Medicis - Promed Summer School on:
Development and Pre-Clinical Evaluation of Radiopharmaceuticals
4th - 8th June, 2018

In vitro Evaluation Cell-Based Assays

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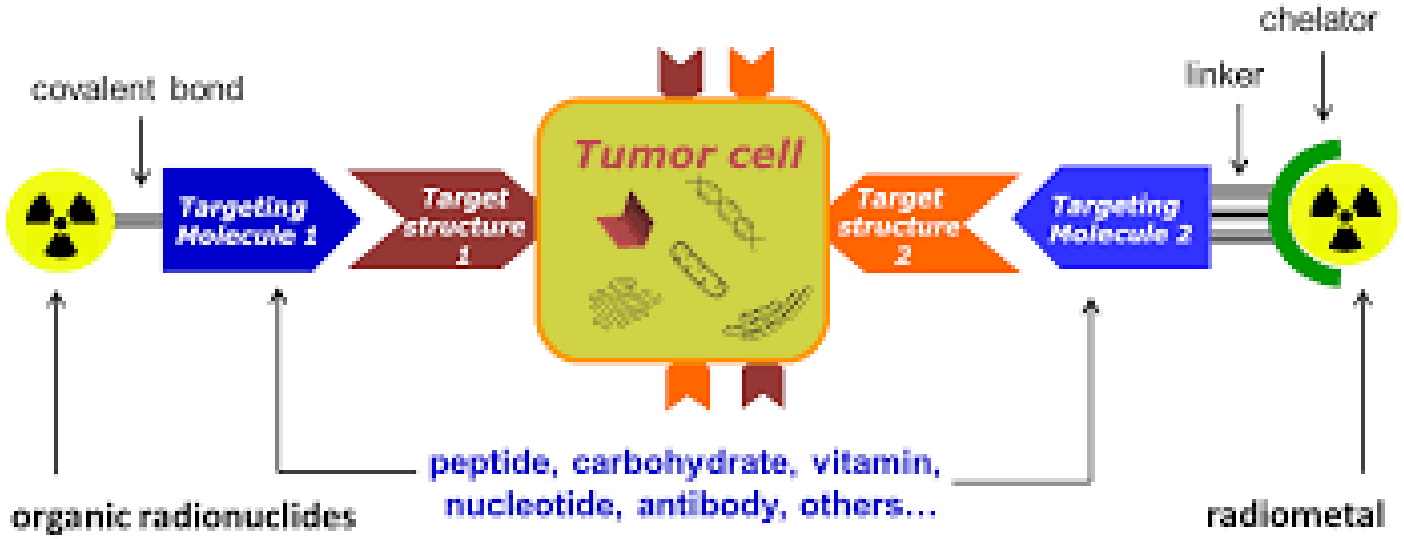
6 June

Radiopharmaceuticals: Preclinical Research and Development Phase



- Target identication and validation
- Selection of targeting molecule
- Chemical synthesis of TM
- Radiolabeling and characterization
- Drug efficacy testing
- (Radio)Chemical and metabolic stability
- *In vitro* tumor cell binding and cellular internalization
- *In vivo* biodistribution and tumor targeting characteristics

Biological Evaluation of Potential Radiopharmaceuticals

- *In vitro* evaluation
- *In vivo* evaluation

Overview of Pre-Clinical Anti-Cancer (Radio)pharmaceutical Development

Cells - based assays

Animal Tumor Models

Human Xenografts

Pharmaceutics & Tox

Human Clinical Trials

Why Cell-Based Assays

Cell culture:

- Tissue from an explant is dispersed, mostly enzymatically into a cell suspension which may then be cultured as a monolayer or suspension culture

Advantages:

- development of a cell line over several generations
- Scale-up is possible
- Absolute control of physical environment
- Homogeneity of sample
- Less compound needed than in animal models

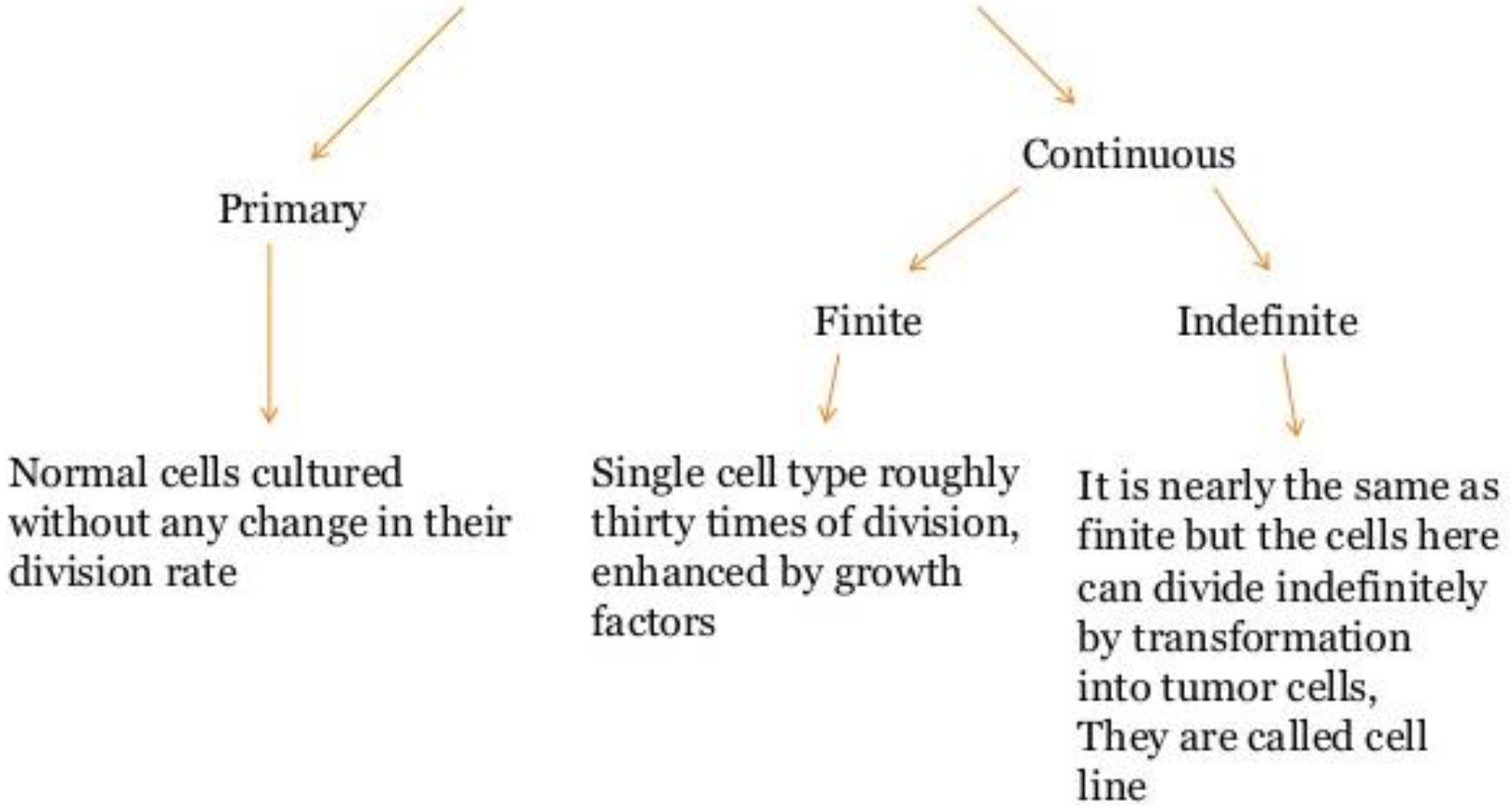
Cell-based assays:

- Refer to any of a number of different experiments based on the use of live cells
- Include a variety of assays that measure cell proliferation, radio(toxicity), target binding and uptake of a radiopharmaceutical, subcellular localization, …
- Offer a more accurate representation of the real-life model since live cells are used

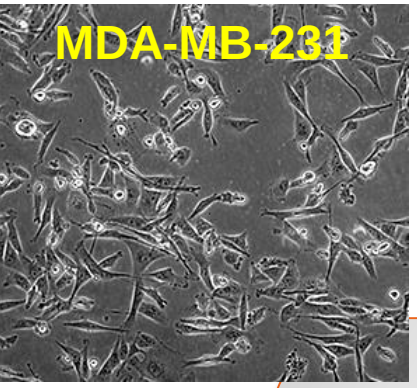
Cell-based assays are a key component in drug development process

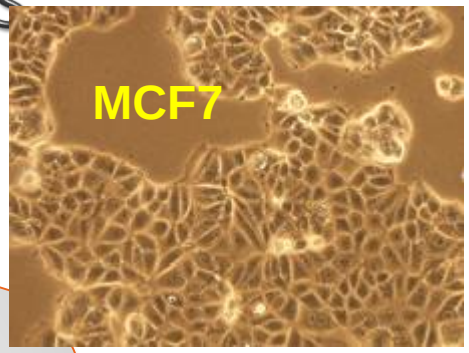
- Cell based assays have emerged as an effective and strong addition to compliment other technologies that are suitable for drug discovery and high throughput screening.
- Cell based assays offer a biologically relevant substitute to predict the response of a drug on an organism
- While initially used mostly for secondary screening, are now progressively being used for primary screens. This has been extremely valuable in screening all types of compounds.
- In addition, they are used in many research areas, providing knowledge about biological targets and pathways in the whole cell.

Types of tissue culture

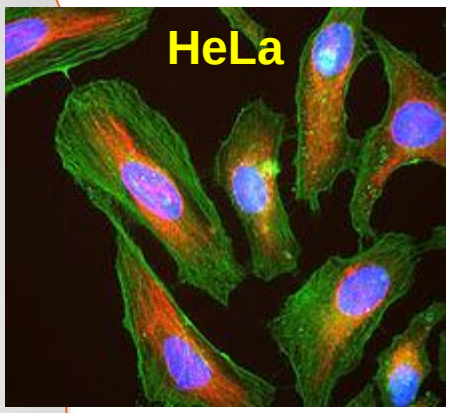


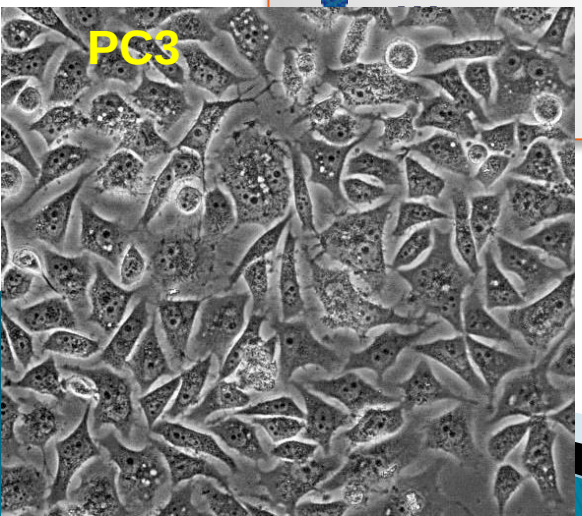
Most Common Cell Lines

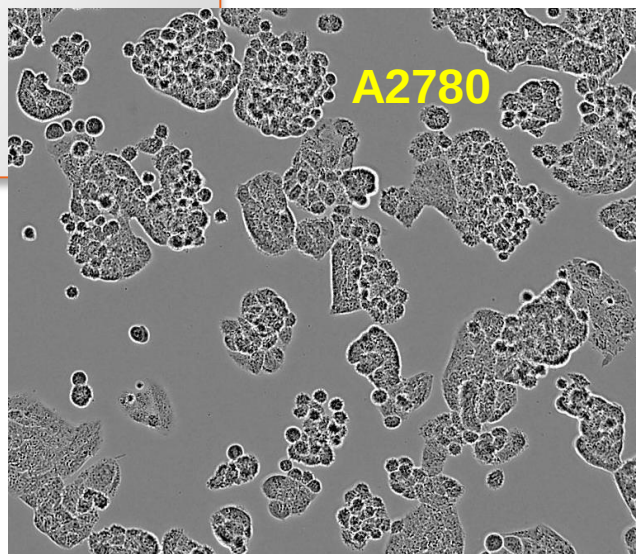




- Breast carcinoma cells (MCF7, MDA-MB-231, MDA-MB-435, T-47D)
- Prostate Carcinoma cells (PC3)
- Melanoma cells (A375, B16F1 (murine))
- Ovarian carcinoma cells (A2780, A2780cisR)
- Cervical carcinoma cells (HeLa)
- Glioblastoma cells (U87MG)
- Colon adenocarcinoma cells (HT29)







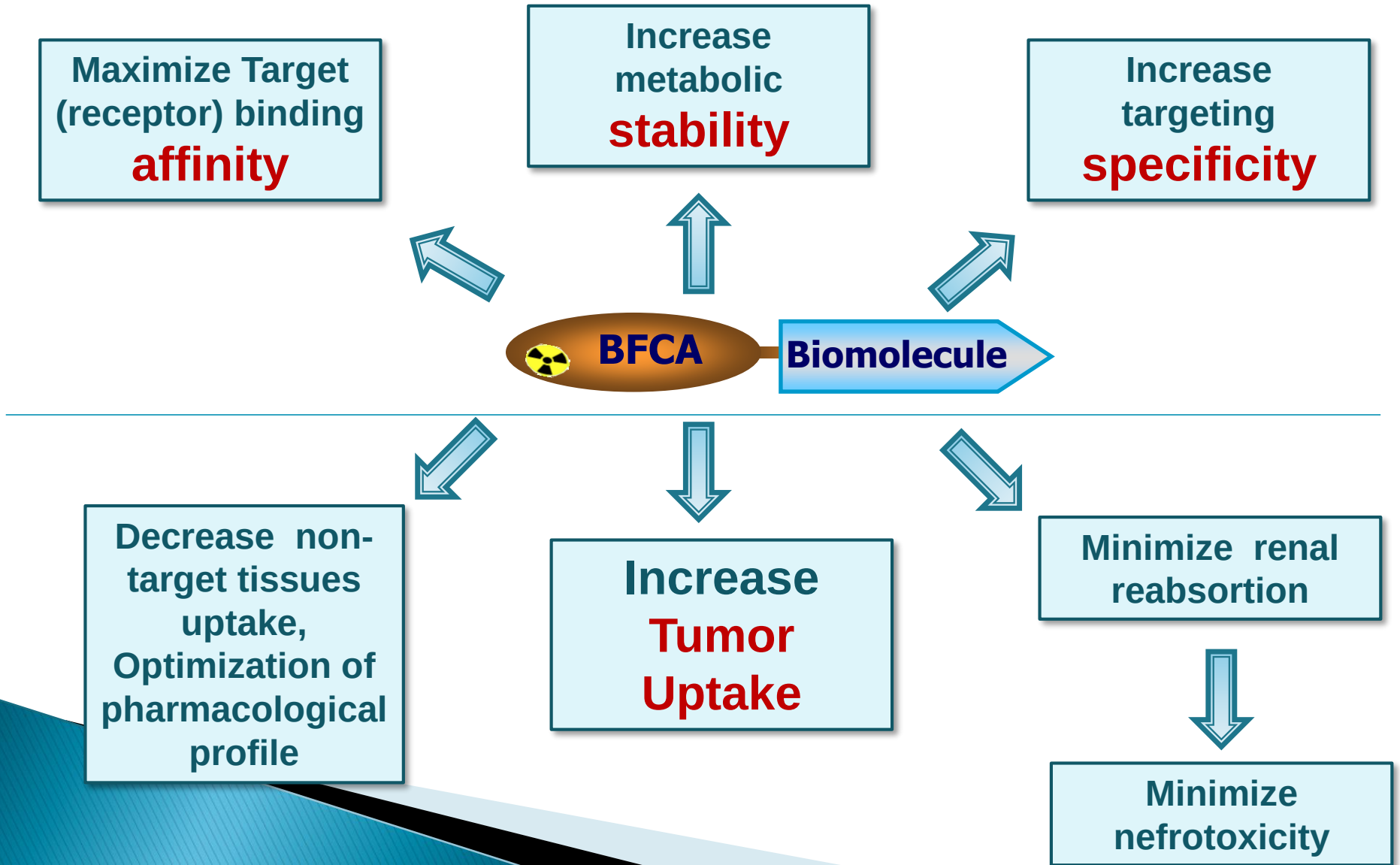
The Targeting Biomolecule

- Peptide
- Antibody
- Small molecule
- Nucleotide
- Enzymatic substrates
- Carbohydrate…

Ideally should presents

- High binding affinity for the target
- High specificity
- Metabolic Stability in vivo
- High target/non-target ratio
- Rapid clearance of non-target tissues
- Tolerance and flexibility in relation to chemical modifications:
  - Radioisotopes or fluorophores
  - Spacers, polymers, metals

Design of new radiopharmaceuticals

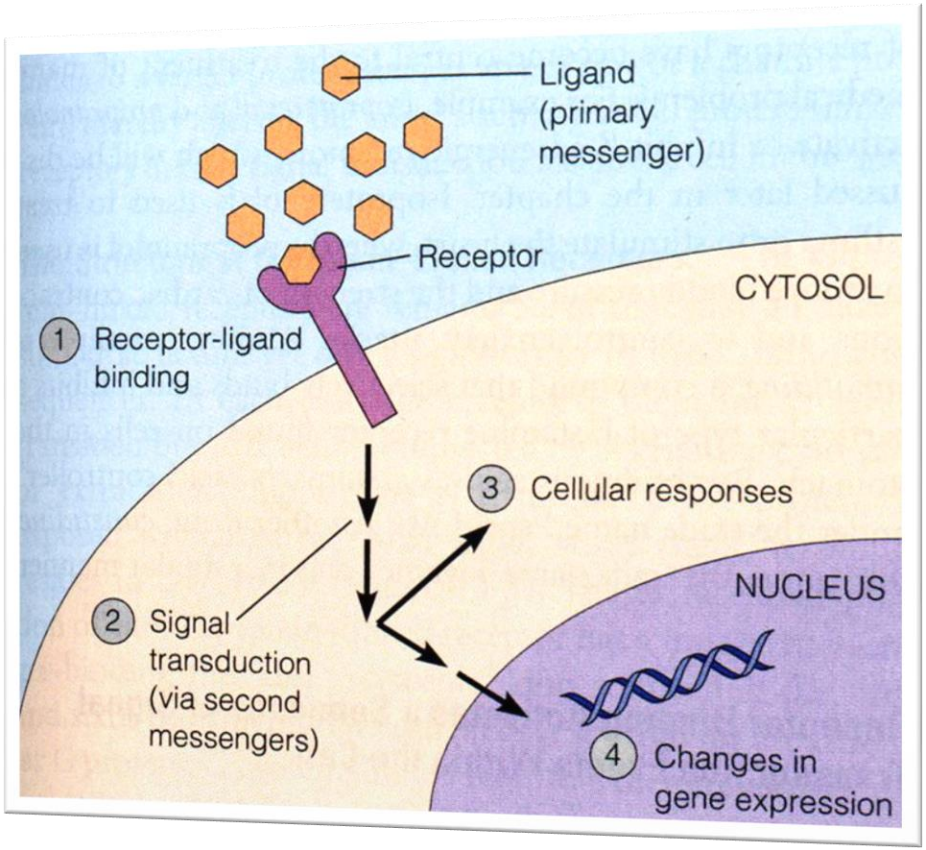


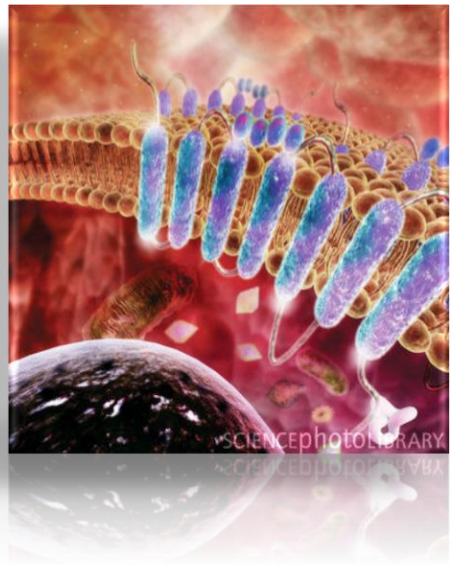
In vitro biological evaluation of potential Radiopharmaceuticals Cell-Based Assays

- Biomolecule-target binding characteristics
- Binding affinity
  - Receptor Saturation Assay
  - Competitive Binding Assay
- Binding specificity
- Cell uptake and internalization studies
- Efficacy
- (Radio)Cytotoxicity

Biomolecule–Target binding

RECEPTORS





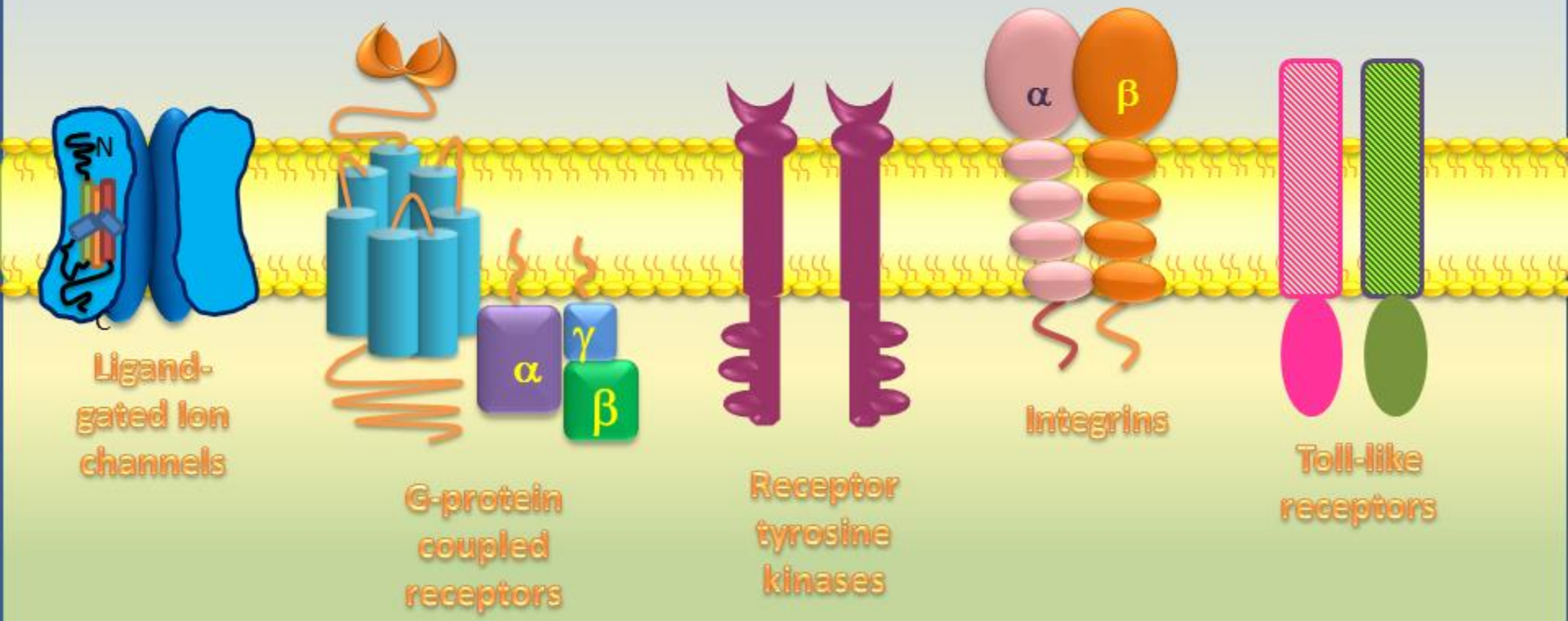
Overexpressed in various pathologies (e.g. tumors) and in several cells lines

Ideal targets for molecular imaging and therapy

Design of specific

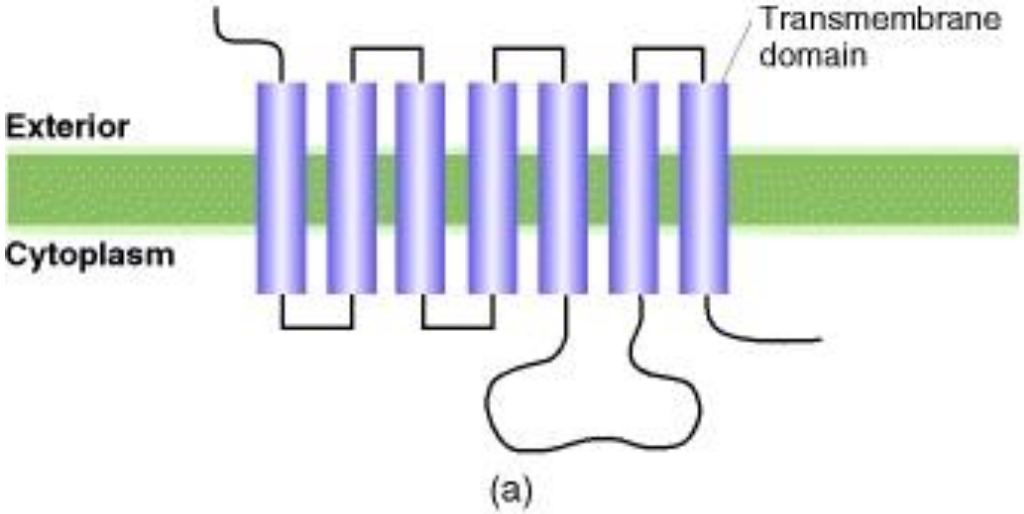
- **Radiopeptides (analogs of endogenous peptides)**
- **Radiolabeled antibodies**

Cell Surface Receptors



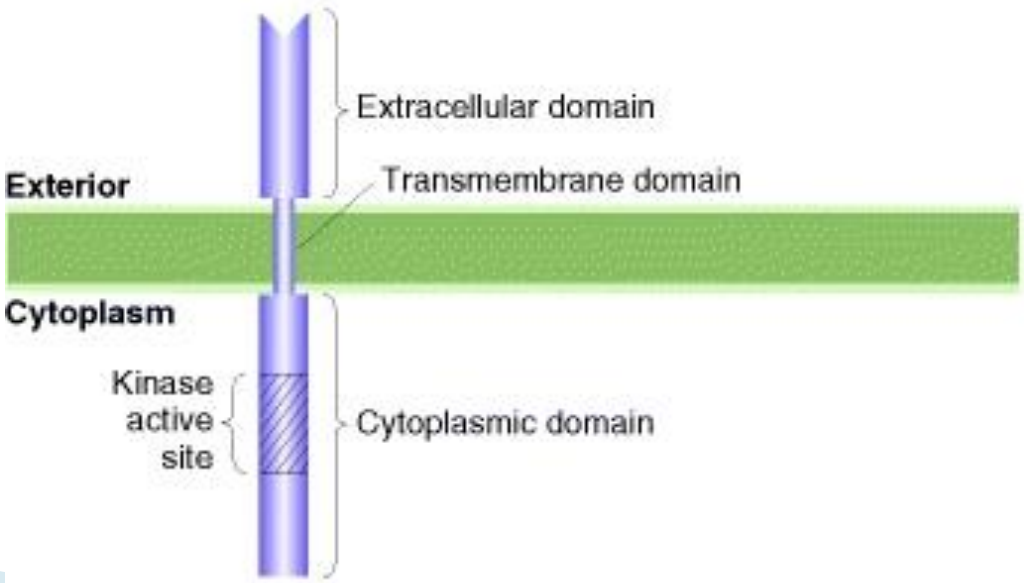
Transmembranar Receptores

G- protein couple receptor (GPCR)



(a)

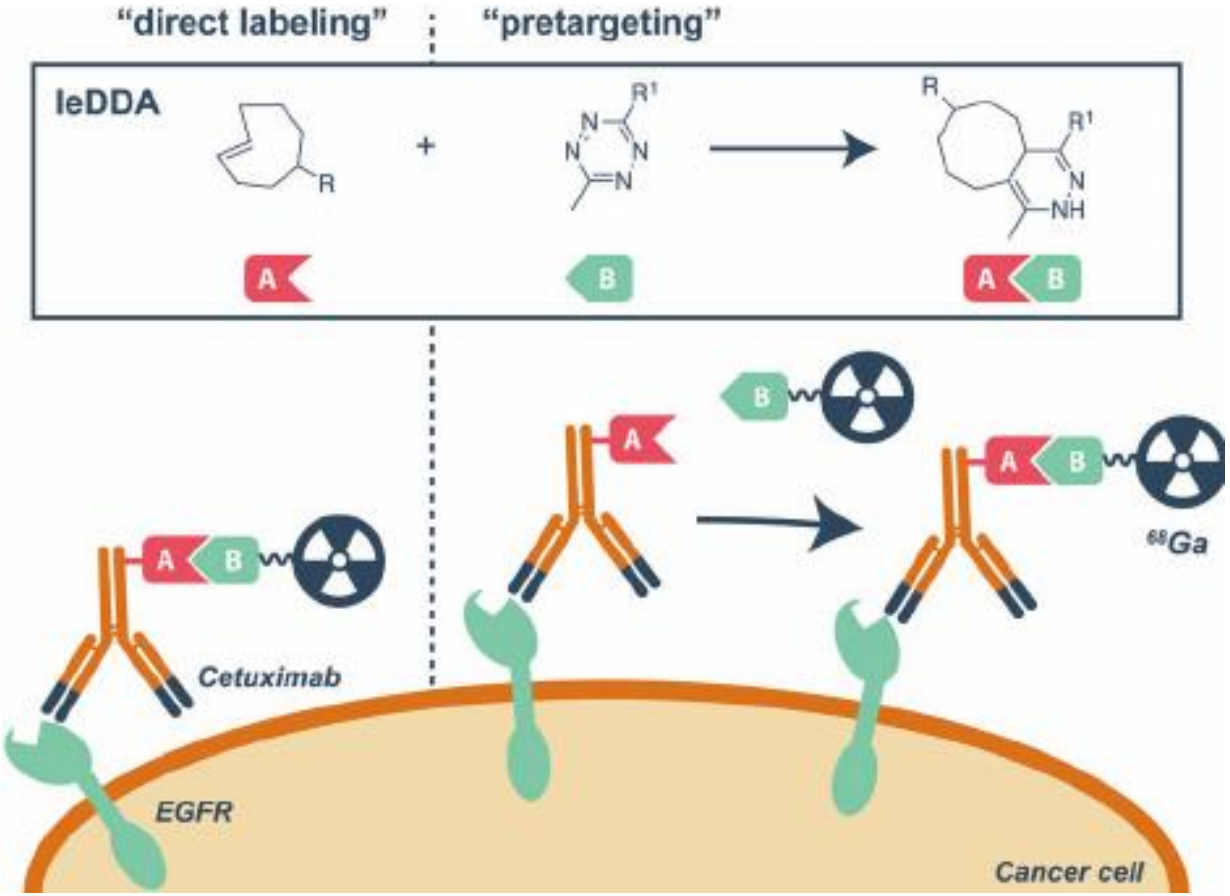
Tirosin Kinase Receptors (RTK)



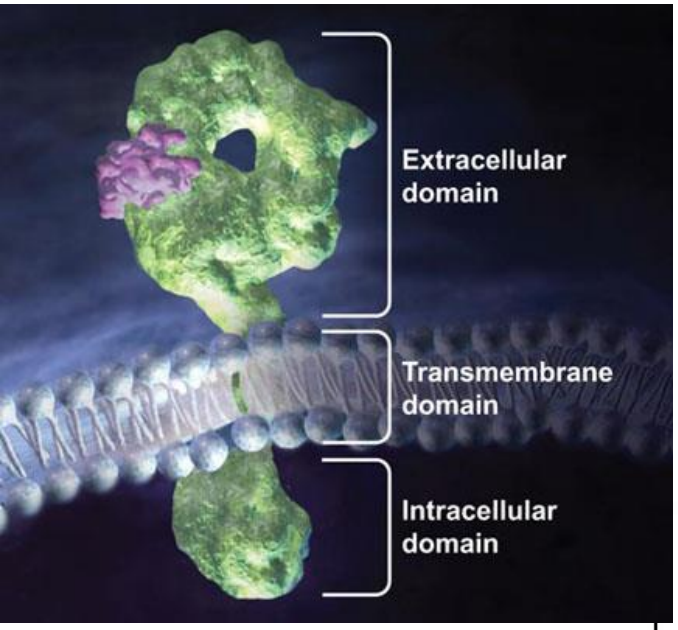
(b)

Antibody– TK Receptor

Cetuximab – EGFR/ErB1



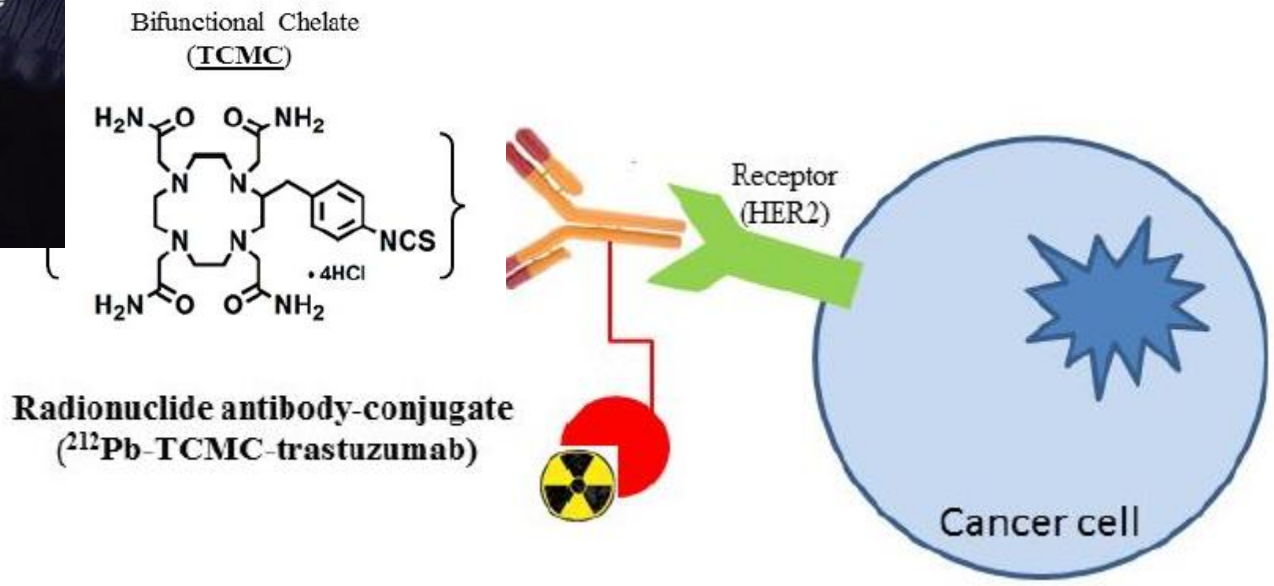
HER2 (RTK) overexpressed in breast cancer

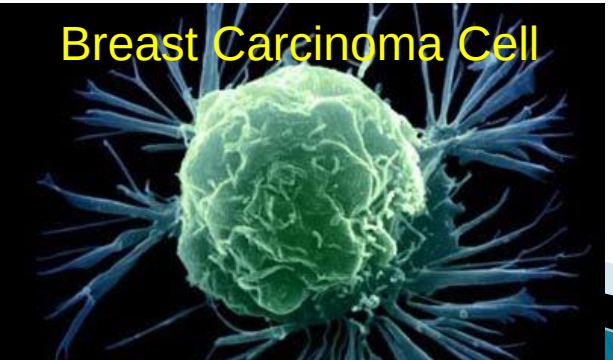


HER Family (TK Receptors):

- HER1 (EGFR, epidermal growth factor receptor, ErbB1)
- HER2 (ErbB2)
- HER3 (ErbB3)
- HER4

Trastuzumab – HER2

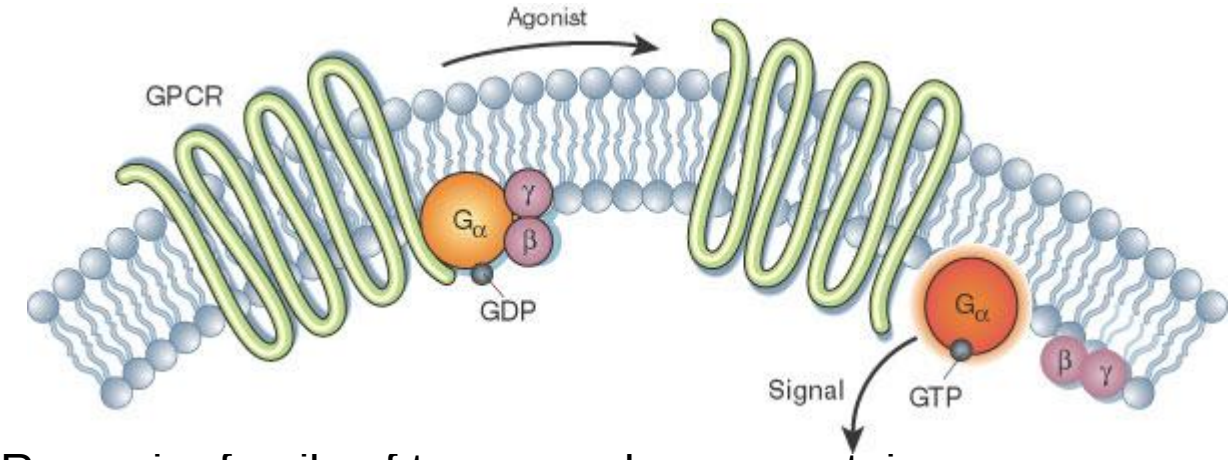




Tumor cells: HER signaling pathways are inappropriately activated, resulting in the rapid growth and spread of cancer cells

http://www.biooncology.com/research-education/her/overview/receptors/index.html

Radiopeptide–GPCRs interaction

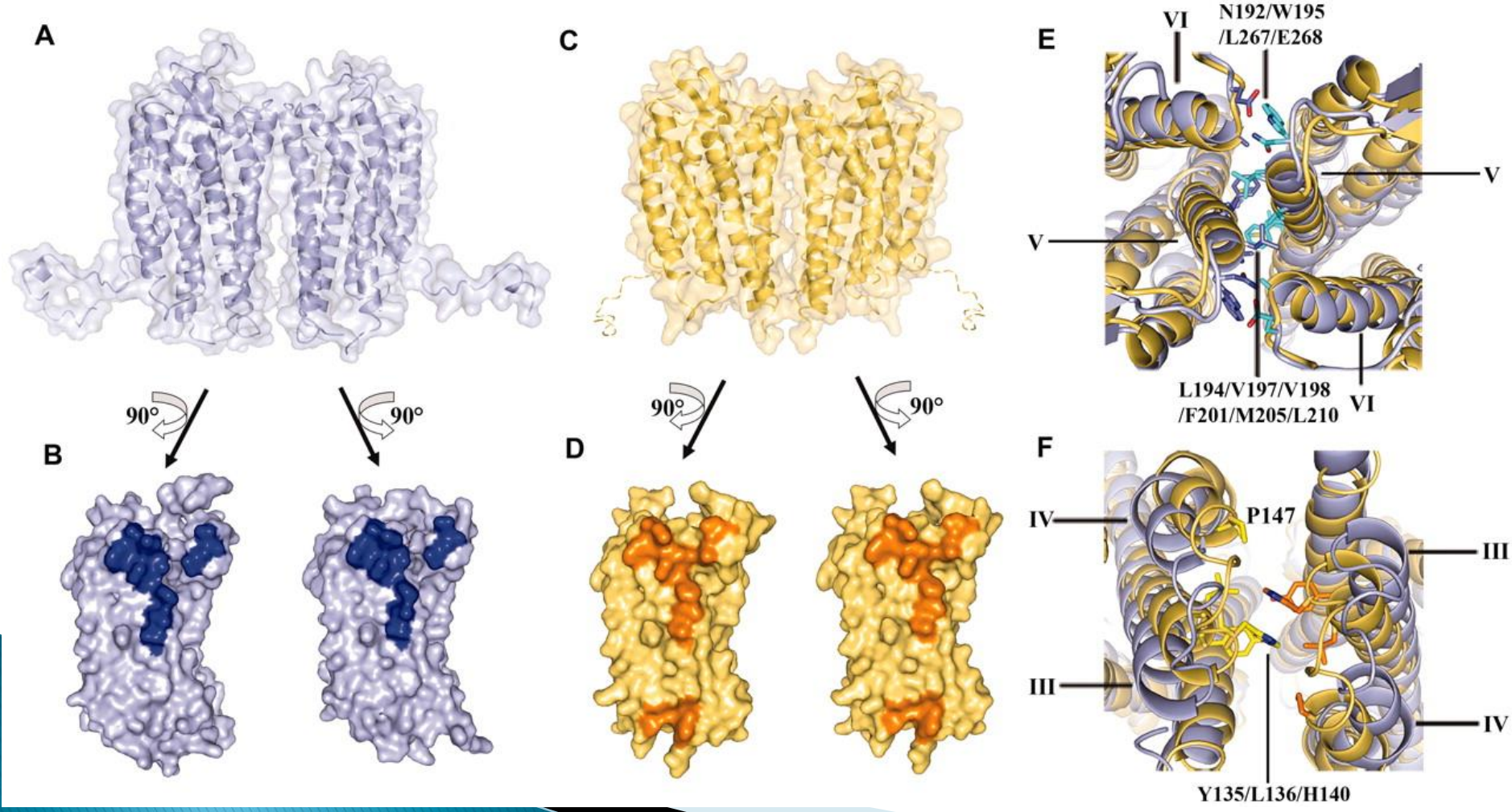


- GPCRs: major family of transmembranar proteins
  Highly regulated and dynamic
- The majority of approved radiopharmaceuticals acts on GPCRs
- “moving target” is very attractive
- For therapeutic applications is fundamental to understand the intracellular pathways and to identify the protein-protein interactions

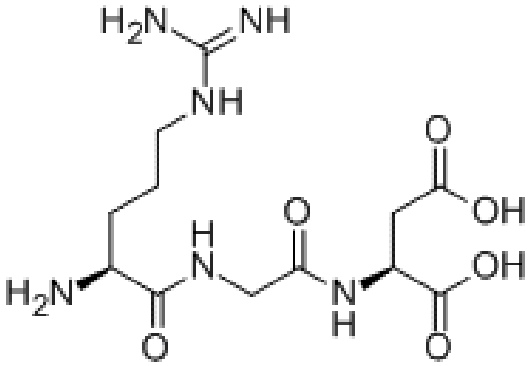
Important diagnostic and therapeutic targets for (radio)pharmaceutical industry

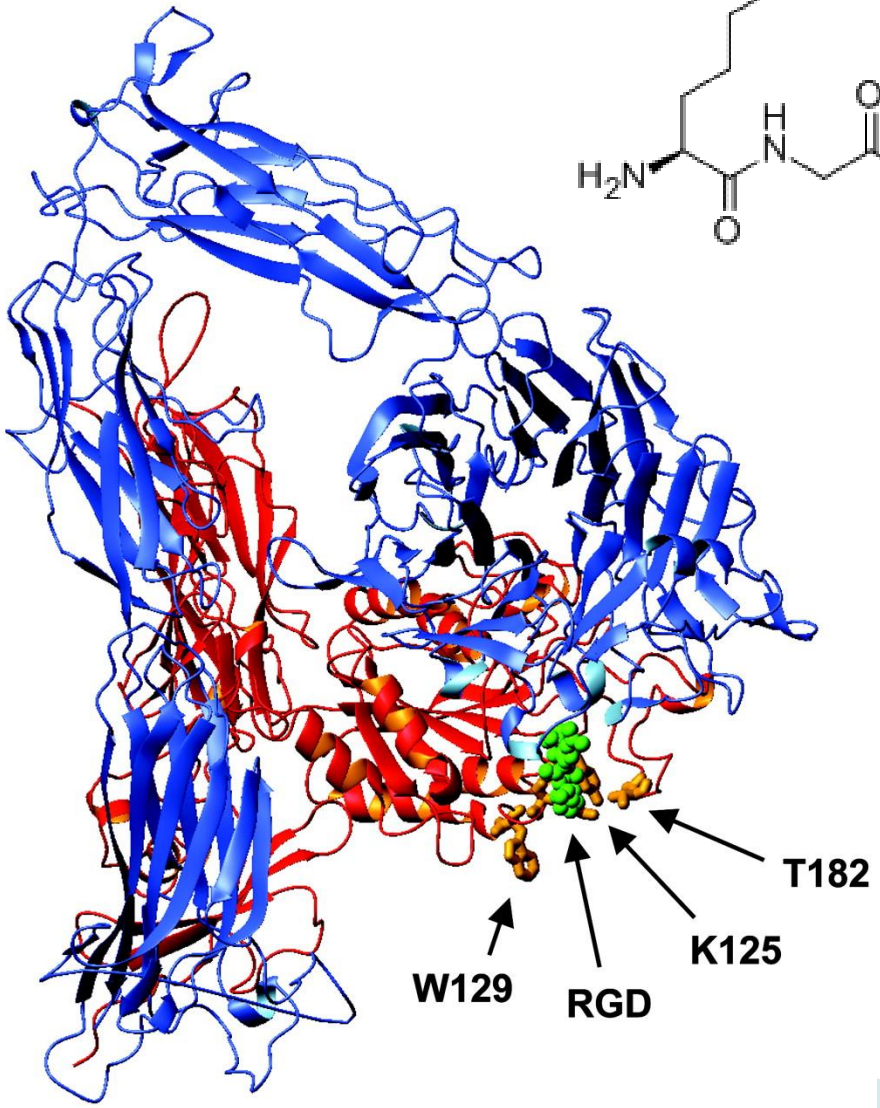
Peptide- CXCR4 Chemokine Binding

Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists



RGD- αvβ3 integrin Binding

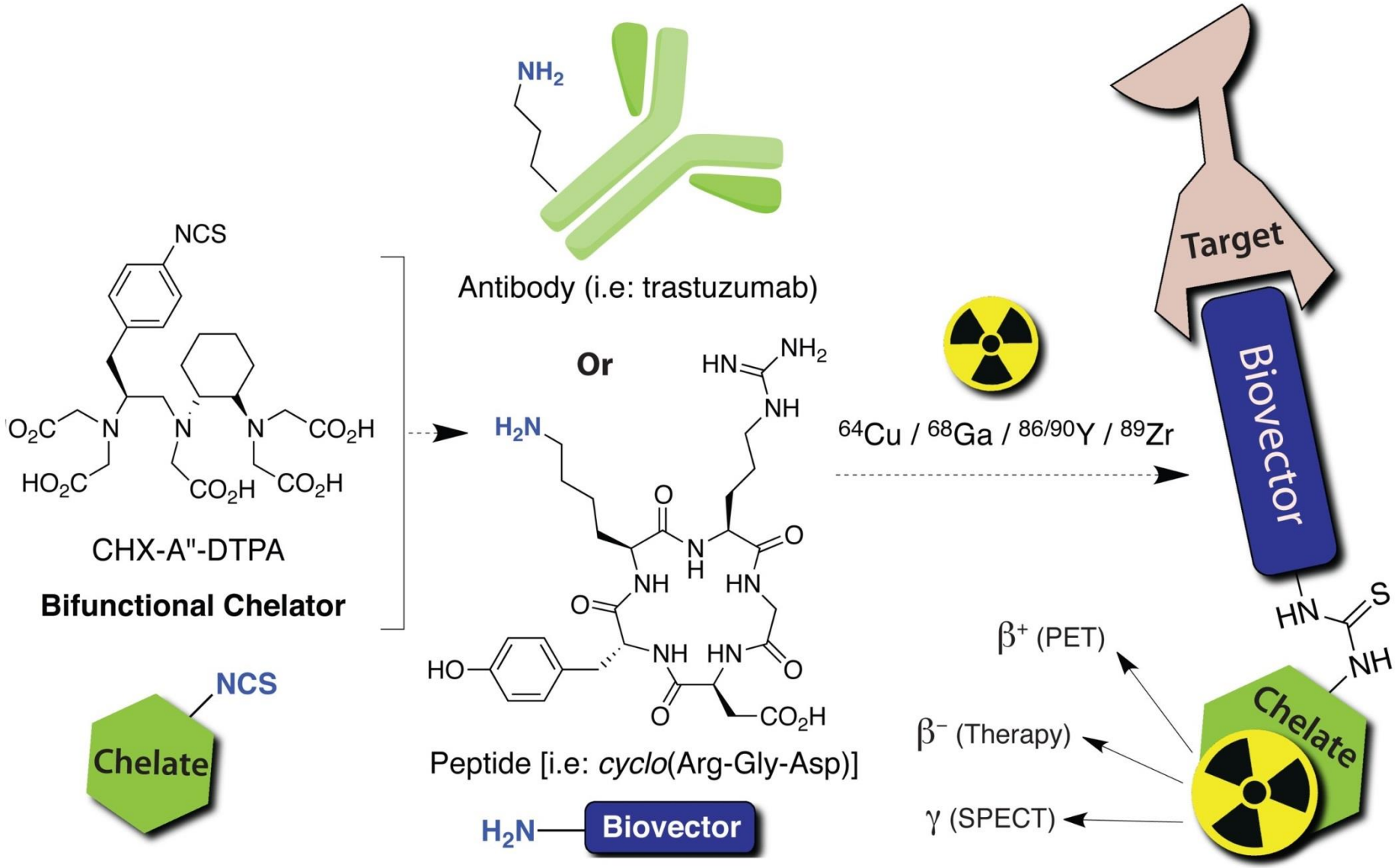




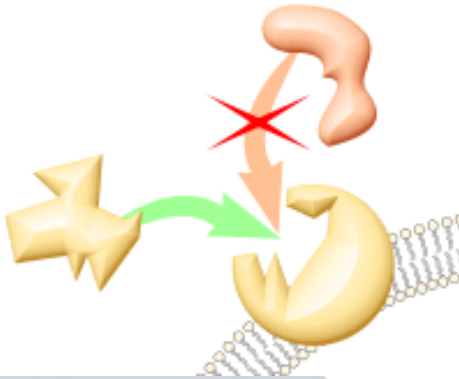
Crystal structure of entire extracellular domain of αVβ3 with amino acids Y122, K125, W129, and K181, which are implicated in the 7E3 epitope (orange).
The RGD peptide is shown in green.

PNAS, 2004 vol. 101 no. 36, 13114

Cartoon illustration of a bifunctional chelate (BFC) based radiopharmaceutical agent conjugated to a biological targeting group (e.g. biovector/vector,



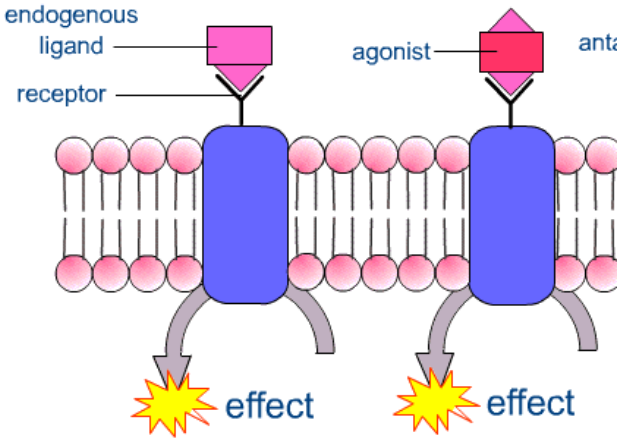
RadioPeptide–Receptor binding: Characterization



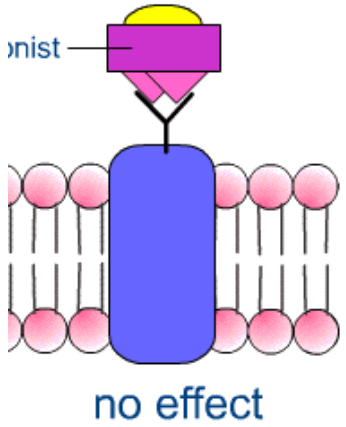
Affinity	• Capacity of a radiopeptide to bind a receptor
Specificity	• Selective binding to a specific receptor subtype
Efficacy	• Ability to produce a biological response upon binding to the receptor
Potency	• Binding affinity/ligand efficacy interplay • Conc.-induced effect relationship

RadioPeptide–receptor binding: Agonist and Antagonist

- **Radiopeptide Agonist**
  - Mimetizes the endogenous peptide
  - Binds, stimulates and activates the receptor (producing a intracellular biological response)
  - **Binding with affinity and efficacy**



- **Radiopeptide Antagonist**
  - Peptide analog structurally similar to the endogenous peptide
  - Able to bind and blocking the receptor (the receptor is not activated and no biological response is produced)
  - **Binding with affinity and no efficacy**



Biomolecule–Receptor binding: Signal Transduction

Reactions inside of the cell (activation or inhibition of biological processes) upon binding of a biomolecule to their specific receptors

Mechanisms include activation of:

- Proteins G
- Tyrosine kinases
- Transcription processes

The activated mechanism depends on:

- stimulus received (ligand, peptide)
- Cell type
- Cell metabolic status
- Presence of pathogens, etc.

Affinity: Radioligand binding assays

- Saturation binding assay
- Competitive binding assay

Radioligand binding Studies

A RADIOLIGAND is a radioactive labeled drug that can associate with a receptor, transporter, enzyme, or any site of interest.

Measuring the rate and extent of binding provides information on the number of binding sites, and their affinity and accessibility for various drugs.

Radioligand binding used to:

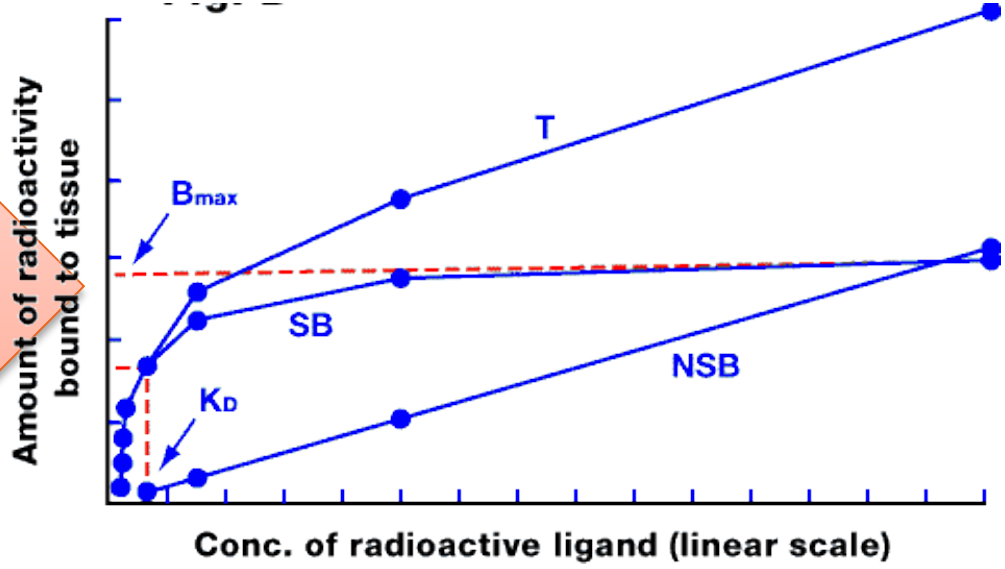
- **Identify novel chemical structures that interact with receptors**
- **Define ligand activity and selectivity in normal and disease tissues**
- **Characterize receptors in their natural environment as well as those transfected into cell lines**
- **Study receptor dynamics and localization**

Radioligand-receptor binding assays: Saturation assay

Measure equilibrium binding of various concentrations of the radioligand. Analyse the relationship between binding and ligand concentration to determine the number of sites, Bmax, and the ligand affinity, Kd

Saturation assay:

- Affinity of Radiopeptide
- **Kd**: [L] for 50% of R occupied
- **Bmax**, binding sites number

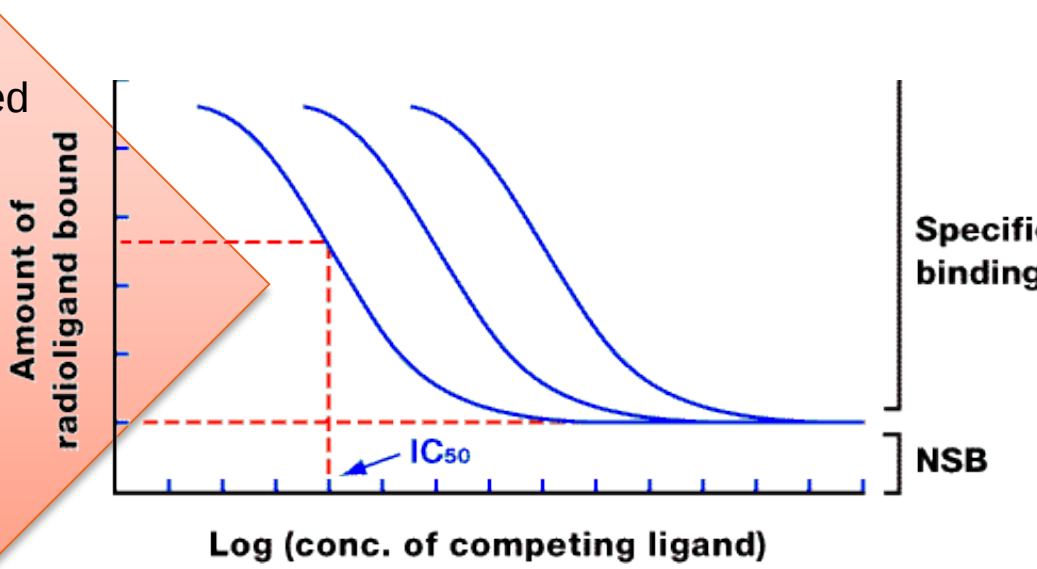


Radioligand-receptor binding assays: Competitive binding assay

Measure equilibrium binding of a single concentration of radioligand at various concentrations of an unlabeled competitor. Analyze these data to learn the affinity of the receptor for the competitor

Competitive binding assay:

- Affinity of non-labeled ligand in competition with a high-affinity radioligand
- **IC50**, [L] 50% of bound
- **Ki,**



(Radio)Peptide–Receptor binding: Affinity

Endogenous Peptide

- Low IC50 (KD)
- High affinity
- Low metabolic stability

Peptide analogs

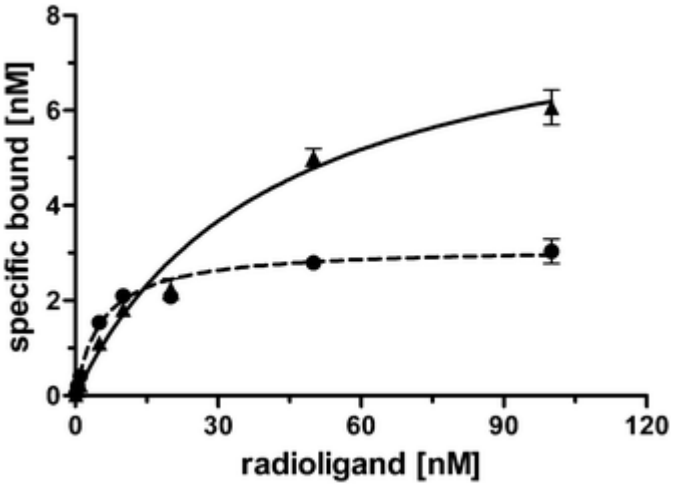
- High affinity
- Structure-affinity studies
- Higher stability

Radiopeptides

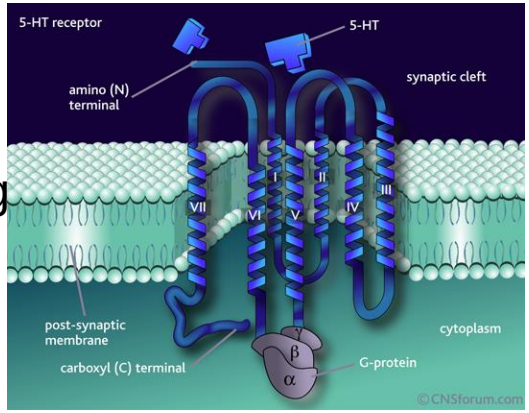
- High affinity
- High R-mediated cell uptake
- High tumor uptake

Saturation binding experiments

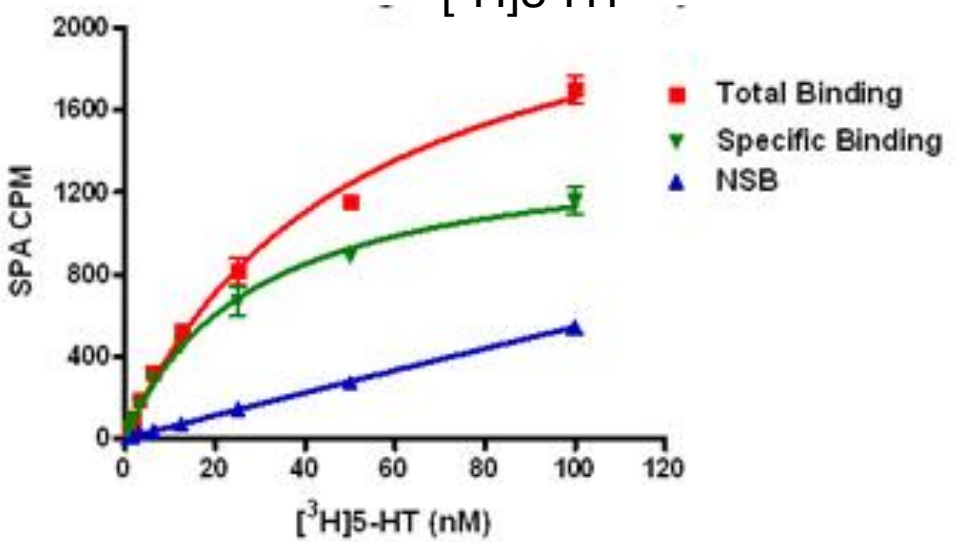
PC3 cells expressing GRP-receptor radiometal conjugates



HEK293 cells stably expressing 5-HT2CRs



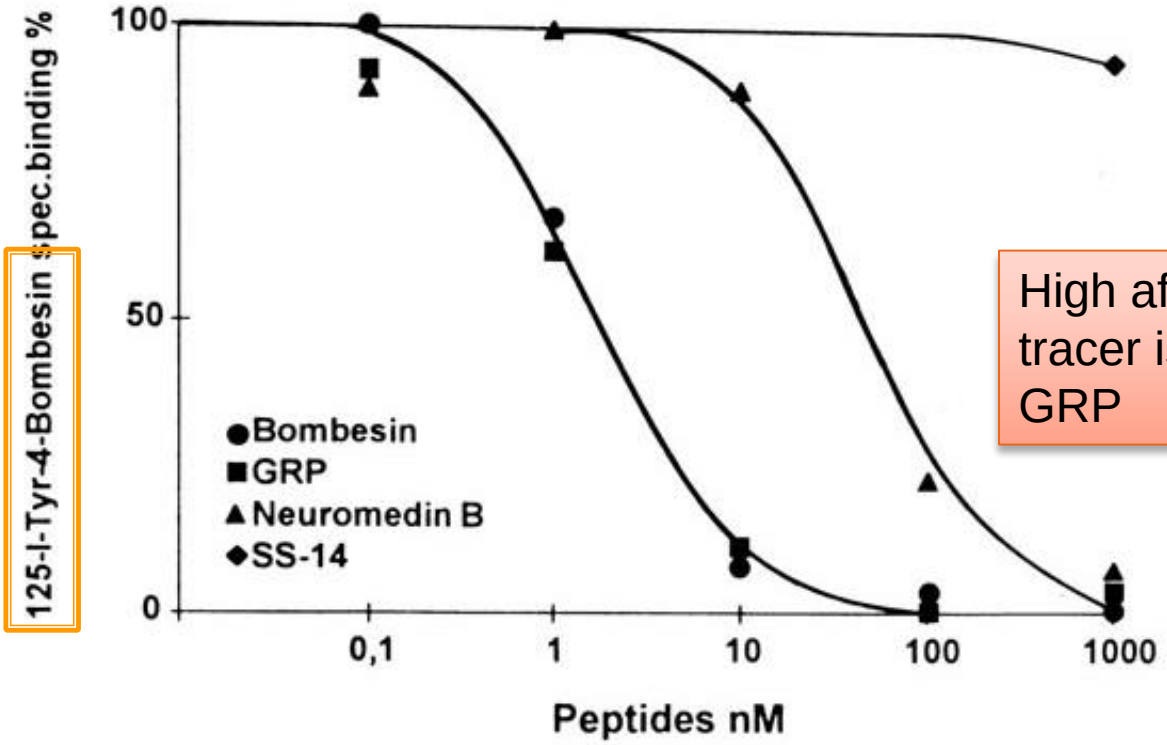
[^{3}H]5-HT



10 µg of membranes prepared from HEK293 cells stably expressing 5-HT2C receptors were incubated with 0.5 mg of WGA SPA beads and increasing conc. of [^{3}H]5-HT in the absence (total binding) or presence of 1000-fold excess unlabeled 5-HT (NSB) overnight at room temperature.

Competitive binding assay: example

GRP receptor expressed in peritumoral vessels in **pancreatic adenocarcinoma**



High affinity displacement of the tracer is found with bombesin and GRP

Displacement experiment. Samples incubated with:

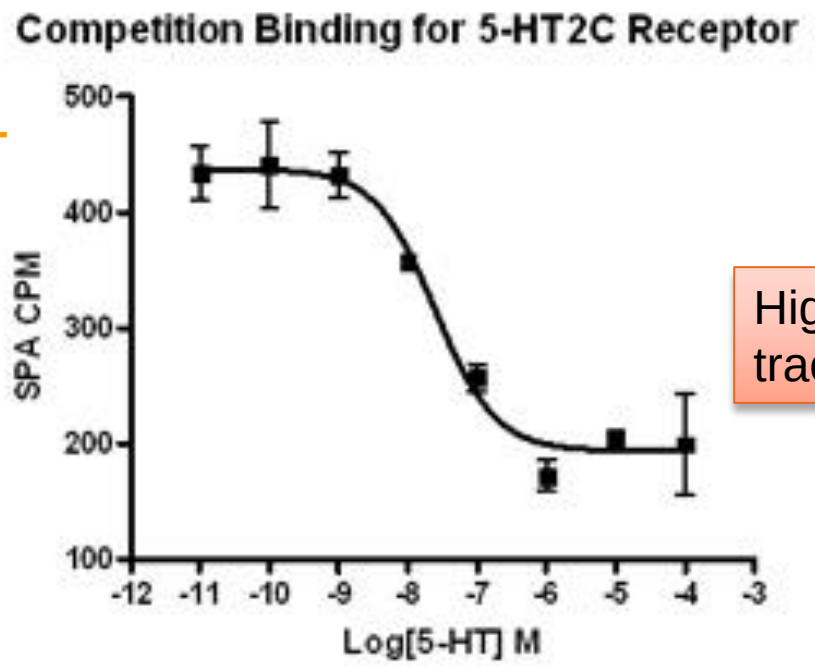
^{125}I-[Tyr^{4}]-bombesin + increasing concentrations of unlabeled:

- Bombesin
- GRP
- Neuromedin B
- Somatostatin-14

Competitive binding assay: example

5-HT2C Receptors stably expressed in HEK293 cells

[^{3}H]5-HT

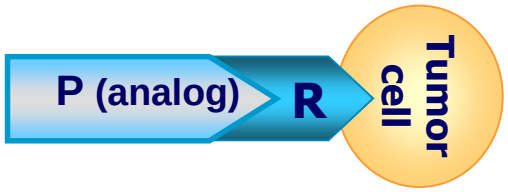


High affinity displacement of the tracer is found with 5-HT

Displacement experiment. Membranes prepared from HEK293 cells stably expressing 5-HT2C receptors incubated with:

[^{3}H]5-HT (25 nM) + WGA SPA beads (0.5 mg) and increasing concentrations of unlabeled 5-HT

Radiopeptide–Receptor binding:
Structural modifications that affect AFFINITY



Modification of endogenous peptide sequence-Peptide analog (ciclization increases affinity)

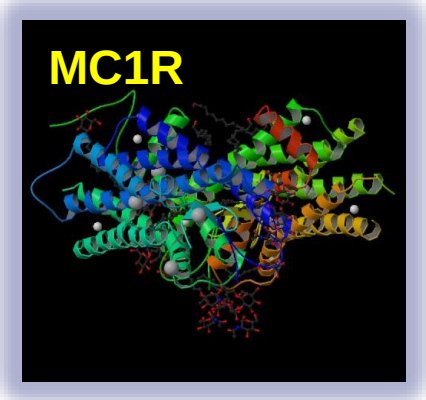
Peptide cyclization: Effect on MC1R binding Affinity (IC_{50} values)

$Ac\text{-}Ser^{1}\text{-}Tyr^{2}\text{-}Ser^{3}\text{-}Met^{4}\text{-}Glu^{5}\text{-}His^{6}\text{-}Phe^{7}\text{-}Arg^{8}\text{-}Trp^{9}\text{-}Gly^{10}\text{-}Lys^{11}\text{-}Pro^{12}\text{-}Val^{13}\text{-}NH_2$

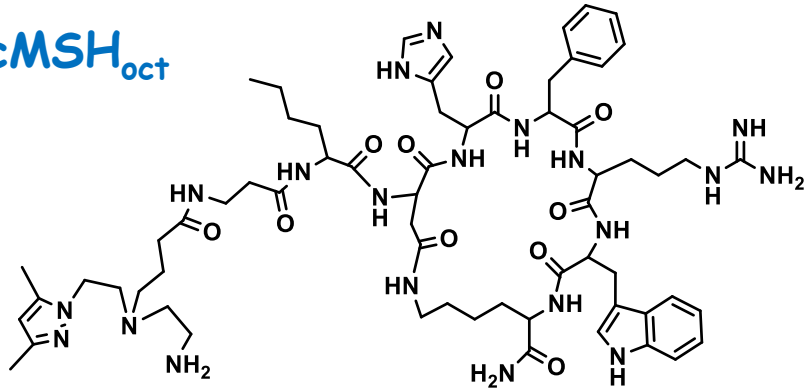
B16F1 murine melanoma cells

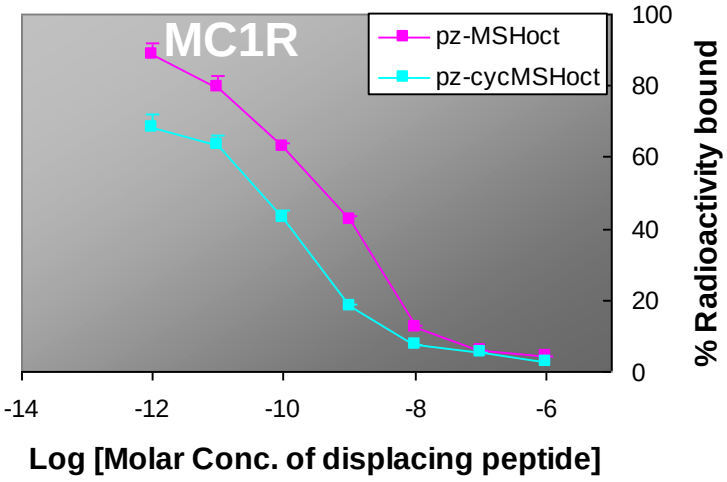
α-MSH (endogenous peptide 13aa)
1.65 nM

Linear pz-MSHoct (peptide 8aa)
1,25 nM



Cyclic pz-CycMSH$_{oct}$
0,21 nM





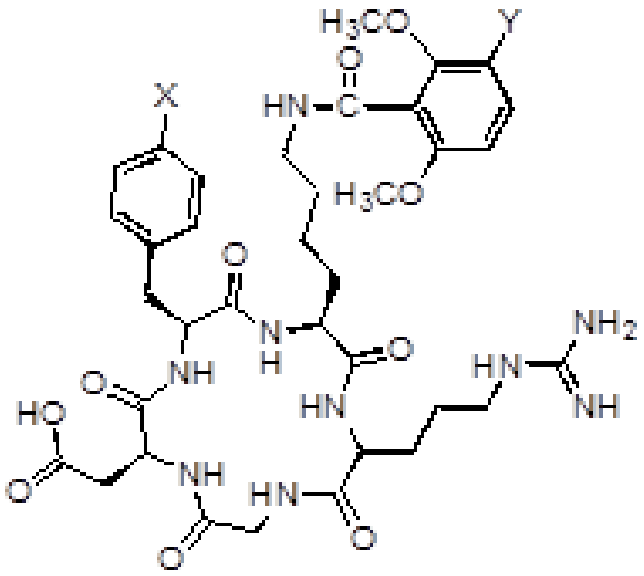
Raposinho P et al. 2008, J Biol Inorg Chem, 13, 449

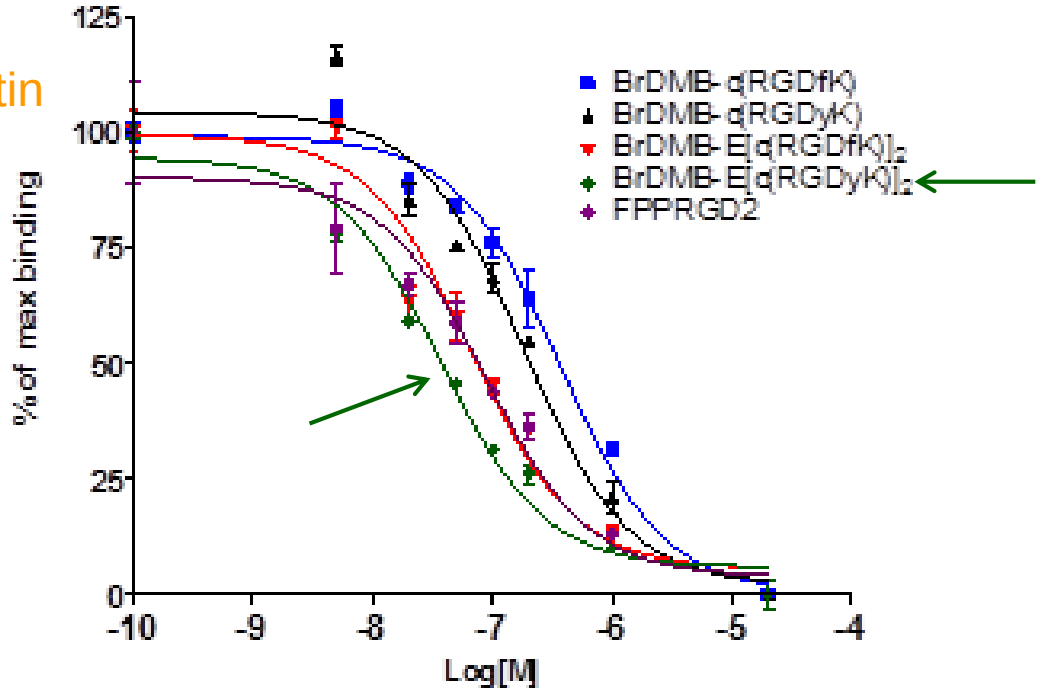
Cyclic RGD derivatives affinity: CBA

High **integrin $\alpha_v\beta_3$** expression in human glioblastoma U87MG cells

A

^{125}I-echistatin





B

X = H, Y = H, DMB-c(RGDfK)
X = OH, Y = H, DMB-c(RGDyK)
X = H, Y = Br, BrDMB-c(RGDfK)
X =OH, Y = Br, BrDMB-c(RGDyK)

Compound	IC50 (nM)
BrDMB-c(RGDfK)	363.3
BrDMB-c(RGDyK)	180.8
BrDMB-E[c(RGDfK)]$_2$	66.5
BrDMB-E[c(RGDyK)]$_2$	37.1
FPPRGD2	87.0

Bicyclic somatostatin-based analogues

AM3 :DOTA-Tyr-cyclo(DAB-Arg-cyclo(Cys-Phe-D-Trp-Lys-Thr-Cys))

119 ± 6
2.3 ± 0.2
4.0 ± 0.03
97 ± 21
27 ± 1
sst_2: agonist;
sst_3: agonist

high rigidity led to agonistic ligands with **good affinity for all 5 ssts**

$^{177}Lu/^{68}Ga$-AM3

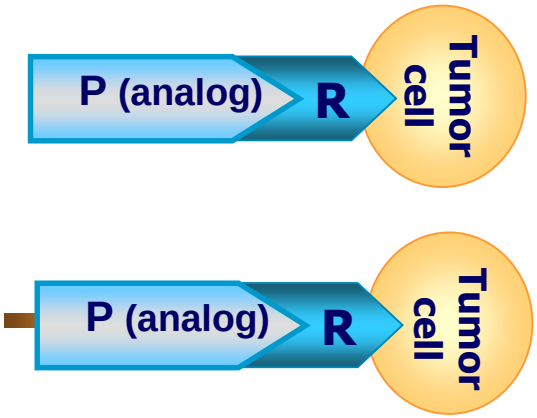
The pharmacokinetic data make this peptide an excellent candidate as an imaging—and especially as a PET—radiotracer.

TERAPIA

- ^{90}Y-DOTATOC
- ^{177}Lu-DOTATATE

Fani1, M et al. J Nucl Med 2010; 51:1771

Radiopeptide–Receptor binding:
Structural modifications that affect AFFINITY



Modification of endogenous peptide sequence-Peptide analog (ciclization increases affinity)

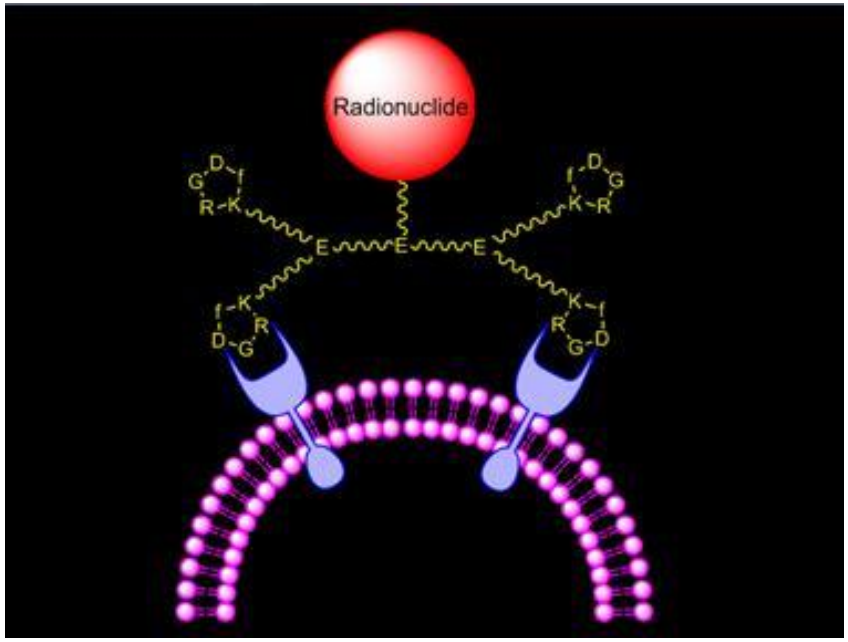
Introduction and nature of a spacer

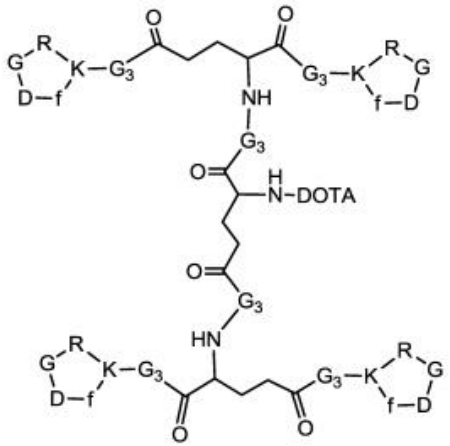
DOTA-chelated neurotensin analogs with spacer-enhanced biological performance for neurotensin-receptor-1-positive

Analog	Sequence	$IC_{50} \pm SD$ (nM)
N0[a]	DOTA-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH	52.3 ± 1.5
N1	DOTA-β-Ala-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH	27.6 ± 1.3
N2	DOTA-5-Ava-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH	20.8 ± 1.4
N3	DOTA-8-Aoc-Lys-Pro-(N-Me)Arg-Arg-Pro-Dmt-Tle-Leu-OH	21.1 ± 1.7
Lu-N0[b]	^{nat}Lu-N0[c]	47.2 ± 1.2
Lu-N1	^{nat}Lu-N1	27.2 ± 1.1
Lu-N2	^{nat}Lu-N2	24.3 ± 1.1
Lu-N3	^{nat}Lu-N3	20.3 ± 1.2
NT	–	22.3 ± 1.2

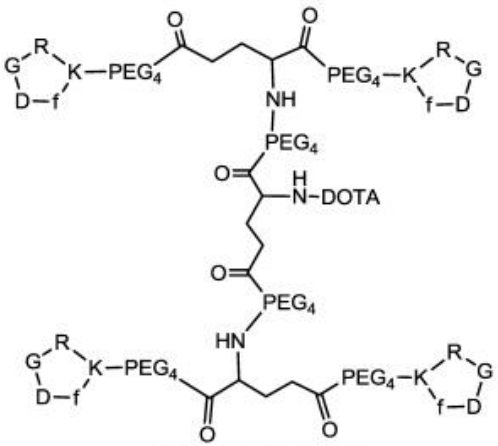
cRGD tetrameric analogs affinity (CBA)

integrin $\alpha_v\beta_3$

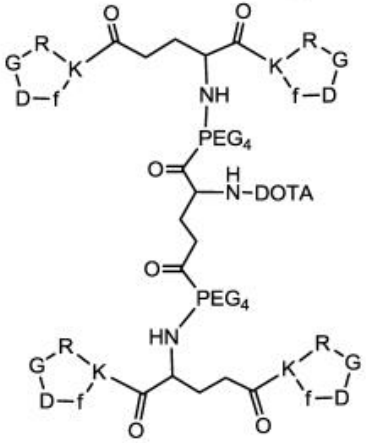




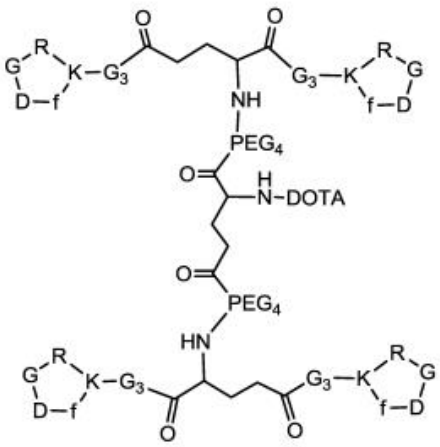
DOTA-6G-RGD$_4$



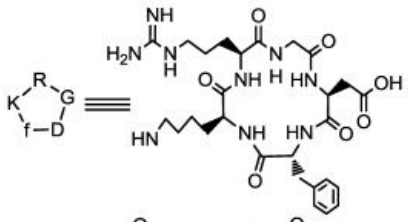
DOTA-6P-RGD$_4$

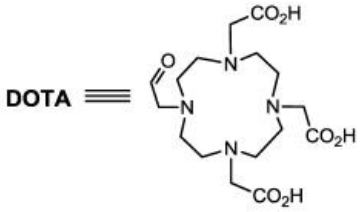


DOTA-2P-RGD$_4$

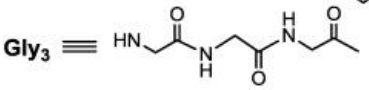


DOTA-2P4G-RGD$_4$

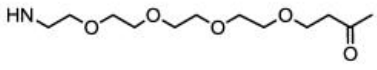




DOTA



Gly$_3$



PEG$_4$

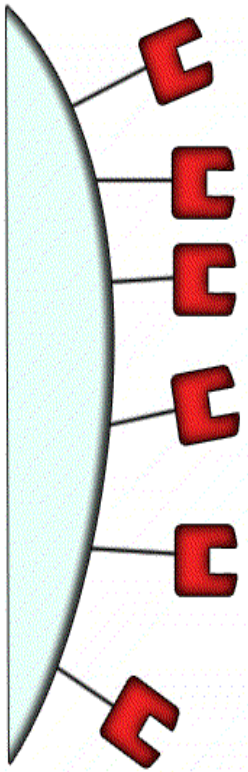
Spacers with different number of:

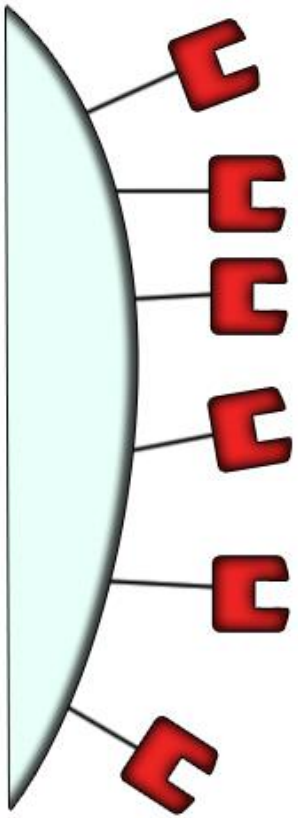
- Gly$_3$
- PEG$_4$

http://www.thno.org/v01p0322.htm

Multivalent vs monovalent binding: Effect on affinity

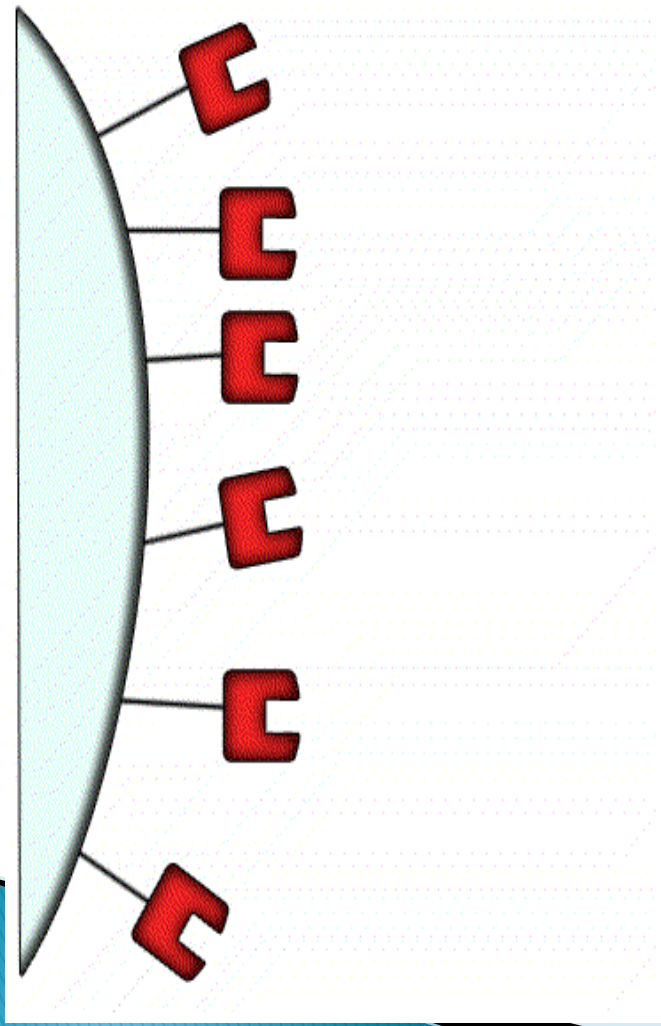
- Use of more than one vector for the targeting of a given system
- Leads to **increased affinity** (avidity), **increased uptake** and **increased retention** in target tissue
- Two main mechanisms are involved:
  - **Higher** local **concentration** of vectors (peptide) leading to a higher probability of binding
  - **Real multivalent simultaneous binding** of more than one vector



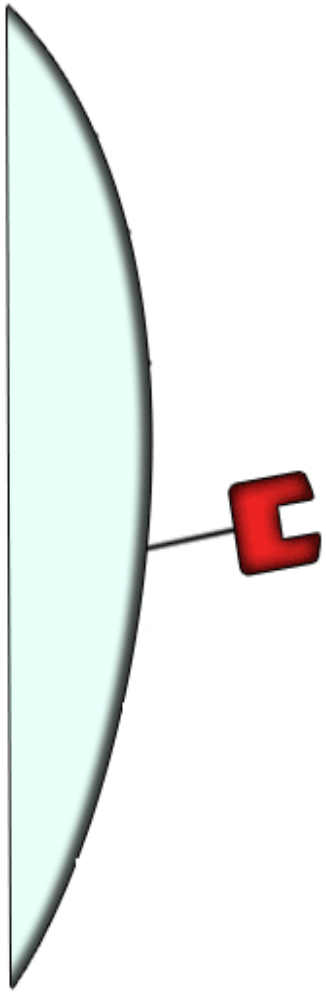


Multivalency: Concentration effect

Rotation effect

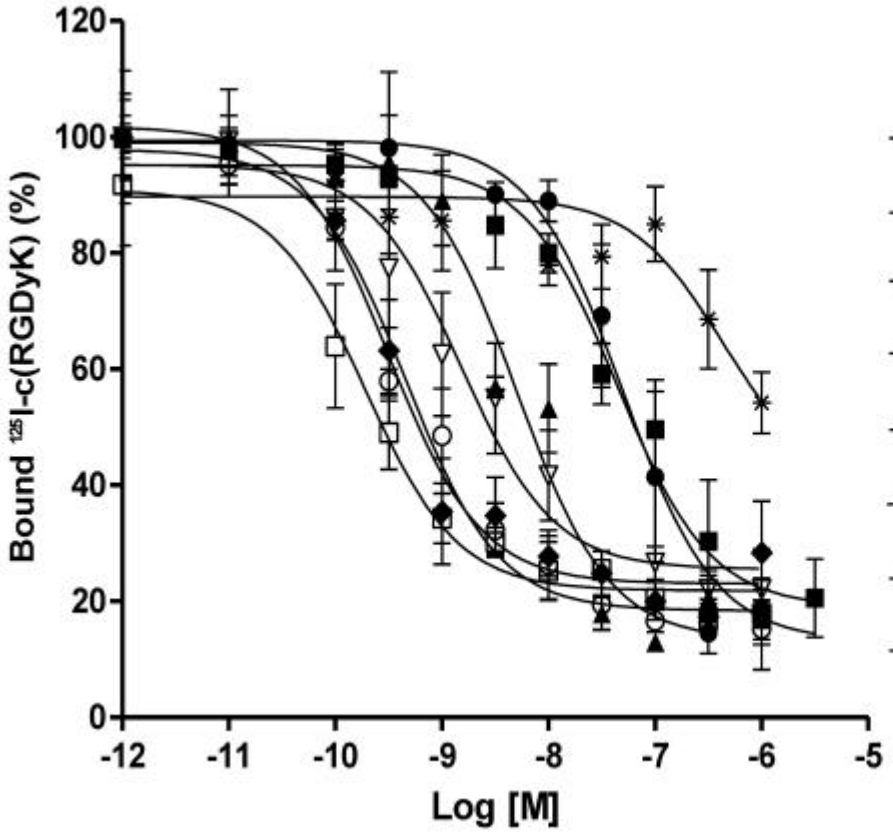


“Bind & Slide” effect



cRGD tetrameric analogs affinity (CBA)

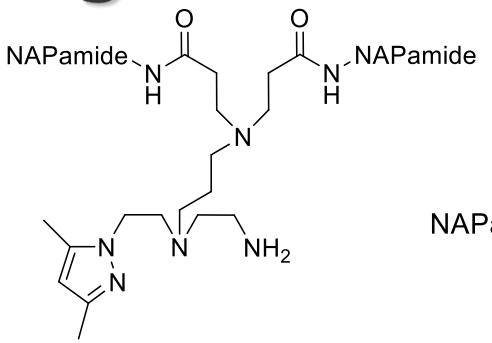
High **integrin $\alpha_v\beta_3$** expression in **human glioblastoma U87MG cells**



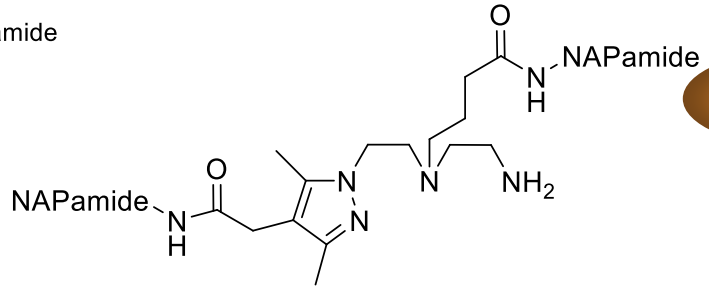
	IC50 (nM)
c(RGDyK)	49.9
DOTA-P-RGD	44.3
DOTA-P-RGD$_2$	5.0
DOTA-3P-RGD$_2$	1.5
DOTA-2P-RGD$_4$	0.5
DOTA-2P4G-RGD$_4$	0.2
DOTA-6P-RGD$_4$	0.3
DOTA-6P-RGK$_4$	437

Competitive inhibition curves of ^{125}I-c(RGDyK) bound to the U87MG human glioma cells

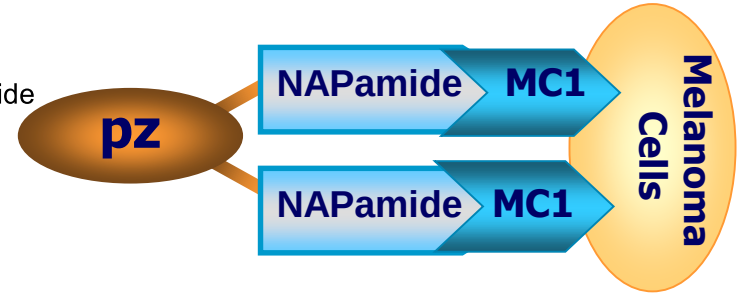
Bivalent conjugates pz-NAPamide$_2$: Higher affinity to MC1R



L^2



L^3

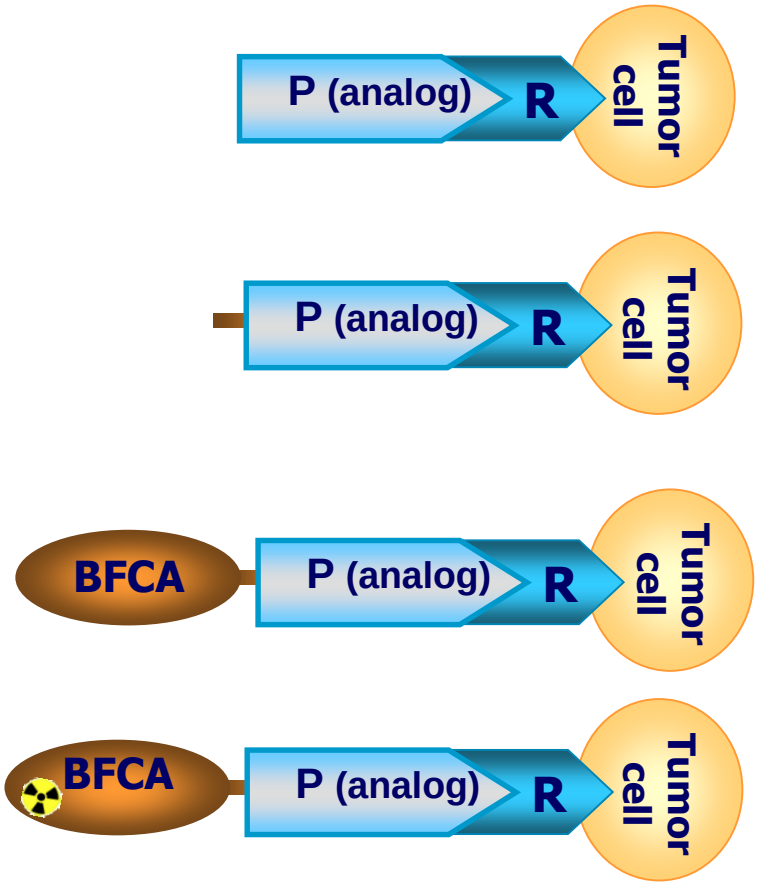


α-MSH Analogs	IC_{50} (nM)
α-MSH	1.65 ± 0.18
NDP-MSH	0.21 ± 0.03
NAPamide	0.78 ± 0.03
Monovalente L^1	0.66 ± 0.13
Bivalente L^2	**0.035 ± 0.018**
Bivalente L^3	**0.16 ± 0.21**
Re-Monovalente 1a	0.033 ± 0.019
Re-bivalente 2a	0.15 ± 0.08
Re-bivalente 3a	1.14 ± 1.13

Nanomolar or sub-nanomolar IC_{50} values

High affinity for all conjugates

Radiopeptide–Receptor binding:
Structural modifications that affect AFFINITY



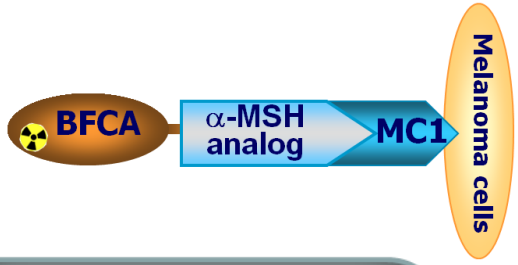
Modification of endogenous peptide sequence-Peptide analog (ciclization increases affinity)

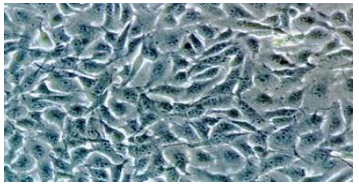
Introduction and nature of a spacer

Conjugation to a BFCA (Charge and nature affects affinity)

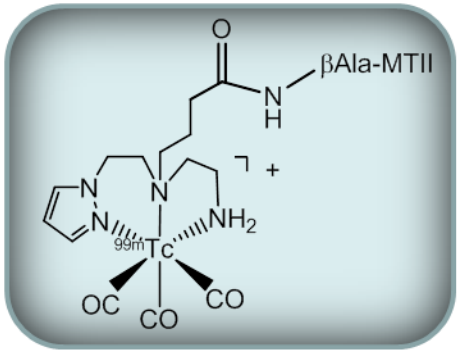
(Cold)Metalation of Peptide conjugates

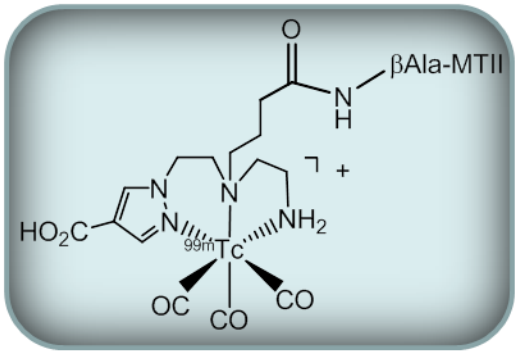
BFCA effect's on the receptor binding affinity

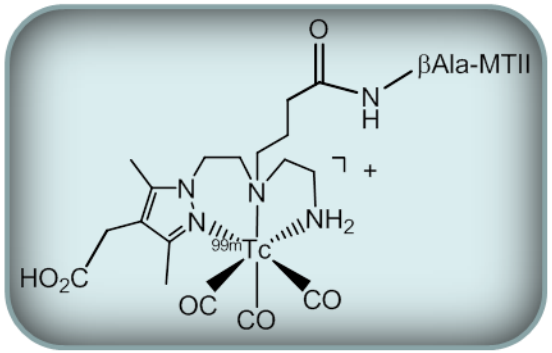




B16F1







IC_{50}(conjugates)

0,023 ± 0,014 nM

0,039 ± 0,009 nM

0,160 ± 0,098 nM

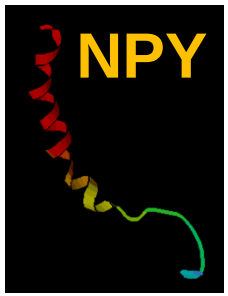
Morais M et al. 2013, *J. Med. Chem.*, 56(5), 1961

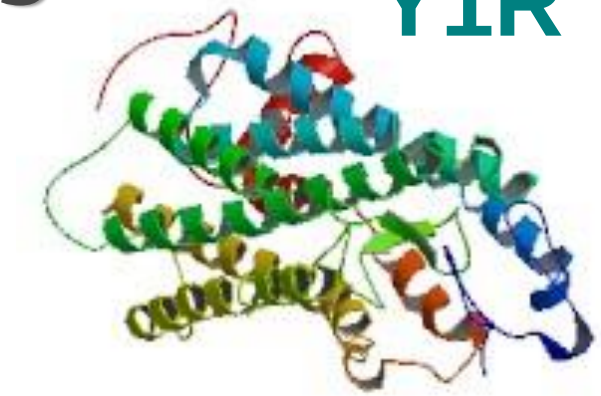
Peptide–receptor binding

- Receptor Subtype Specificity

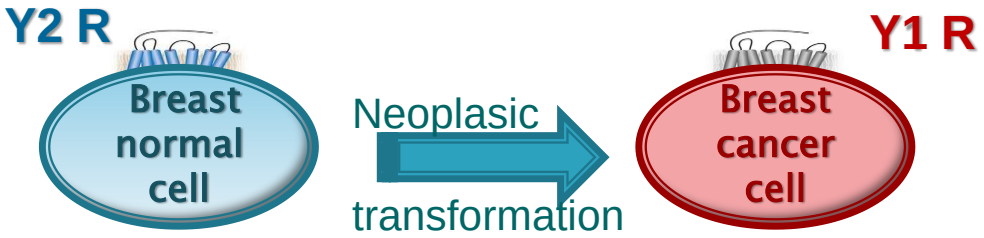
Evaluation of the affinity of different analogs to different receptors or receptors subtype

Peptide–Receptor binding: Specificity





- Looking for new targets for tumor diagnostic or therapy is better to find a difference between the normal and neoplasic tissue
- NPY receptor subtype Y1 is expressed in remarkably high incidence and density on breast tumors while Y2 subtype is mostly expressed in healthy tissue
- NPY analogs have been designed in order to be specific for Y1R



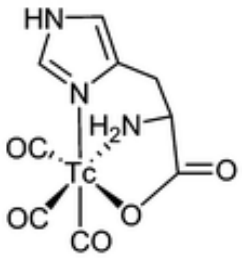
NPY analogs–Y1 Receptor binding: Specificity

Y1R analog

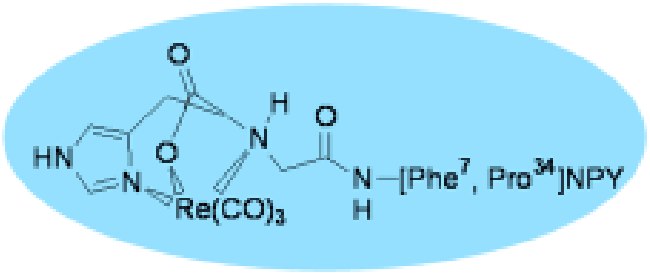
[Phe^7,Pro^{34}]-NPY

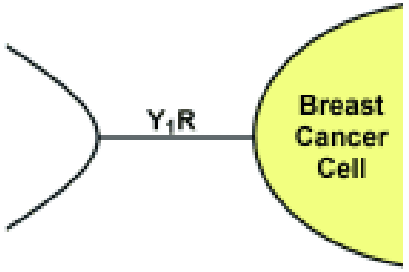
YPSKPDFPGEDAPAEDMARYYSALRHYINLITRPRY-NH_2

BFCA



$[^{99m}Tc(CO)_3(N^{\alpha}His)]$





		IC_{50} [nM] SK-N-MC (Y1R)	MCF-7 (Y1R)	SMS-KAN (Y2R)	HEC-1b-hY_5
1c	$Re(CO)_3$-(N^{α}His-ac)-NPY	3.9±0.3	17.0±6.5	3.2±1.3	29.8±1.9
1d	$Lys^4(Re(CO)_3$-(N^{α}His-ac))-NPY	10.5±3.9	8.5±6.5	6.1±2.6	27.3±5.1
2c	$Re(CO)_3$-(N^{α}His-ac)-[Phe^7, Pro^{34}]NPY	11.8±2.6	26.9±5.2	106.3±22.2	>1000
2d	$Lys^4(Re(CO)_3$-(N^{α}His-ac))-[Phe^7, Pro^{34}]NPY	1.3±0.1	5.2±1.0	97.5±11.9	208.4±8.0

High affinity and selectivity to Y1R

Khan IU et al. 2009 Angew Chem Int Ed, 48,1

Cellular uptake assays

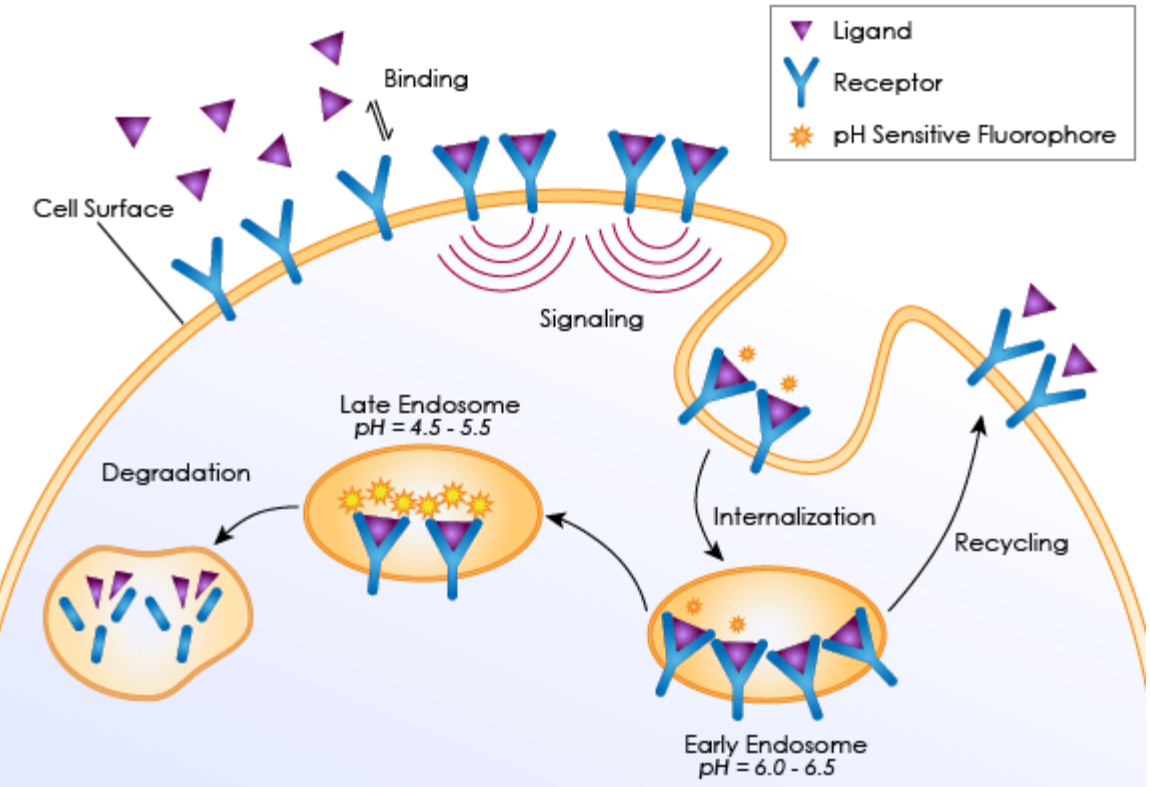
- Radioactive-based assay
  Immunofluorescence-based assay

Uptake/Internalization Cell-studies

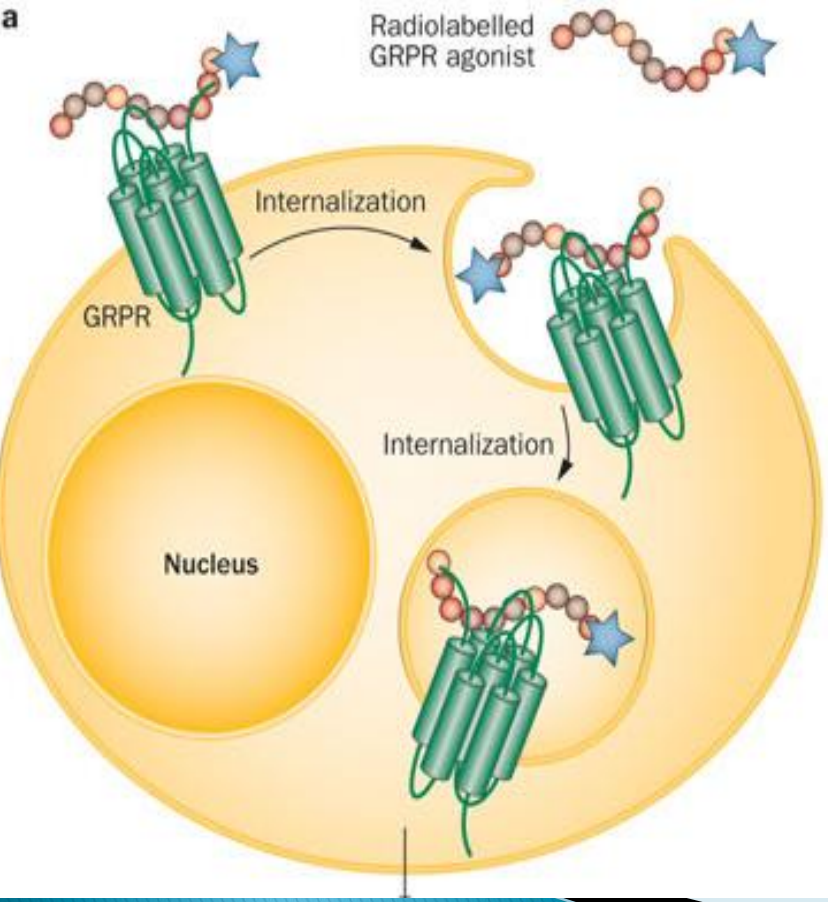
- Cellular Uptake/Internalization
- Receptor Blockade studies
- Cellular Retention
- Nuclear/ Mytochondria uptake
- …

Rdiopeptide /ligand Internalization

Receptor Internalization Assays measures the absorption of membrane receptors into the cell via endocytosis. The event is activated by the binding of ligand to surface receptors that signals the formation of plasma membrane-formed inward vesicles to enclose the target receptors. After the vesicles are formed and internalized, they are redirected to fuse with early endosomes (pH 6.0-6.5) that can recycle the receptors back to the plasma membrane, or they can be degraded via late endosomes and lysosomes (pH 4.5-5.5).



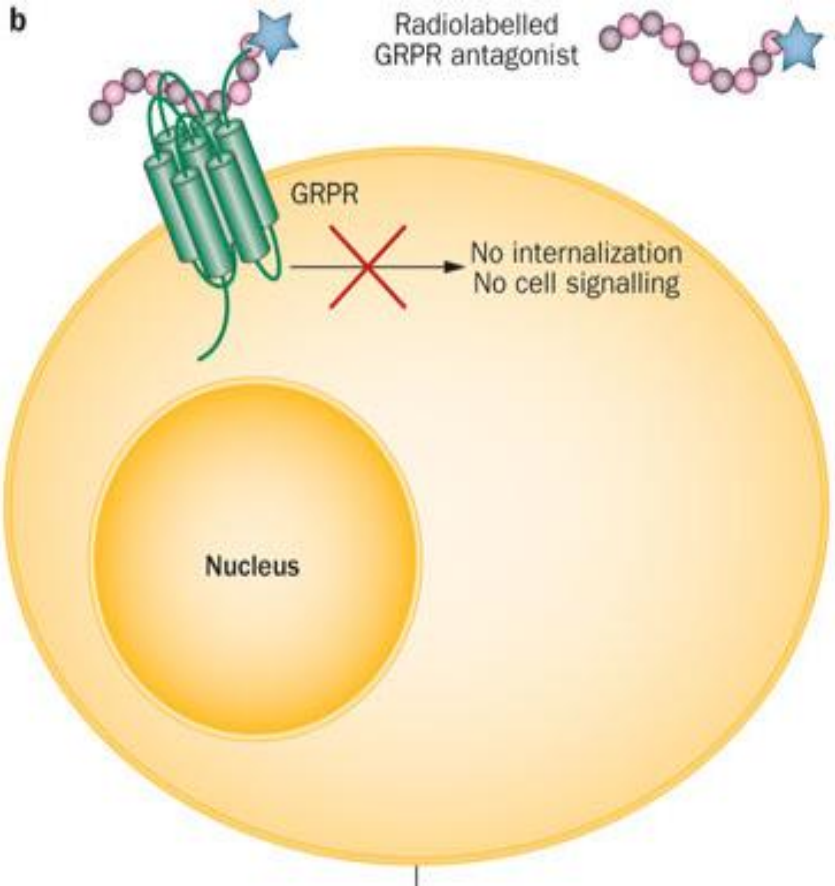
Radiolabeled agonist–Receptor binding: Internalization



- Radiolabeled Agonist incubates with cells overexpressing receptors at 37ºC
- Binds to the receptor and internalized
- Surface-bound fraction is removed with a acidic pH buffer
- Internalized fraction is recovered after cells lyses with NaOH 1M
- The activity in both fractions is measured

- Receptor blockade Assay with non-labeled agonist: Specific Receptor-mediation internalization
- Cellular uptake:
  - Surface-bound + internalized

Radiolabeled antagonist–receptor binding: NO Internalization



- Radiolabeled antagonist incubates with cells overexpressing receptors at 37ºC
- Binds to the receptor,
- Blocks the receptor, keeping at surface
- No internalization and no cell signaling
- Surface-bound fraction is removed with a acidic pH buffer
- Internalized fraction is recovered after cells lyse with NaOH 1M
- The activity in both fractions is measured

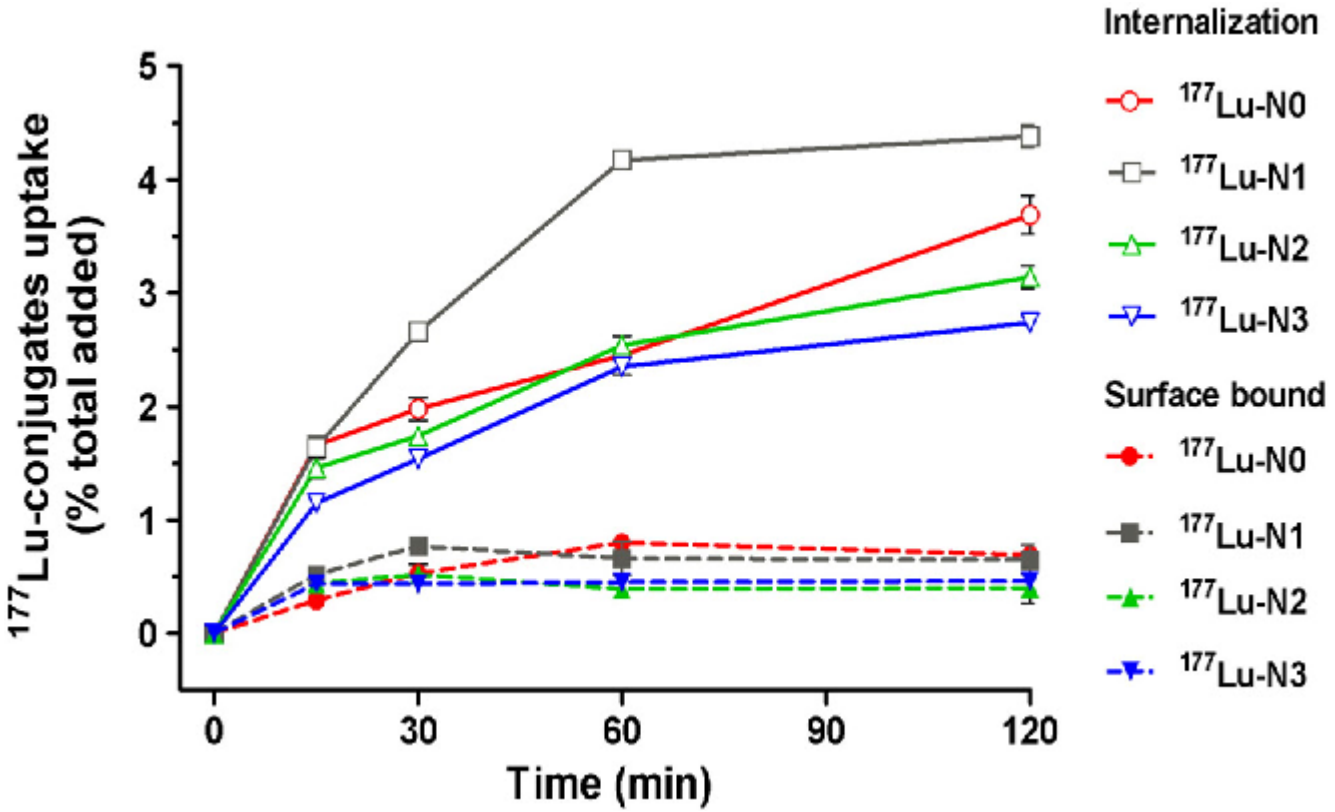
- Cellular uptake:
  - **Surface-bound** + internalized

Cellular Retention

- Radiolabeled agonist/receptor internalized at 37ºC
- After a period of internalization, the radioligand is removed
- Cells incubate with cell culture medium where they release the radioligand
- At different time points the medium is removed and activity released (externalized from the cell) measured
- The activity still inside the cell (retention fraction) is recovered by NaOH 1M lyse
- The activity in both fractions is measured

- Calculated % cellular retention

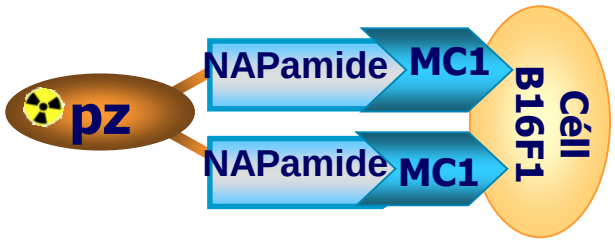
^{177}Lu-DOTA-Neurotensin analogs: Binding and Internalized NTR-1

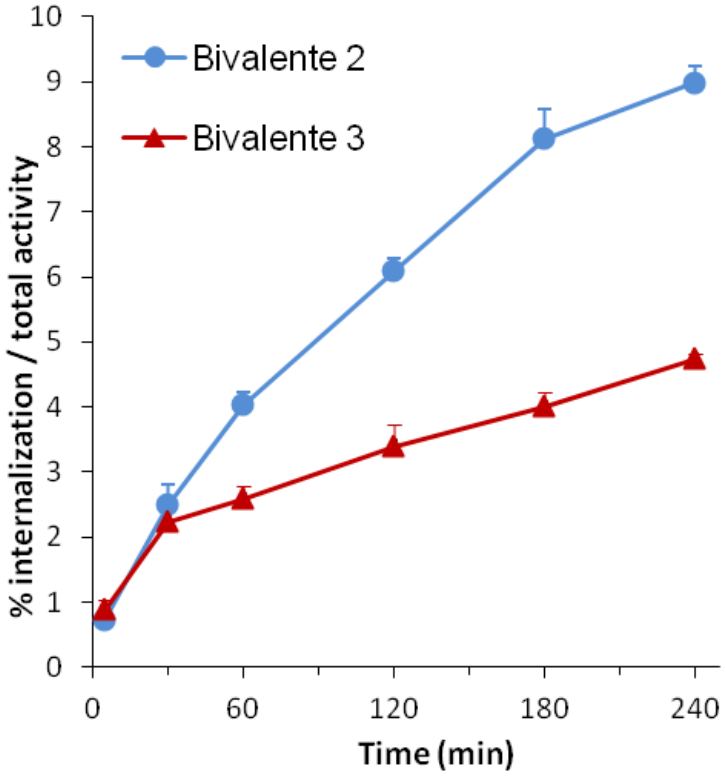


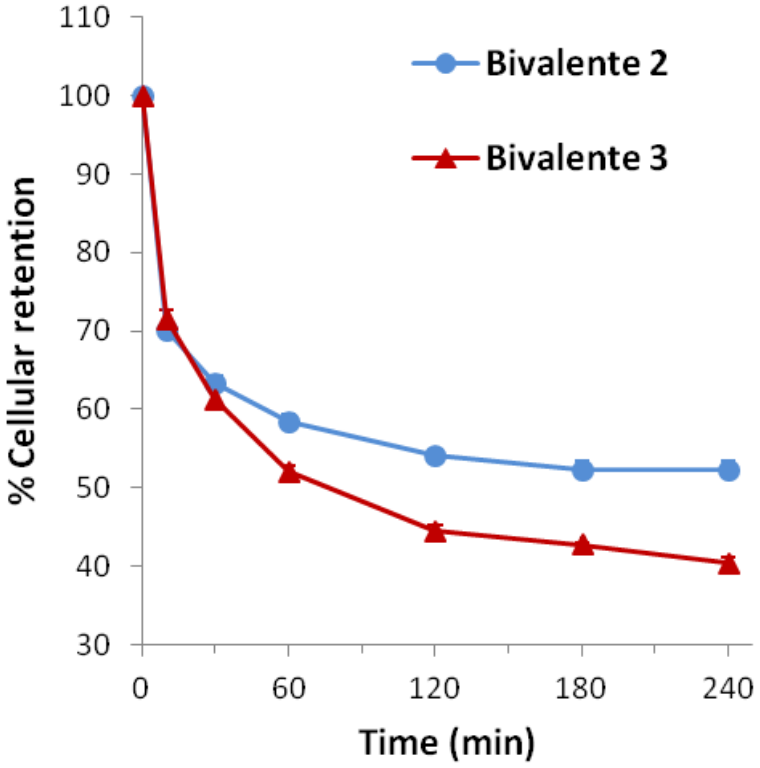
Spacer-enhanced biological performance for neurotensin-receptor-1-positive tumor targeting

Yinnong Jia, Wen Shi, 2015 Nuclear medicine and biology

Bivalent Radiopeptides ^{99m}Tc-pz-NAPamide$_2$: Internalization and retention in B16F1 cells

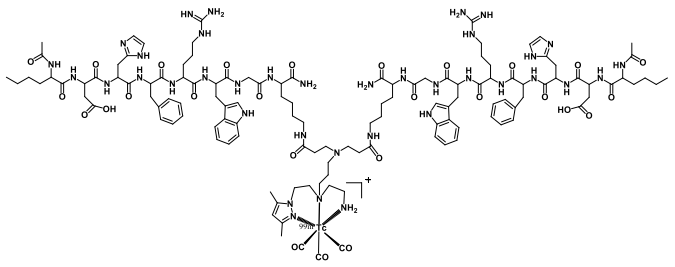






Bivalent 2:
Higher internalization and retention

Bivalent Radiopeptides ^{99m}Tc-pz-NAPamide$_2$: Internalization (B16F1 cells)



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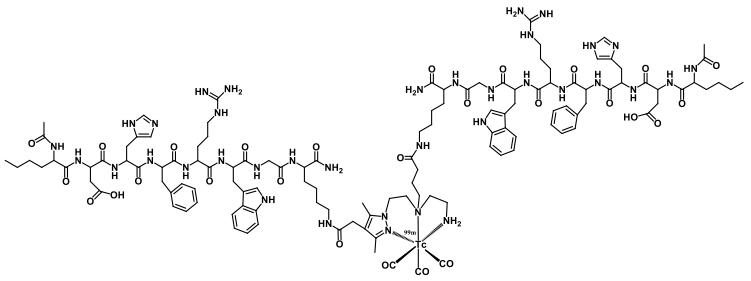
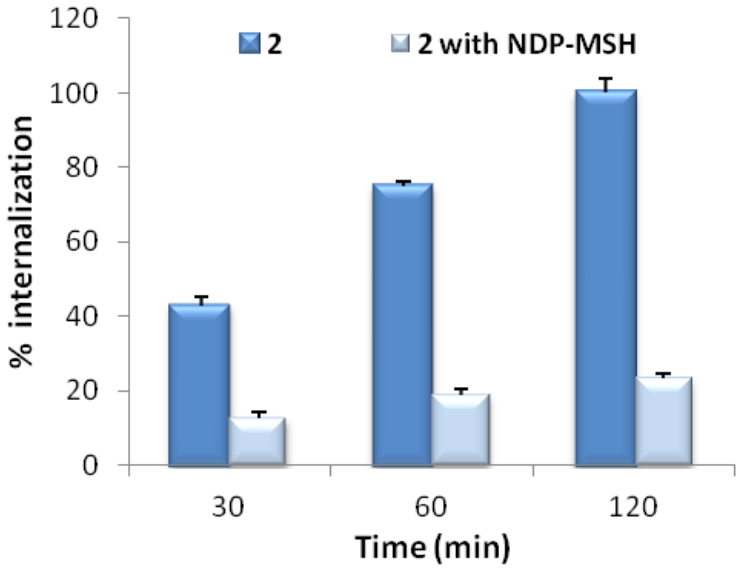
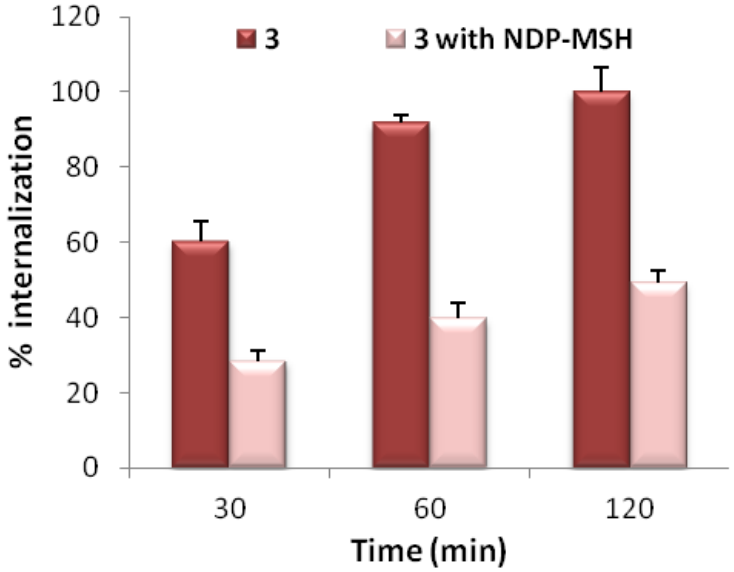


NDP

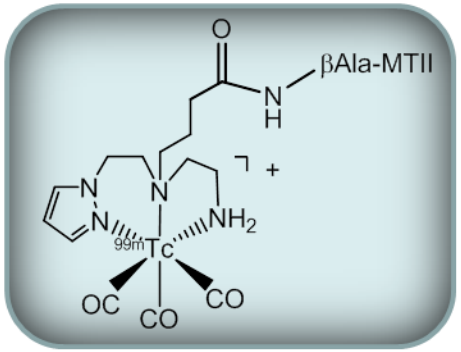


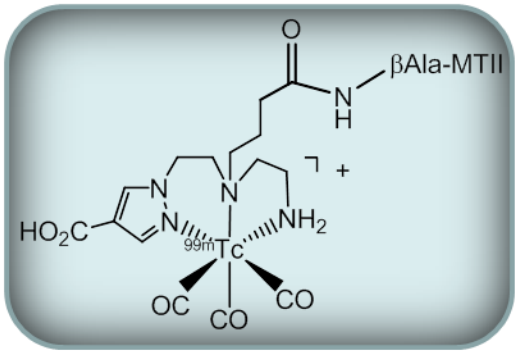


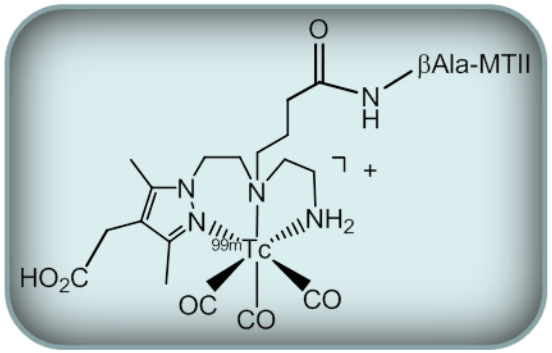
Co-incubation with high [NDP] reduced celular internalization (**2**: 70-76%; **3**: 50-56%)

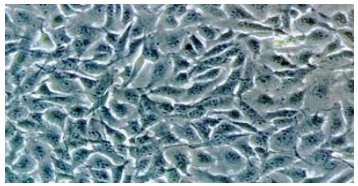
Specific MC1R-mediated internalization

Radiopeptide–Receptor binding:
Internalization–MC1R, blocking study

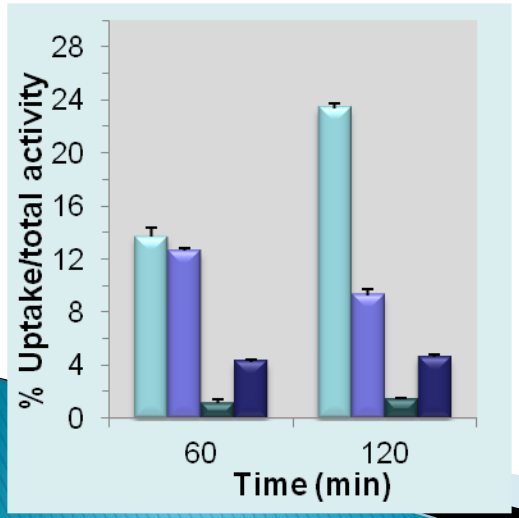


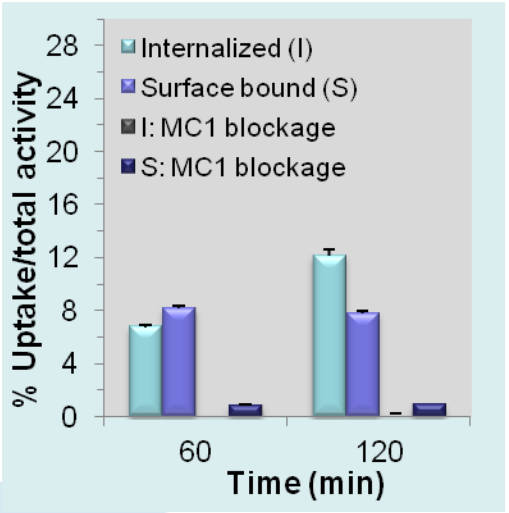


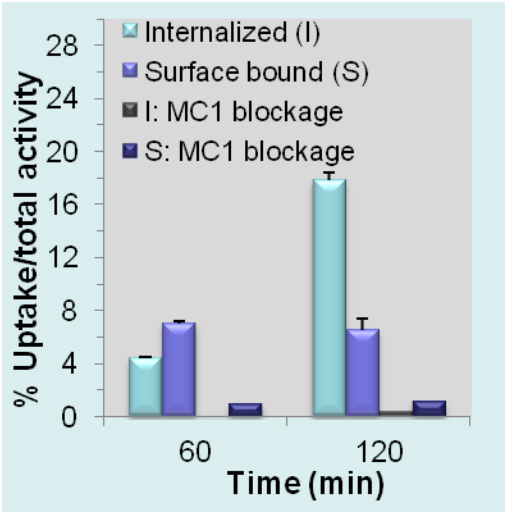




B16F1:
melanoma murine cells







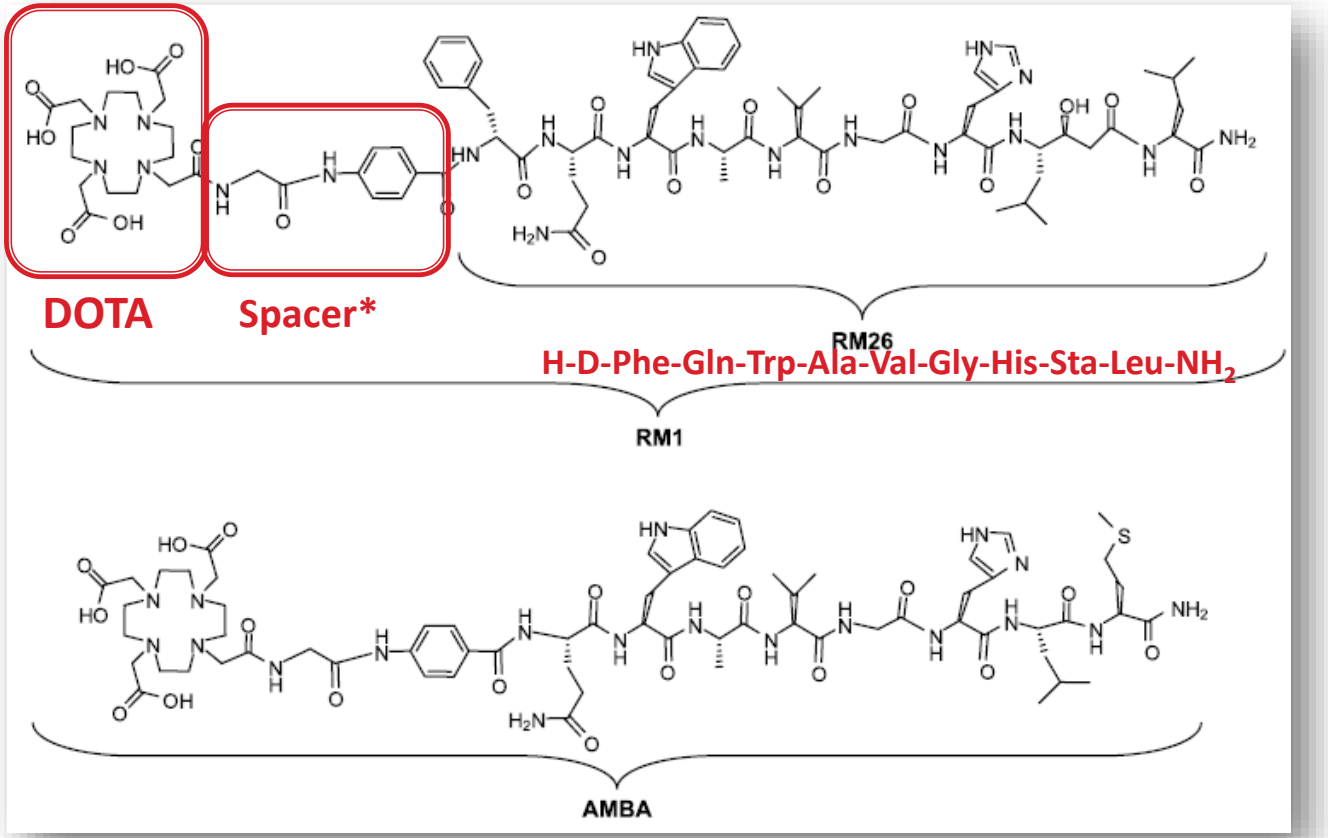
Morais M et al. 2013, *J. Med. Chem.*, 56(5), 1961

Bombesin agonists vs antagonists: Receptor affinity and internalization

Antagonist $^{nat/111}$In-RM1 vs agonist $^{nat/111}$In-AMBA

Antagonist

Agonist



*glycine-4-aminobenzoyl

Mansi R, *et al.* Clin Cancer Res, 2009

Bombesin agonists vs antagonists: Receptor affinity and internalization

	IC_{50} (nmol/L)
RM26	5.6
RM1	35
^{nat}In-RM1	14
^{nat}In-AMBA	0.8

	$B_{máx.}$ (nmol/L)
^{111}In-RM1	2.4
^{111}In-AMBA	0.7

PC3 cells

Internalization (4h)

	Internalized	Membrane-bound
• ^{111}In-RM1:	4.66 ± 0.08%	21.8 ± 0.93%
• ^{111}In-AMBA:	29 ± 2.3%	4.33 ± 0.27%

Imunofluorescence: no internalization of ^{nat}In-RM1

Ca2+ Release

Antagonists:

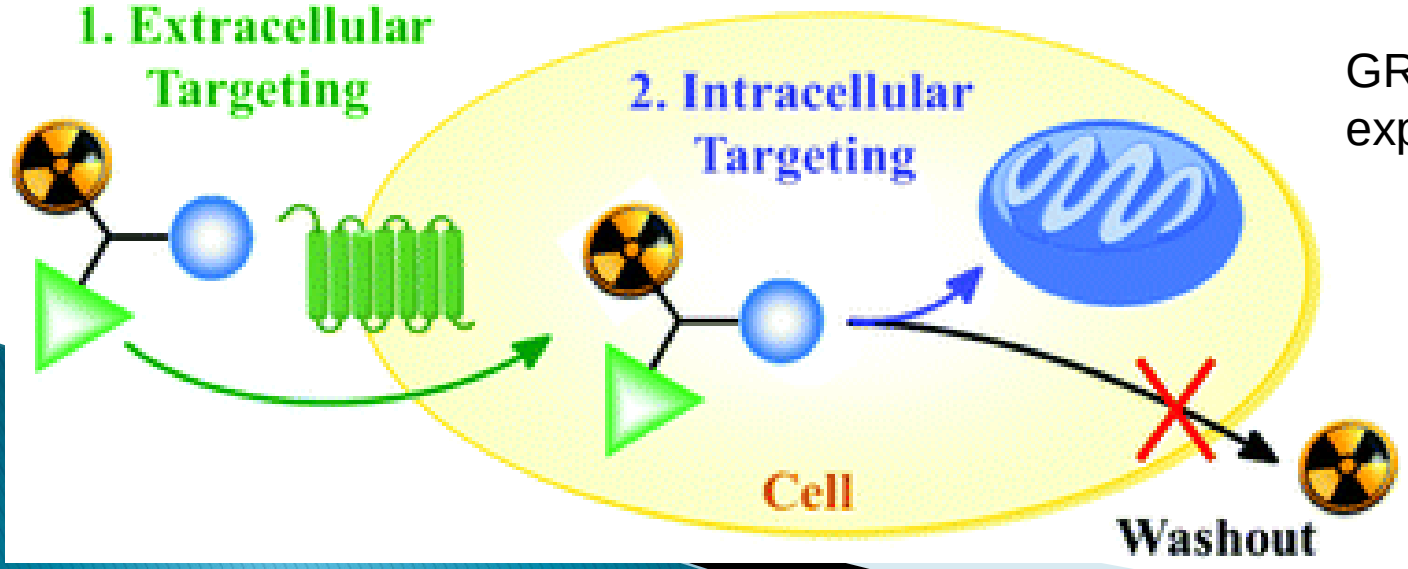
- **No effect (to 10 *µ*mol/L)**
- **Agonist effect reduced (competitive antagonism)**

Mansi R, *et al.* Clin Cancer Res, 2009

Radiopeptide–Receptor binding: Cellular internalization and retention

Dual-targeting conjugates designed to improve the efficacy of radiolabeled peptides

- Rapid washout of internalized radiopeptide may represent a limitation for clinical applications.
- Dual-targeting peptide radioconjugate designed to enhance the cellular retention of radioactivity.
- Trifunctional conjugate comprised of :
  - a Tc-99m SPECT reporter probe,
  - a cell membrane R-specific peptide,
  - a second targeting entity directed towards mitochondria



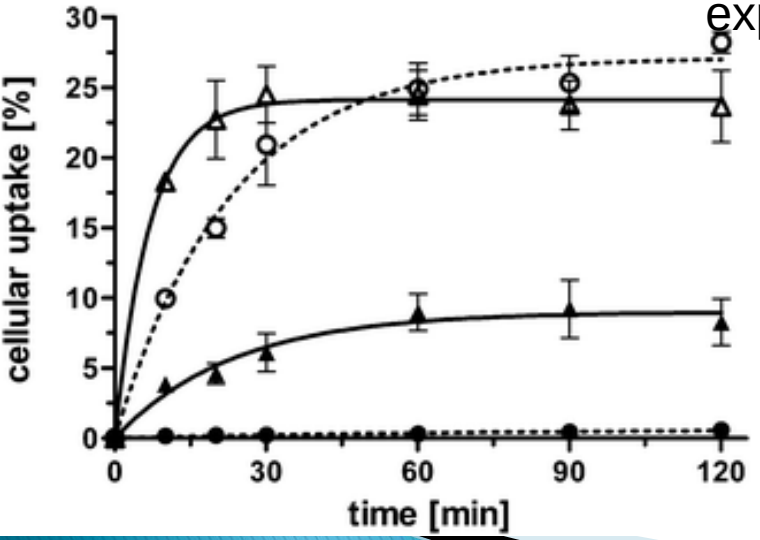
GRP-receptor expressing PC-3 cells

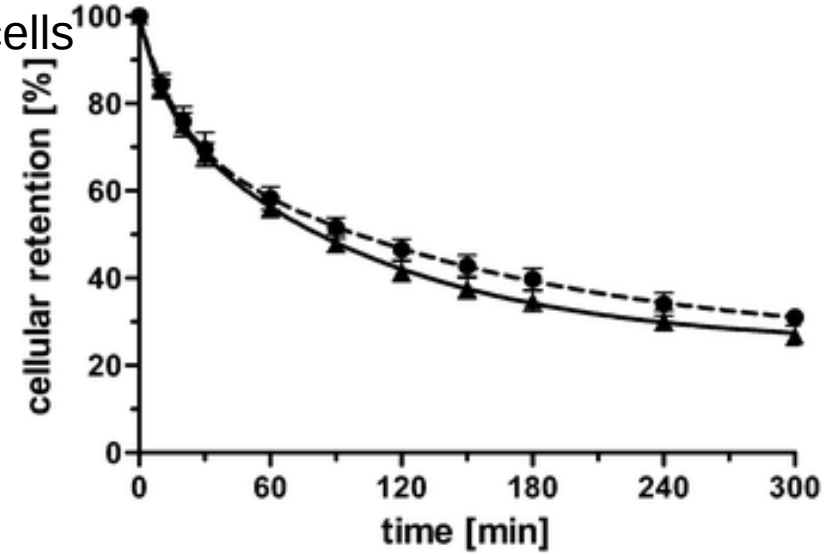
Radiopeptide–Receptor binding: Cellular internalization and retention

- The specificity of the first generation of dual-targeting conjugates towards its extracellular target was demonstrated
- But intracellular targeting could not be confirmed probably due to NSB or hindered passage through the membrane of the organelle.

Novel approach with potential to improve the efficacy of RP by enhancing the intracellular retention of radioactivity

GRP-receptor expressing PC-3 cells





http://pubs.rsc.org/en/content/articlelanding/2012/ob/c2ob26127h#!divAbstract

Radiolabeled Antibody Internalization

Radiolabeled Antibody internalization

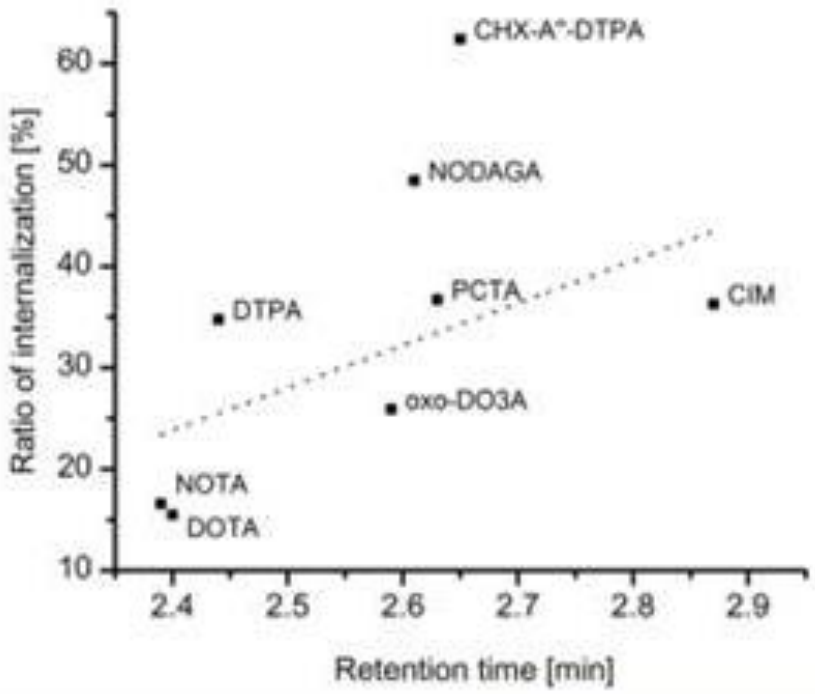
Antibodies specific for cell-surface receptors induce receptor-mediated endocytosis when they bind.

For the development of therapeutically potent anti-cancer antibody drugs, it is often important:

- to identify antibodies that internalize into cells efficiently,
- rather than just binding to antigens on the cell surface.
- Such antibodies mediate receptor endocytosis,
- resulting in receptor downregulation on the cell surface
- potentially inhibiting receptor function and tumor growth.

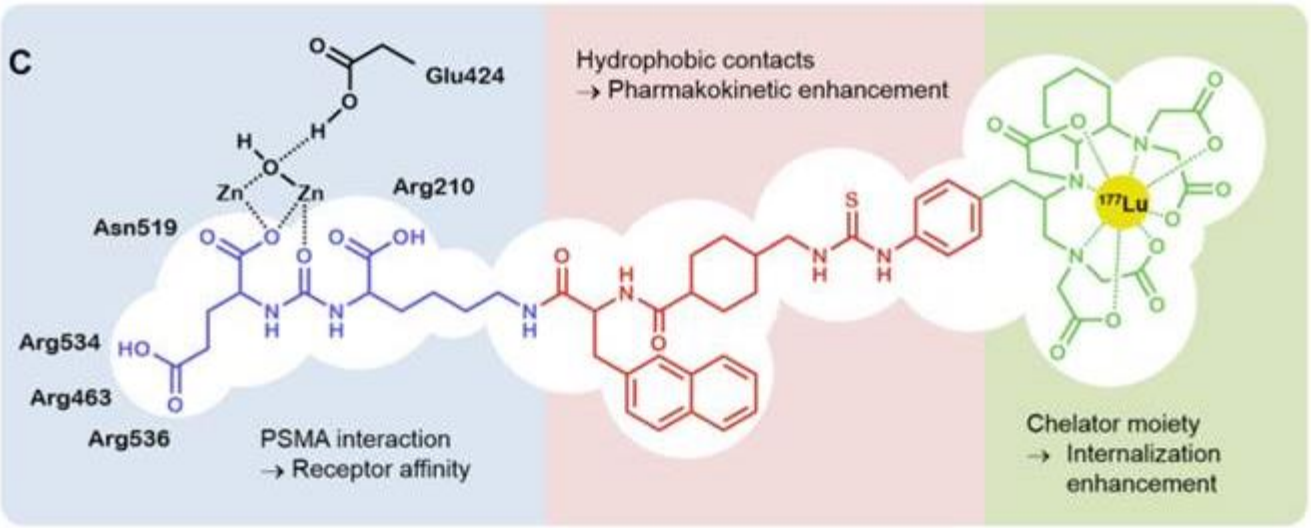
Efficient antibody internalization is a prerequisite for the delivery of cytotoxic drugs into target cells and is critical for the development of antibody–drug conjugates.

Radiolabeled Antibody Internalization



Comparison of internalization ratio and retention time

compound coupled to CHX-A''-DTPA performed best in both assays and may therefore be a promising new compound.

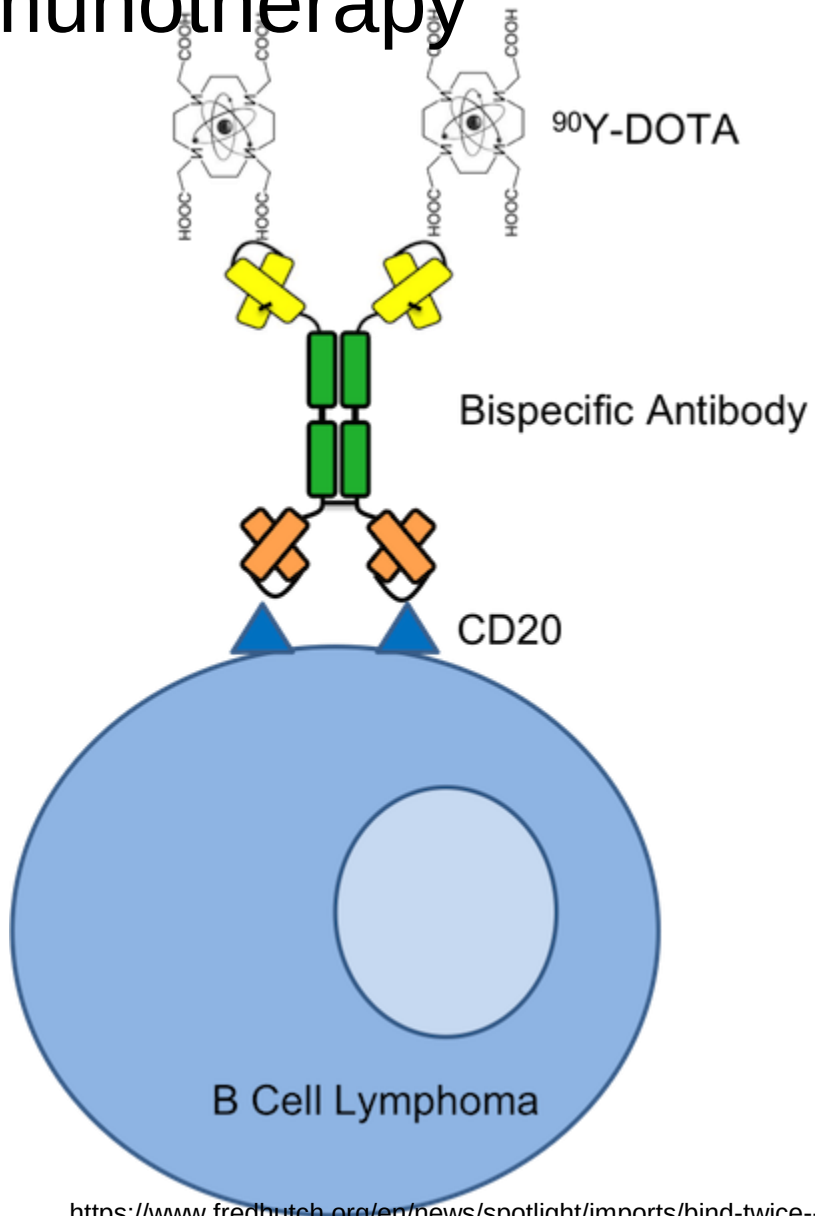


Suggested **model of action** of the CHX-A''-DTPA-coupled ligand at the PSMA receptor:

- urea-based binding motif and the linker region seem to play primarily a crucial **role** in targeting the tumor,
- the **chelating** entity may have an essential part by **enhancing internalization** of the compound.

Bind twice, kill once: bispecific antibody for radioimmunotherapy

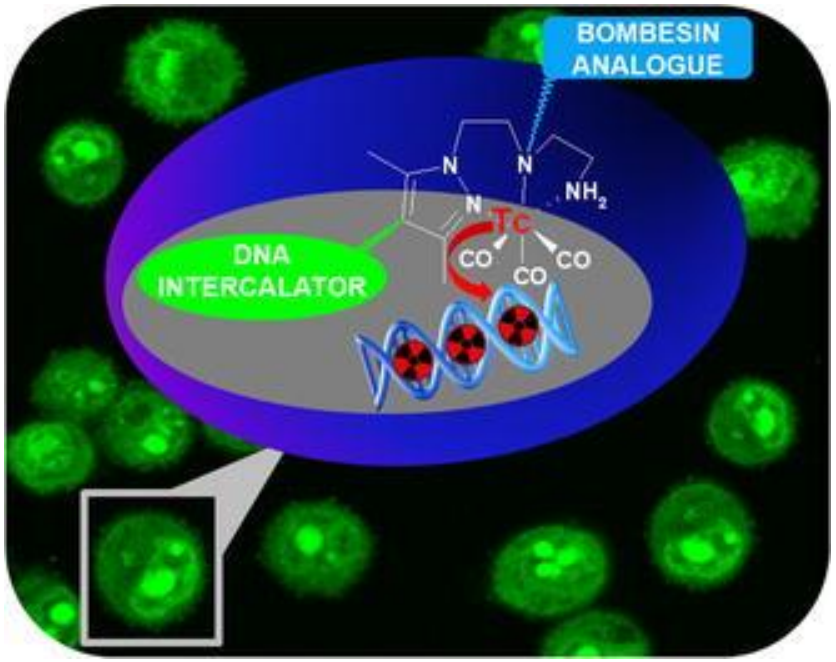
The newly engineered bispecific antibody specifically binds to CD20 antigen at the surface of the cancer B cell,
while also directly binding to the radiolabeled compound that will mediate cell death.
Figure provided by Dr. Damian Green



https://www.fredhutch.org/en/news/spotlight/imports/bind-twice--kill-once--bispecific-antibody-for-radioimmunotherap.html

Nuclear internalization

Nuclear targeting with cell-specific multifunctional tricarbonyl M(I) (M is Re, ^{99m}Tc) complexes: synthesis, characterization, and cell studies



intercalator and bombesin peptides internalize and target the nucleus of gastrin releasing peptide receptor positive PC3 human prostate tumor cells.

Mitochondria internalization and damage

Dual-targeting pro-apoptotic peptide to selectively target cancer cells and specifically damage mitochondria to lead the programmed cell death

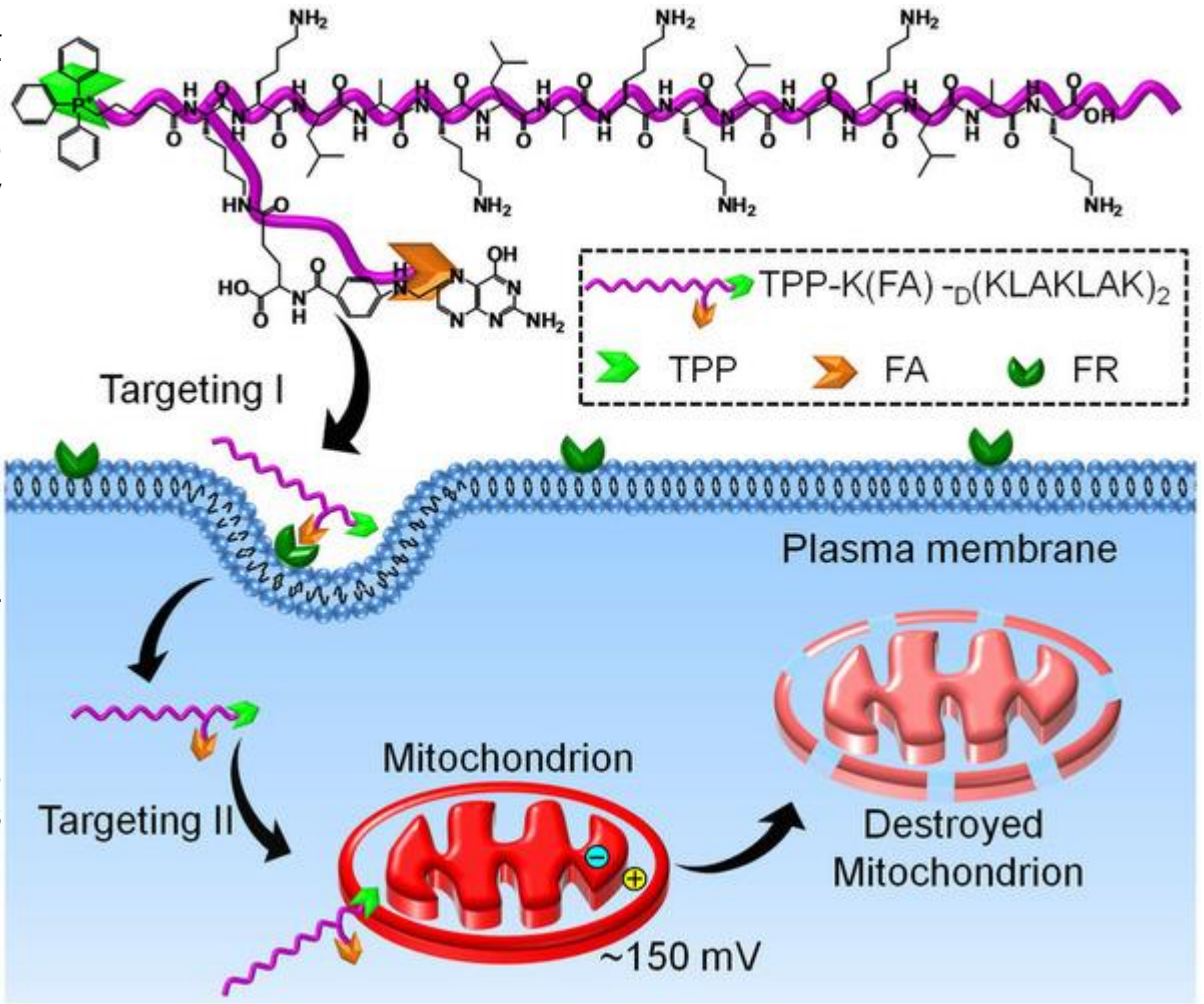
Mitochondria are vital organelles to eukaryotic cells. Damage to mitochondria will cause irreversible cell death or apoptosis.

Functionalized pro-apoptotic peptide demonstrates a dual-targeting capability using folic acid (FA) (targeting agent I) and triphenylphosphonium (TPP) cation (targeting agent II).

FA is a cancer-targeting agent, which can increase the **cellular uptake** of the pro-apoptotic peptide via receptor-mediated endocytosis.

TPP cation is the **mitochondrial** targeting agent, which specifically delivers the pro-apoptotic peptide to its particular subcellular mitochondria after internalized by cancer cells.

The pro-apoptotic peptide accumulates in mitochondria and causes its serious damage. This dual-targeting strategy has the potential to effectively transport the pro-apoptotic peptide to targeted cancer cell mitochondria, inducing mitochondrial dysfunction and triggering the mitochondria-dependent apoptosis to efficiently eliminate cancer cells.

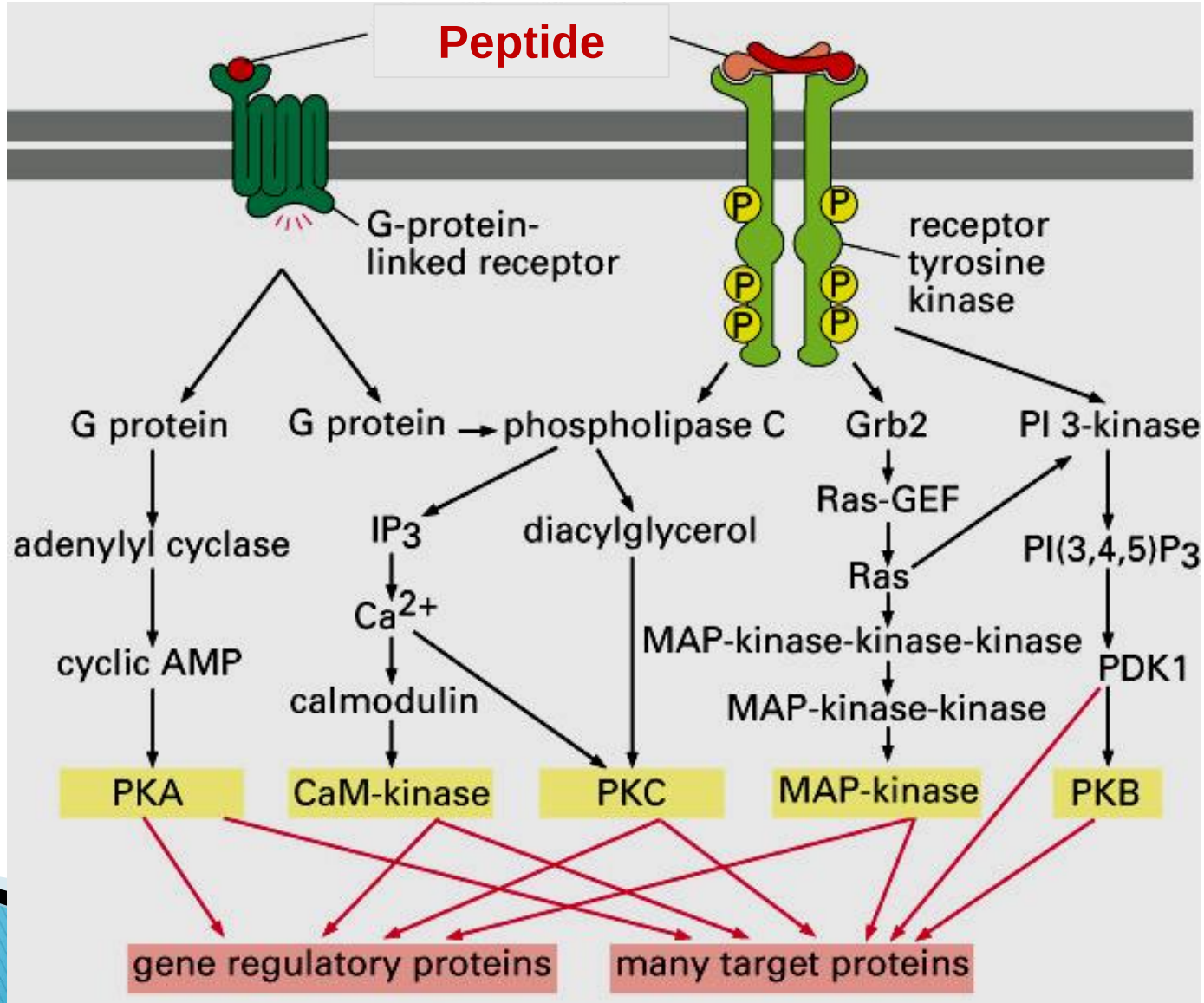


http://www.nature.com/srep/2013/131216/srep03468/pdf/srep03468.pdf

Peptide-receptor binding

- Efficacy and Potency

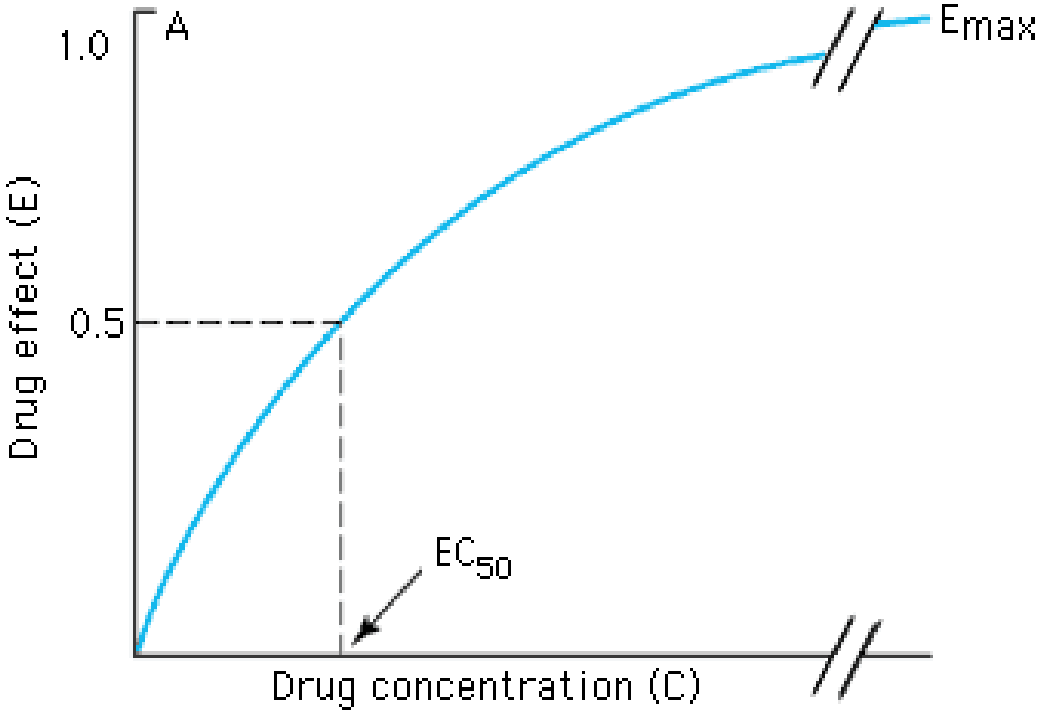
Peptide receptor binding: Efficacy, receptor activation



Graded dose-response curve

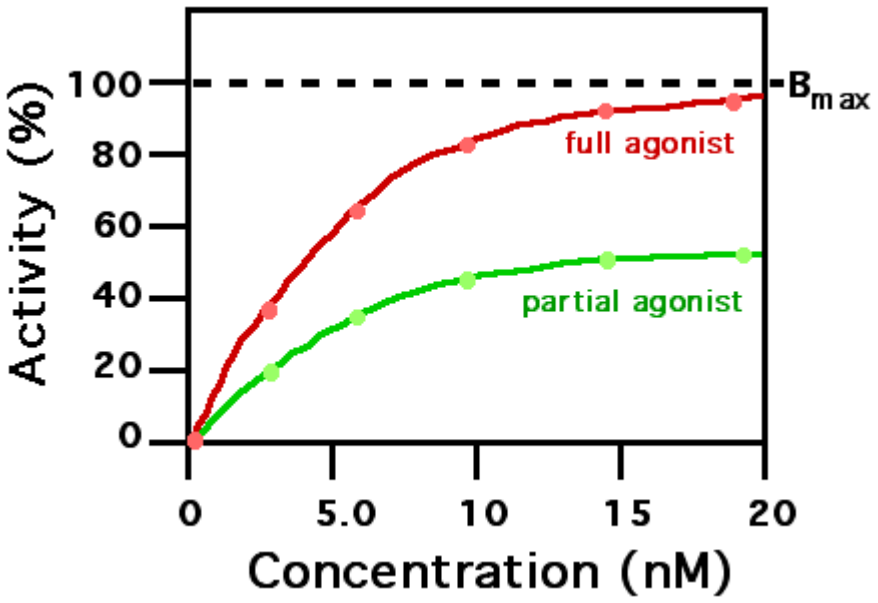
- Effector: molecules that translate the peptide receptor interaction into a change in cellular activity. Ex: ***adenylyl cyclase***
- When the response of a particular receptor-effector system is measured against increasing concentrations of a drug (peptide), the graph of the response versus the drug concentration or dose is called a graded dose-response curve.

- The efficacy (Emax) and potency (EC50) parameters are derived from these data.



Efficacy

- Efficacy (maximal efficacy): is the maximal effect (Emax) an agonist can produce if the dose is taken to very high levels.
- Efficacy is determined mainly by the nature of the receptor and its associated effector system.
- It can be measured with a graded dose-response curve.
- By definition, partial agonists have lower maximal efficacy than full agonists.



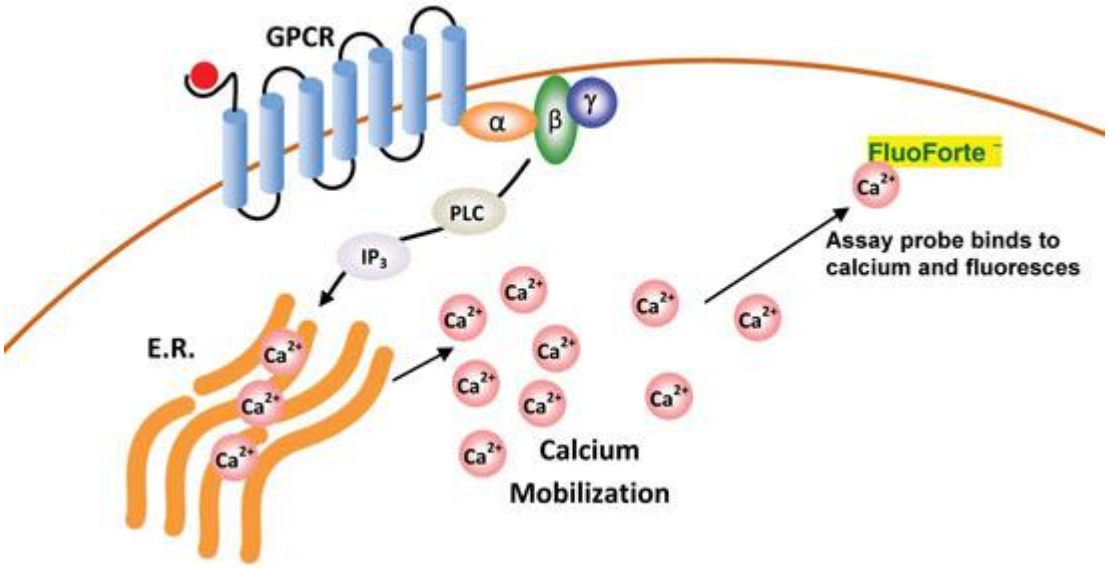
Potency

- Potency denotes the amount of an agonist needed to produce a given effect.
- In graded dose-response measurements, the effect usually chosen is 50% of the maximal effect (EC50).
- Potency is determined mainly by the affinity of the receptor for the agonist peptide.
- In quantal dose-response measurements ED50, TD50, and LD50 are typical potency variables (median effective, toxic, and lethal doses, respectively, in 50% of the population studied).
- Potency can be determined from either graded (EC50) or quantal dose-response curves (ED50, TD50, and LD50), but the numbers obtained are not identical.

Agonism /antagonist potency Calcium assays

Calcium-mobilization assay

FluoForte® Calcium assay kit for microplates



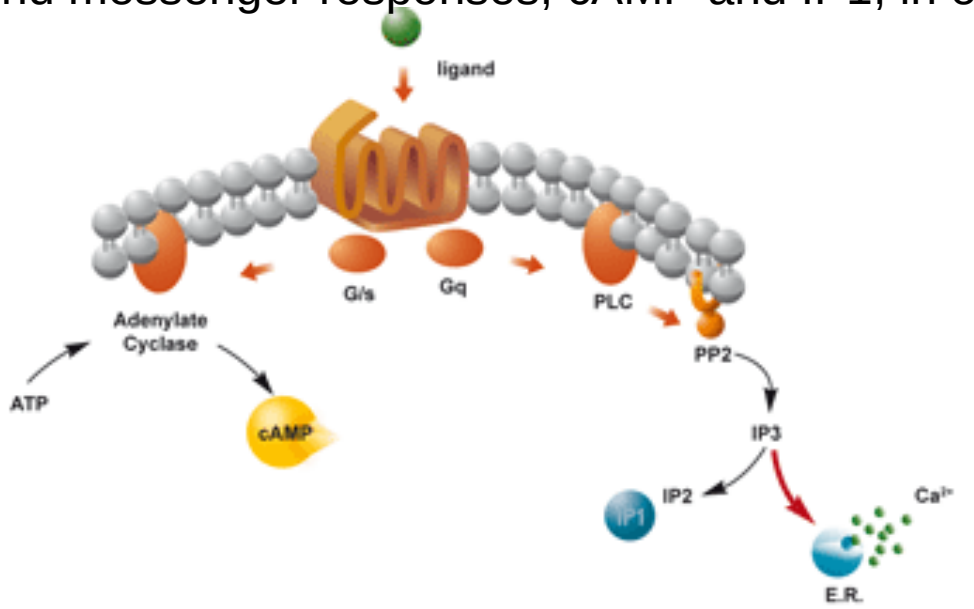
Measurement of intracellular calcium provides valuable information on the activation status of GPCRs and ion channels. Stimulated receptor releases intracellular calcium, causing the calcium-sensitive dye in the kit to fluoresce.

HitHunter® Calcium No WashPLUS Assay Platform

FLIPR Calcium 5 Assay Kit

Agonism /antagonist potency cAMP assays

The HTplex™ Assay from Cisbio measures GPCR activation via two second messenger responses, cAMP and IP1, in one experiment.



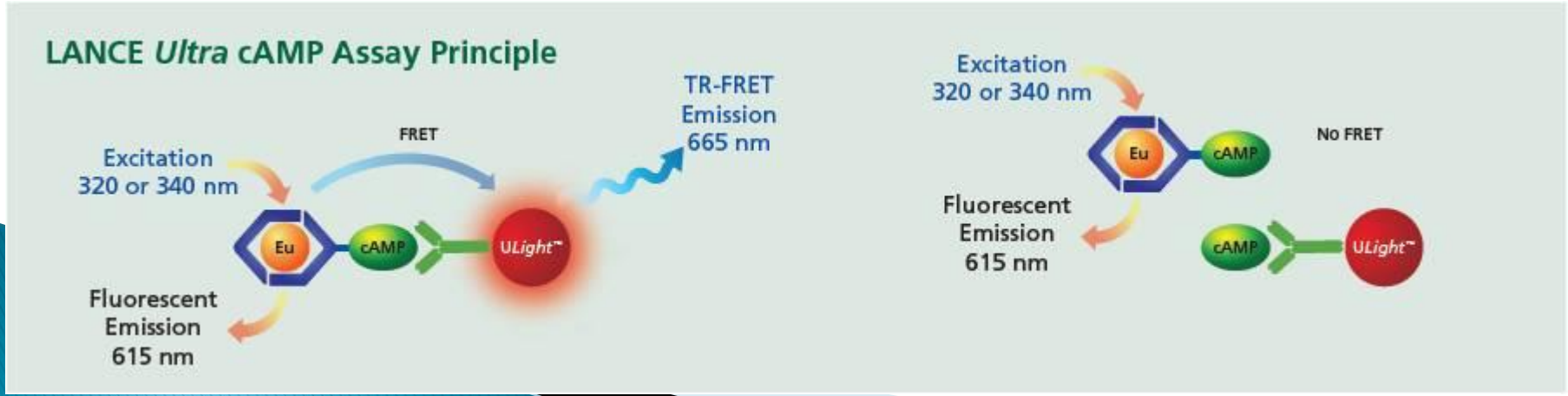


Table 2-1. G proteins and their receptors and effectors

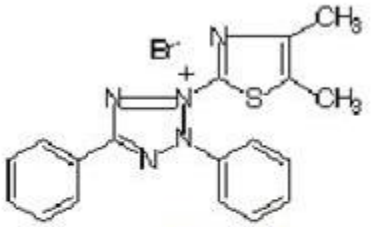
G Protein	Receptors for:	Effector/Signaling Pathway
G_s	β-Adrenergic amines, glucagon, histamine, serotonin, and many other hormones	↑ Adenylyl cyclase , ↑ cAMP
G_{il}, G_{i2}, G_{i3}	α_2-Adrenergic amines, acetylcholine (muscarinic), opioids, serotonin, and many others	Several, including: ↓ Adenylyl cyclase , ↓ cAMP Open cardiac K^+ channels , ↓ heart rate
G_{olf}	Odorants (olfactory epithelium)	↑ Adenylyl cyclase , ↑ cAMP
G_o	Neurotransmitters in brain (not yet specifically identified)	Not yet clear
G_q	Acetylcholine (eg, muscarinic), bombesin, serotonin ($5\text{-}HT_{1C}$), and many others	↑ Phospholipase C, ↑ IP_3, ↑ diacylglycerol, cytoplasmic Ca^{2+}
G_{t1}, G_{t2}	Photons (rhodopsin and color opsins in retinal rod and cone cells)	↑ cGMP phosphodiesterase (phototransduction)

(Radio)Cytotocicity

- MTT Assays

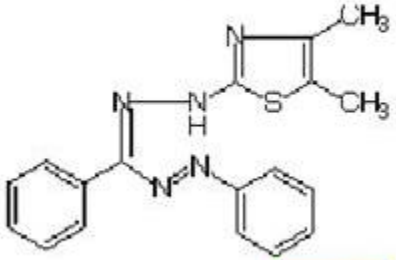
Evaluation of Cytotoxicity: MTT assay

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide



MTT **yellow**

Live cells → Mitochondrial reductase present → converts



MTT formazan **violet**

Absorbance read at 690 nm and subtract background at 570 nm.

http://en.wikipedia.org/wiki/File:MTT_Plate.jpg

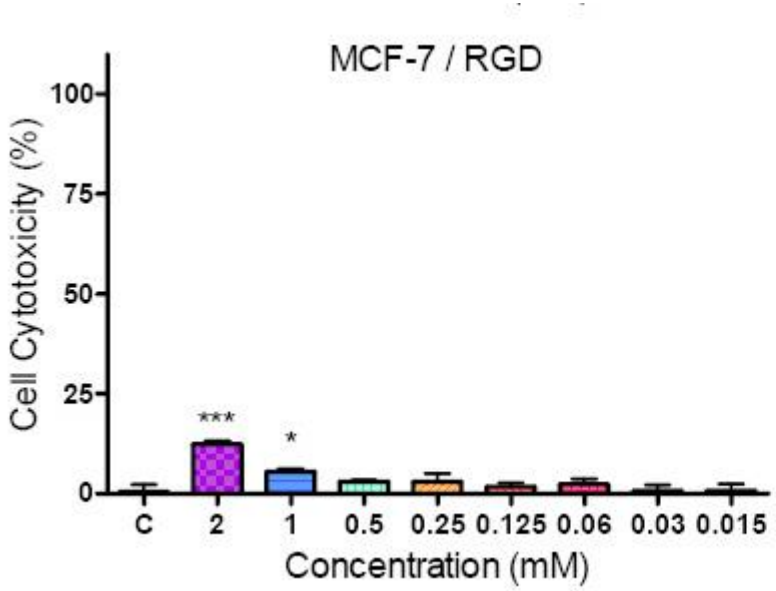
MTT test at different concentrations

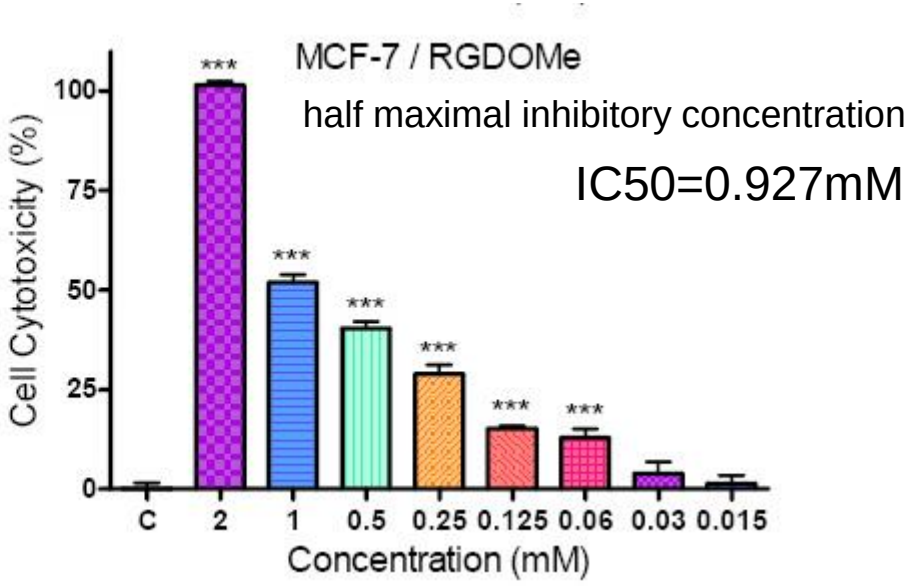
The (radio)cytotoxic dose can be determined

Evaluation of cell cytotoxicity (MTT)

- RGD-containing peptides: versatile applications (tumor imaging and therapy, drug delivery vector, targeted gene transfer, and biomaterial or tissue engineering)
- Significant progress has been made in the discovery and development of integrin $\alpha_v\alpha_3$-specific linear and cyclic RGD peptide analogs such as cilengitide and c(RGDfK) for cancer therapy, as well as targeted delivery of cancer imaging and therapeutic agents (Ex [99mTc]apticide used in imaging deep vein thrombosis) .
- Modifications of RGD peptides (polymerisation, coupling with carriers and substitution by peptidomimetics,) enhance the anti-tumour properties and lengthen the degradation time *in vivo* .
- RGD peptides-cytostatic agents conjugates exhibit an antitumour and antiangiogenic synergetic effect.
- RGD-cytotoxic drugs were developed and showed promising activities *in vitro* and *in vivo*

Effect of RGD and RGD-OMe on growth of MCF-7 cells after 24 h of treatment.





half maximal inhibitory concentration

IC50=0.927mM

The cell growth inhibitory effects of RGD-OMe are significantly higher than those of RGD. Evidently, the modification in the carboxylic group of RGD with simple esterification increases the cell growth inhibitory effects of the parent compound.

http://www.biodiscoveryjournal.co.uk/Article/Cytotoxic-effects-of-novel-RGD-analogues#.U2wH1vldVqU

Cell-based studies: Conclusions

IC50 studies , internalization,externalization (retention) studies

are absolutely necessary for

A very first characterization of the binding behavior of a new tracer;

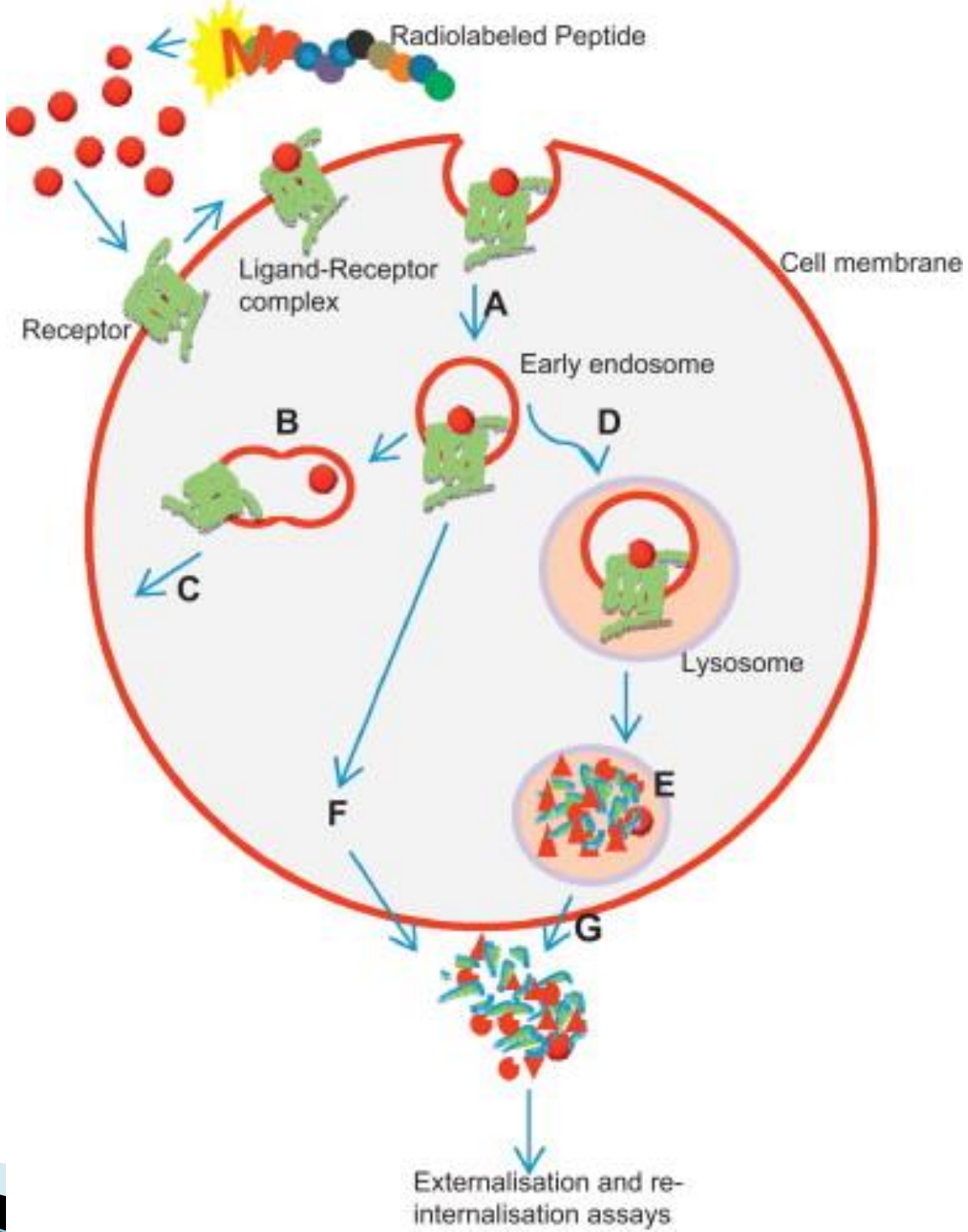
but

will never give a more that a very preliminary picture of the real in vivo situation:

- metabolism and formed species,
- specific uptake in other tissues,
- unspecific uptake,
- clearance kinetics,
- clearance route,
- retention in (excretion, but also other) organs, plasma protein binding,
- uptake by blood cells,
- penetration of the blood brain barrier...

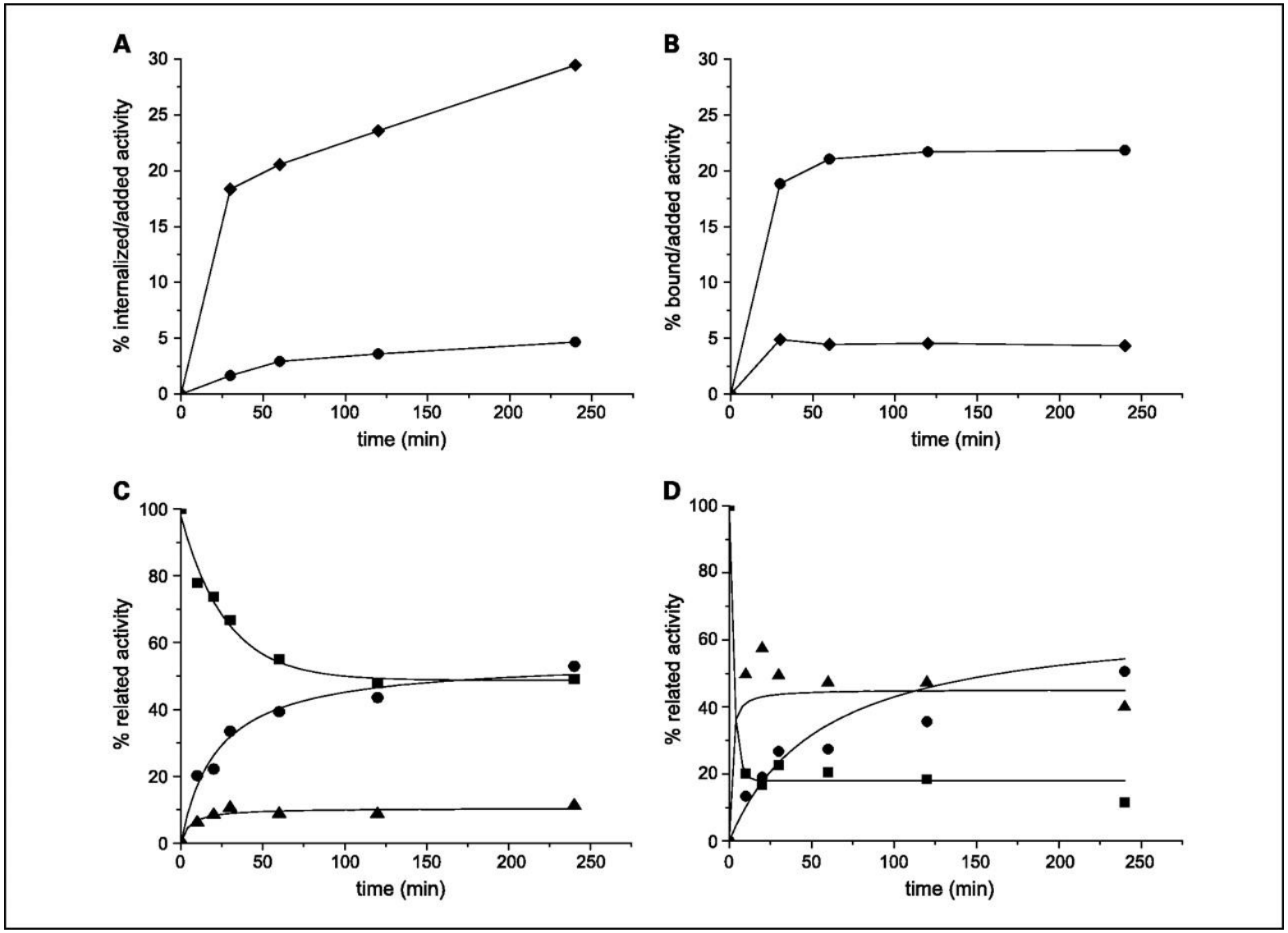
Thanks for attention

Questions?

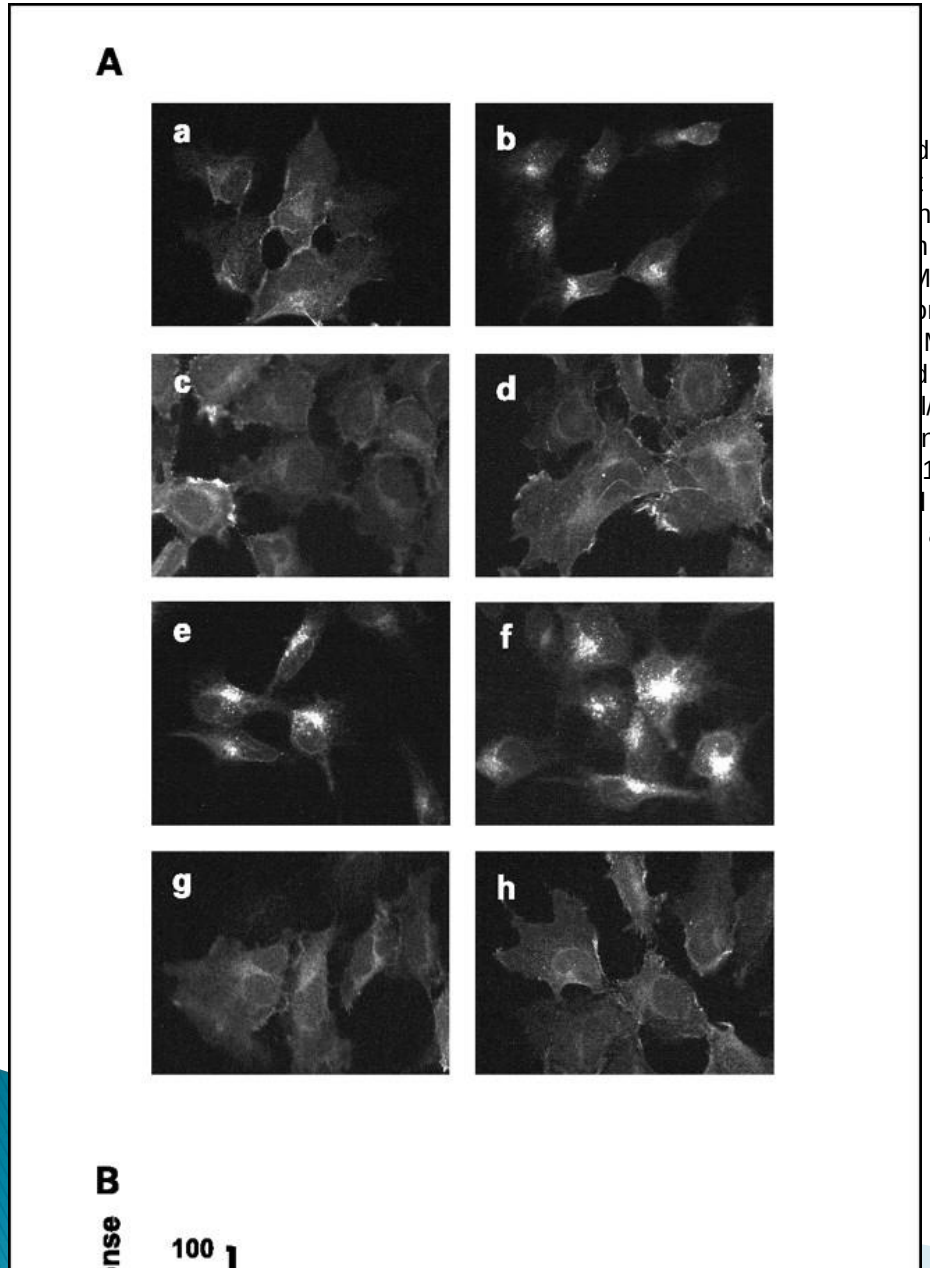

Radiolabeled Peptide
Ligand-Receptor complex
Receptor
Cell membrane
A
Early endosome
B
C
D
Lysosome
E
F
G
Externalisation and re-internalisation assays

Immunofluorescence microscopy

The agonist and antagonist properties of the bombesin analogues were confirmed by immunofluorescence-based internalization assay using HEK-GRPR cells. **Figure 3A** illustrates that 10 nmol/L bombesin can trigger internalization of the receptors. [^{nat}Lu]-AMBA at 1,000 nmol/L also induces internalization of GRPRs, whereas [^{nat}In]-RM1 and RM26 were not able to stimulate GRPR internalization. However, when given at a concentration of 1,000 nmol/L together with 10 nmol/L bombesin, both peptides are able to prevent bombesin-induced receptor internalization.



Internalization of [111In]-RM1 (•) significantly lower compared with [111In]-AMBA (⧫) in PC-3 cells (A). Conversely, higher percent of [111In]-RM1 (•) remained bound to the cell membrane of PC-3 cells in comparison with [111In]-AMBA (⧫; B). The fate of the GRPR-bound [111In]-RM1 (C) and [111In]-AMBA (D). At the specified times, the amount of radioactivity present as free (•), surface-bound (▪), and internalized ( ) ligand.



duced by bombesin is efficiently antagonized by the bombesin analogues RM2
[natLu]-AMBA. HEK-GRPR cells were treated for 30 min either with vehicle (n
(b), a concentration inducing a submaximal internalization effect. d, f, and h,
the presence of 1 μmol/L of the analogues RM26 (d), [natLu]-AMBA (f), and [
M26 (c), [natLu]-AMBA (e), and [natIn]-RM1 (g) when given alone at a concentr
on with the peptides, the cells were processed for immunofluorescence microsc
Methods. B, dose-response curves of bombesin analogues determined by the
d in Materials and Methods. PC-3 cells were treated either with bombesin at co
l/L and 10 μmol/L (•) alone, or with bombesin at concentrations ranging betwee
nce of 10 μmol/L of the analogues RM1 (▲), or [natIn]-RM1 (⧫), or the bombes
1, and RM26 behave like antagonists shifting the dose-response curve of bom
l alone at 1 and 10 μmol/L RM1 (▵), [natIn]-RM1 (×) and RM26 (□) have no eff
are expressed as percentage of maximum calcium response induced by ionor

Approaches to improve metabolic stability of a statine-based GRP receptor antagonist

February 2017Volume 45, Pages 22–29

Abstract

The bombesin receptor family, in particular the gastrin-releasing peptide receptor (GRPr), is an attractive target in the field of nuclear oncology due to the high density of these receptors on the cell surface of several human tumors. The successful clinical implementation of ^{64}Cu-CB-TE2A-AR06, ^{68}Ga-RM2 and ^{68}Ga-NODAGA-MJ9, prompted us to continue the development of GRPr-antagonists. The aim of the present study was to assess if N-terminal modulations of the statine-based GRPr-antagonist influence the binding affinity, the pharmacokinetic performance and the *in vivo* metabolic stability.

Methods

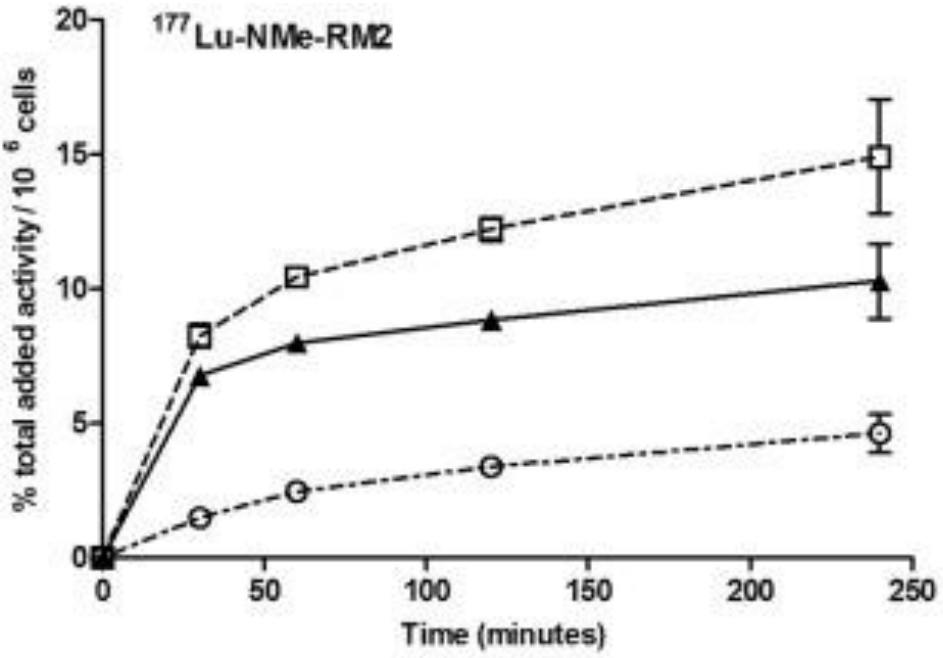
The GRPr-antagonist (D-Phe-Gln-Trp-Ala-Val-Gly-His-Sta-Leu-NH_2) was functionalized with the chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) *via* the spacer 4-amino-1-carboxymethyl-piperidine (Pip) and the amino acid N-Methyl-β-Ala, to obtain NMe-RM2 and labeled with ^{68}Ga and ^{177}Lu. The GRPr affinity of the corresponding metalloconjugates determined using [^{125}I–Tyr^4]-BN as radioligand. *In vitro*evaluation included internalization studies using PC3 cells. The ^{68}Ga-conjugate was evaluated in PC3 xenografts by biodistribution and PET studies, while investigations on the metabolic stability and plasma protein binding were performed.

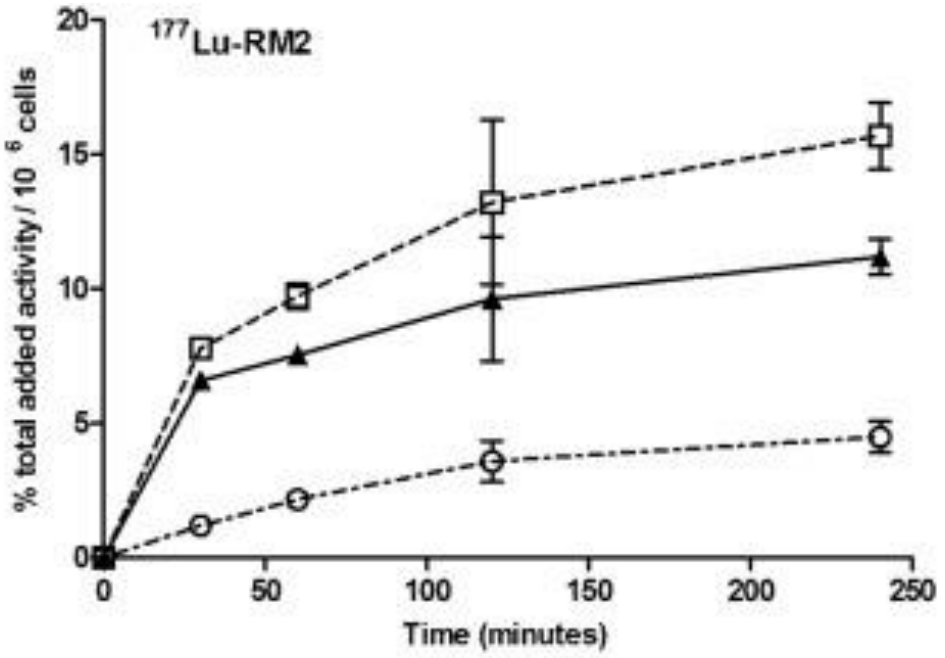
Results

The half maximum inhibitory concentrations (IC_{50}) of the metalloconjugates, using [^{125}I–Tyr^4]-BN, are in the low nanomolar range. PC3-cell culture binding studies of both metallated NMe-RM2 and RM2 show high GRPr-bound activity and low internalization. Metabolic studies showed that ^{68}Ga-NMe-RM2 and ^{68}Ga-RM2 are being cleaved in a similar fashion into three metabolites, with a good proportion of about 50% of the remaining blood activity at 15 min post injection (p.i.) being represented by the intact radiotracer. ^{68}Ga-NMe-RM2 was shown to target specifically PC3 xenografts, with high and sustained tumor uptake of about 13% IA/g within a time frame of 3 h. The PET images clearly visualized the tumor.

Conclusions

The relatively high percentage of the remaining intact radiotracer in blood 15 min post injection sufficiently enables *in vivo* targeting of GRPr positive tumors, finding which has been also shown in clinical trials.





specific cell-associated

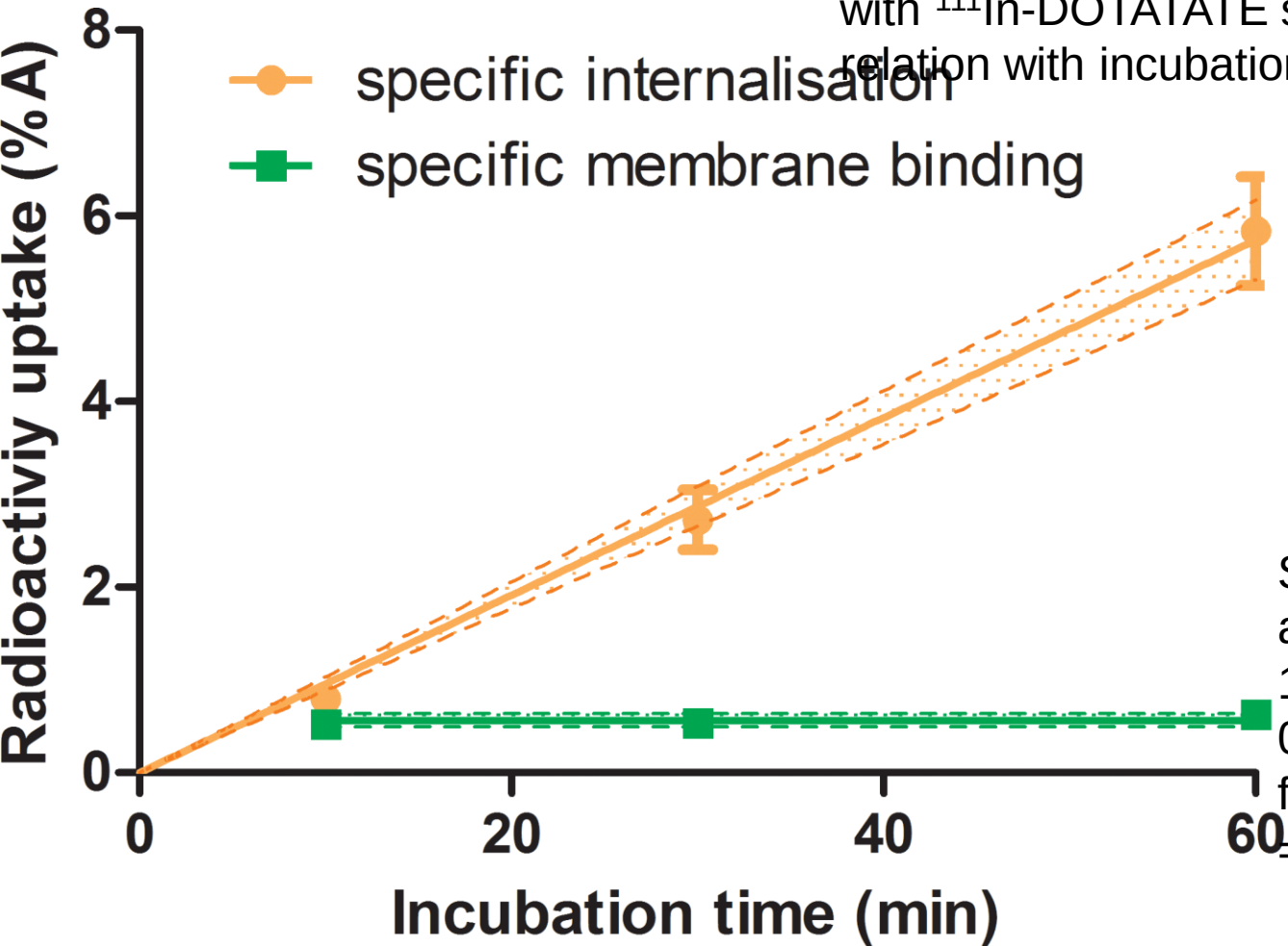
specific surface-bound

specific internalized

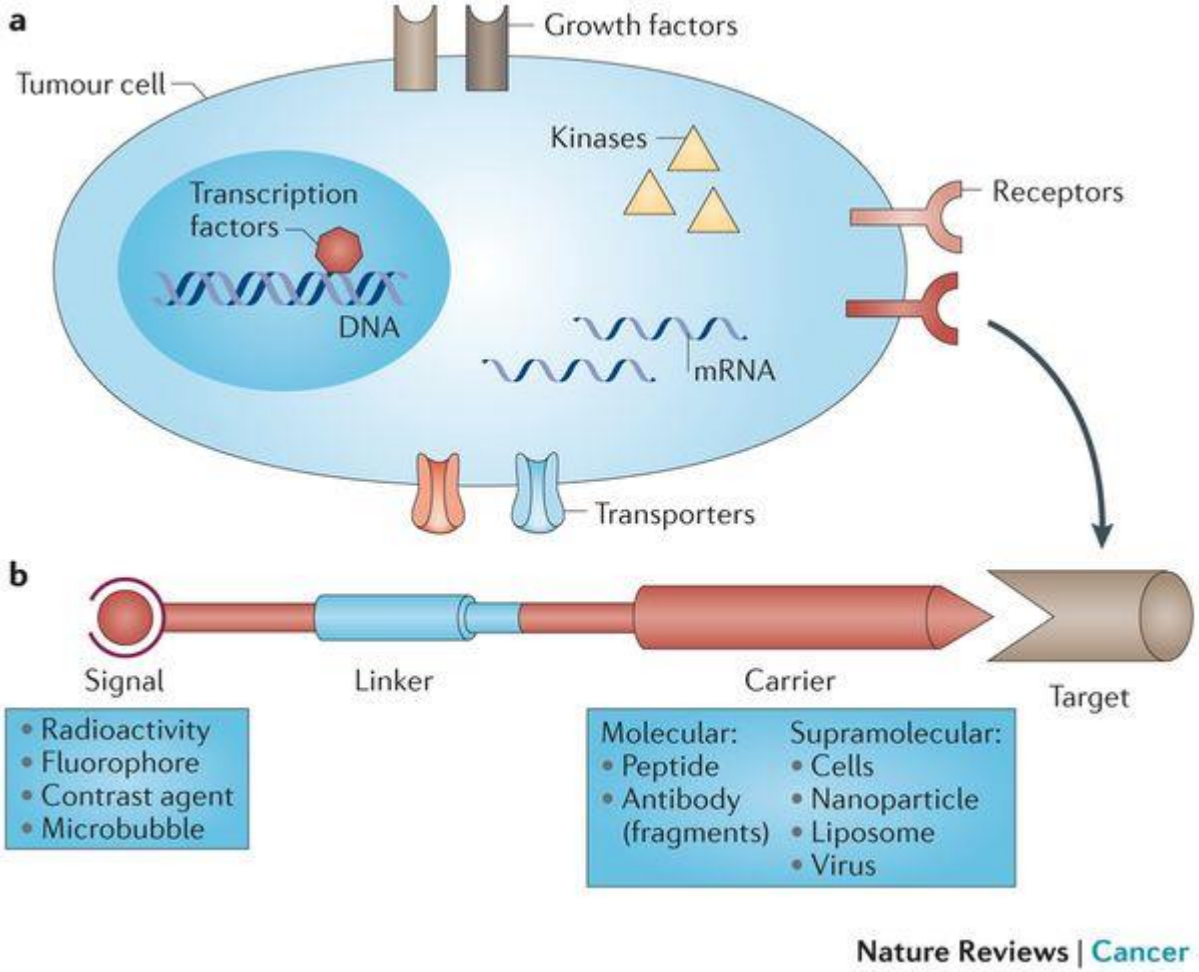
Internalization in $SSTR_2$ positive CA20948 cell line

Internalization was performed to determine the amount of radioligand bound to the receptors, at the cell membrane and internalized into the cell. Optimized concentration of ^{111}In-DOTATATE for internalization on CA20948 was 1 nM. At this concentration, the f_{int} increased linearly as a function of incubation time. However, the f_{mem} remained constant at a level of 0.008%A as a function of incubation time, see Fig 2.

Cellular internalization determined with ^{111}In-DOTATATE showed a linear relation with incubation time.



Specific membrane binding and specific internalization o 1 nM 111In-DOTATATE on 0.5x106 cells of CA20948 as function of incubation time, n = 3.


a
Growth factors
Tumour cell
Kinases
Transcription factors
Receptors
DNA
mRNA
Transporters
b
Signal
Linker
Carrier
Target
• Radioactivity
• Fluorophore
• Contrast agent
• Microbubble
Molecular:
• Peptide
• Antibody (fragments)
Supramolecular:
• Cells
• Nanoparticle
• Liposome
• Virus
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