin	cRGD
Transferrin receptor	Transferrin
CD19/20	?
HER2	Herceptin
EGFR	EGF
VEGFR	VEGF
Folate receptor	Folate
Vasopressin	Antagonist G
CD44	Hyaluronic acid
GD2	14G2a antibody

Ligand for αvβ3 integrins

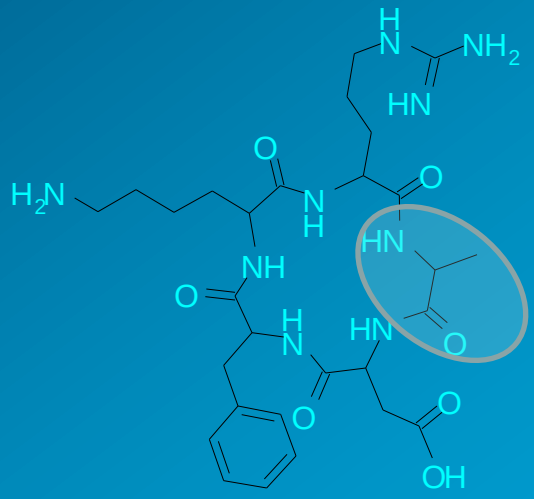
Cyclic RGD-Peptide

Cyclic structure lends stability



glycine

Control cyclic RAD-Peptide



alanine

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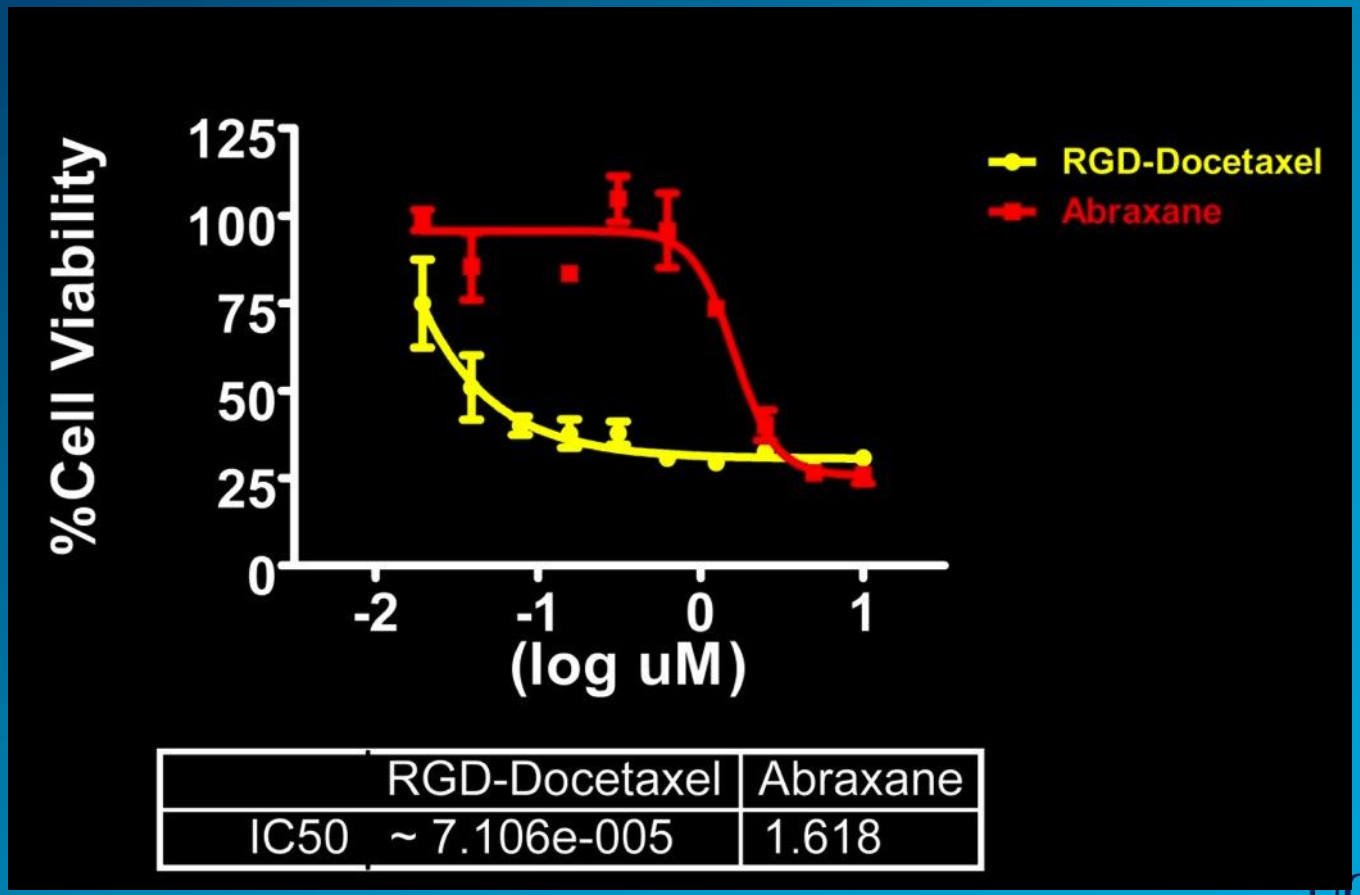
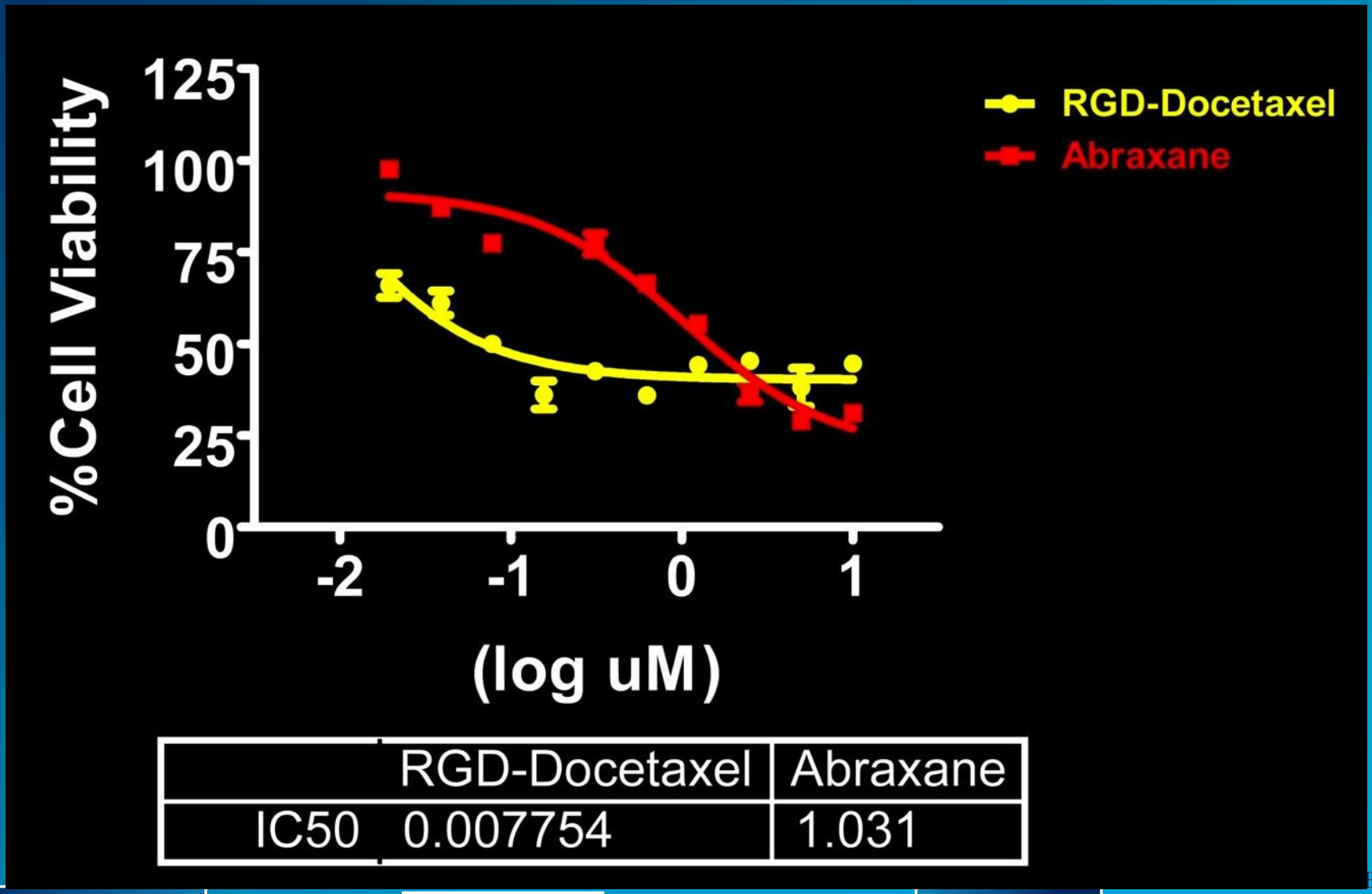
Comparison of avβ3-targeted NPs containing Docetaxel vs. Abraxane on Breast Carcinomas in vitro

4T1



	RGD-Docetaxel	Abraxane
IC50	~ 7.106e-005	1.618

Targeting NPs to R40P pancreatic carcinomas in vitro



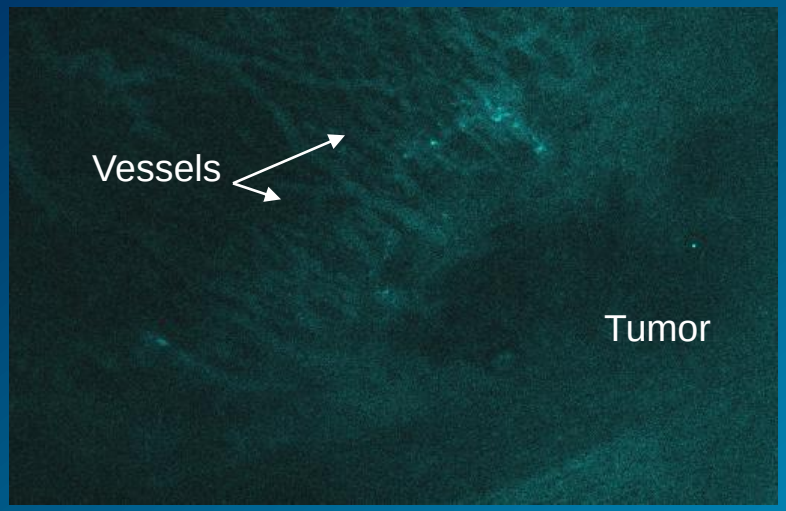
	RGD-Docetaxel	Abraxane
IC50	0.007754	1.031

Verify binding of particles to tumors using intravital system

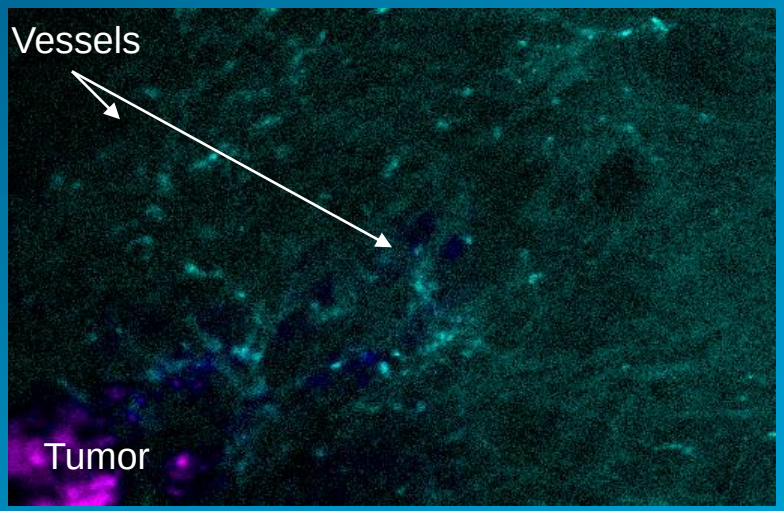
RAD Nonspecific

RGD Specific (αvβ3)

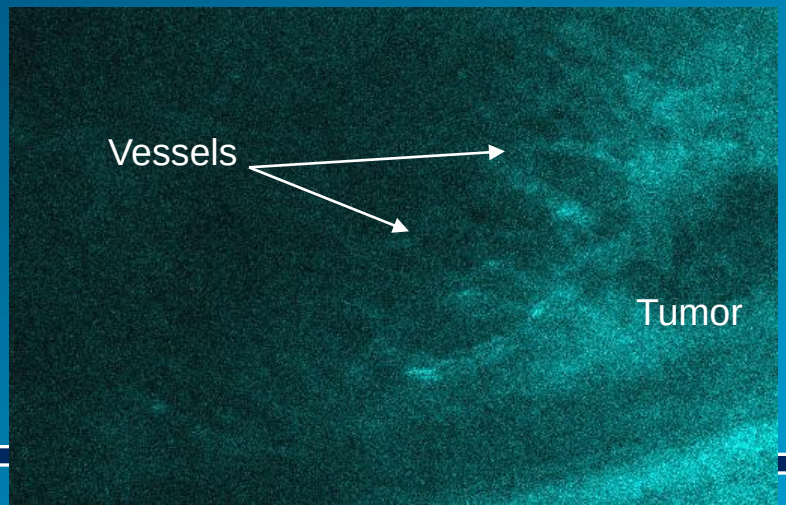
2 h



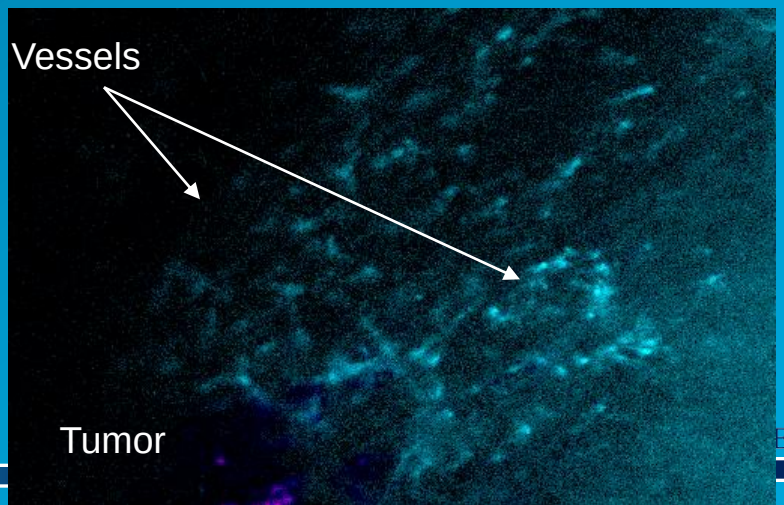
2 h

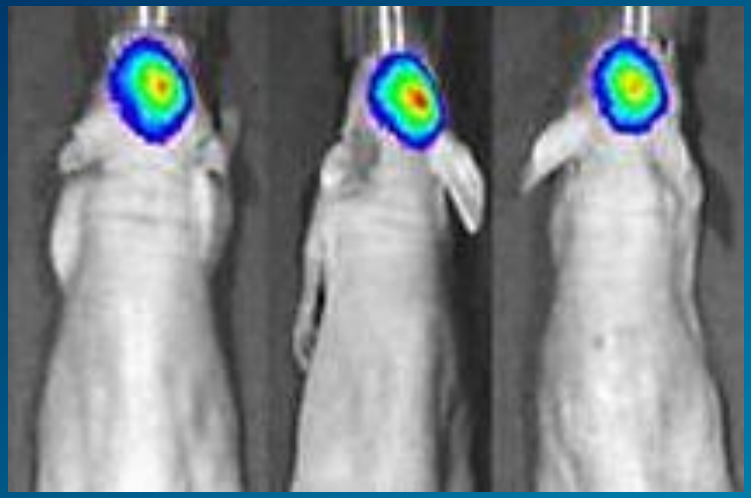


4 h



4 h





Mice with orthotopic GBM treated with liposomal stauro

Days after tumor innoculation	Control % alive	Liposomal Stauro % alive	Targeted Liposomal Stauro % alive
25	90	90	100
35	0	80	70
60	0	10	40
100	0	0	20

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Summary and Conclusions

Targeting the tumor stem-like compartment with compounds that can cross the BBB may help suppress the expansion of GBM in patients.

Nanoparticle based reformulation of compounds that have failed preclinical development may prove useful for GBM.


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