Preclinical evaluation of radiopharmaceuticals

PRISMAP Young Scientist Event

INFN, Padova, Italy

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Filipa Mendes

C²TN - Center for Nuclear Sciences and Technologies

Instituto Superior Técnico, University of Lisboa, Portugal

Outline

Basic aspects of preclinical evaluation of diagnostic and therapeutic radiopharmaceuticals:

- Overview of the several steps of in vitro and in vivo evaluation strategies
- Cellular and animal models and techniques appropriate to study (targeted) radiopharmaceuticals

IAEA RADIOISOTOPES AND RADIOPHARMACEUTICALS SERIES

No.

GUIDANCE FOR PRECLINICAL STUDIES WITH RADIOPHARMACEUTICALS

To provide the general principles and baseline preclinical study protocols to characterize safety, efficacy and quality of intended research radiopharmaceuticals under development.

IAEA 2021
Draft

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Korde *et al.*
EJNMMI Radiopharmacy and Chemistry (2022) 7:18
https://doi.org/10.1186/s41181-022-00168-x

EJNMMI Radiopharmacy and Chemistry

REVIEW Open Access

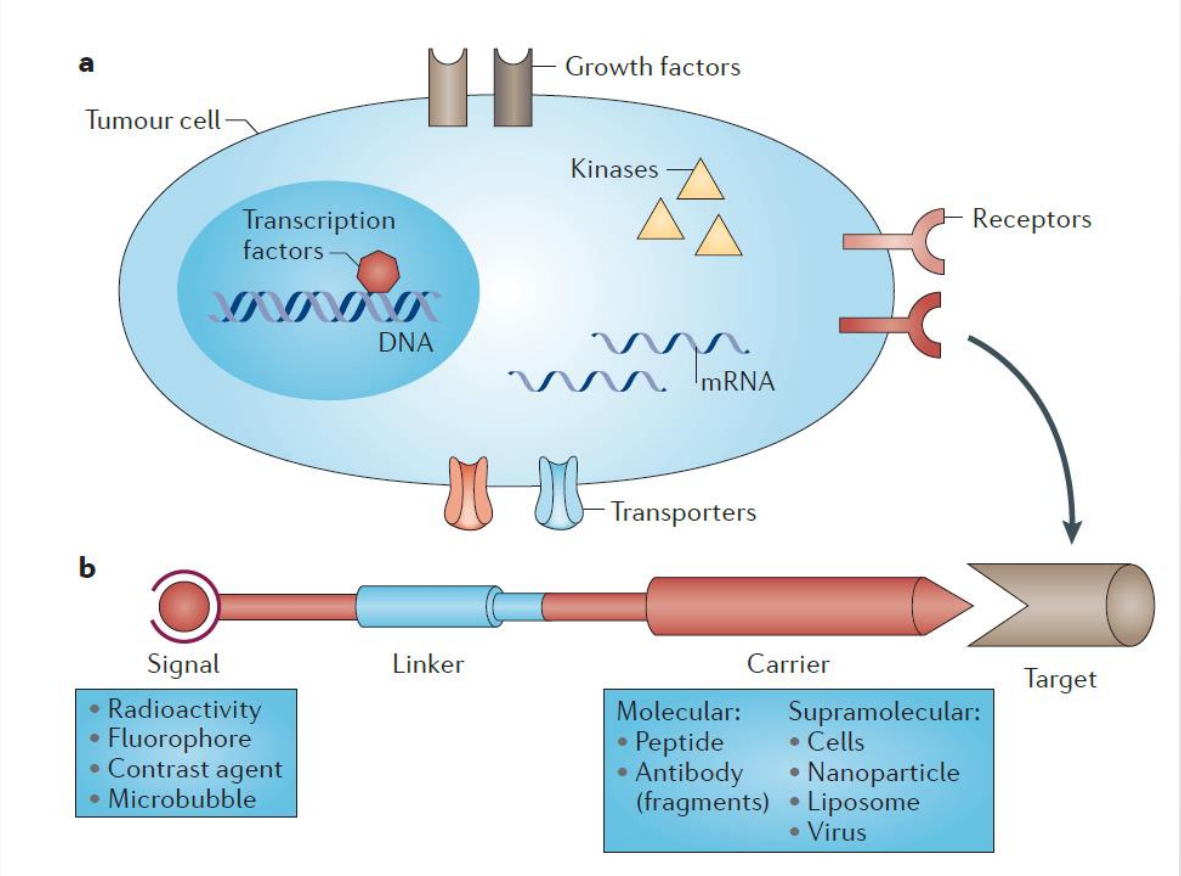
Practical considerations for navigating the regulatory landscape of non-clinical studies for clinical translation of radiopharmaceuticals

Aruna Korde[1], Renata Mikolajczak[2], Petra Kolenc[3,4], Penelope Bouziotis[5], Hadis Westin[6], Mette Lauritzen[7], Michel Koole[8], Matthias Manfred Herth[9,10], Manuel Bardiès[11], Andre F. Martins[12,13], Antonio Paulo[14], Serge K. Lyashchenko[15], Sergio Todde[16], Sangram Nag[17], Efthimis Lamprou[18], Antero Abrunhosa[19], Francesco Giammarile[1] and Clemens Decristoforo[20*]

Molecular targets in (tumour) cells

Changing times in medicine - Paradigm shift in disease detection and treatment from a focus on morphology, function, and pathophysiology to **genetic and molecular events**

In this way, molecular nuclear medicine will play an increasing role in disease definition, preventive medicine, and treatment planning and monitoring – Personalized medicine



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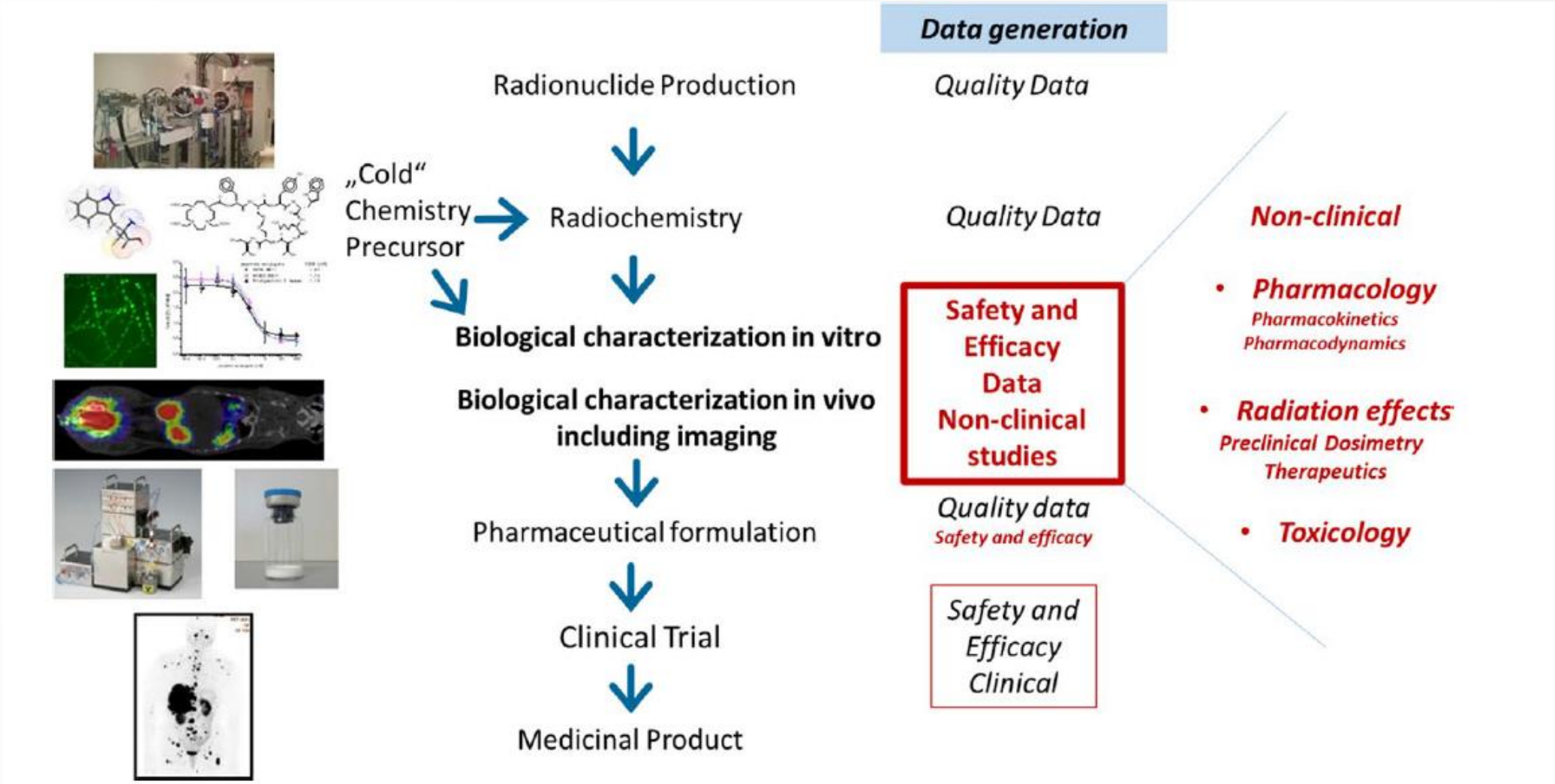
NatRevCancer, 2014, 14:481

Path to clinical translation of radiopharmaceuticals



EJNMMI Radiopharmacy and Chemistry, 2022, 7:18

Before Clinical translation

Before any radiopharmaceutical is cleared for use in humans, it must undergo rigorous testing:

To provide in-depth characterization of its behaviour, both physico-chemical and biological
To assess its safety and suitability for the intended clinical application

Studies include:

- Stability and affinity measurements, or determination of the radiopharmaceutical's target engagement
- Drug´s biodistribution profile, identification of metabolic pathways and metabolites
- Estimation of radiation doses
- Additionally, even though most radiopharmaceuticals are applied only in tracer doses in terms of drug content, which usually cause no or only very limited pharmacological effect, currently toxicology testing is required for all radiopharmaceuticals

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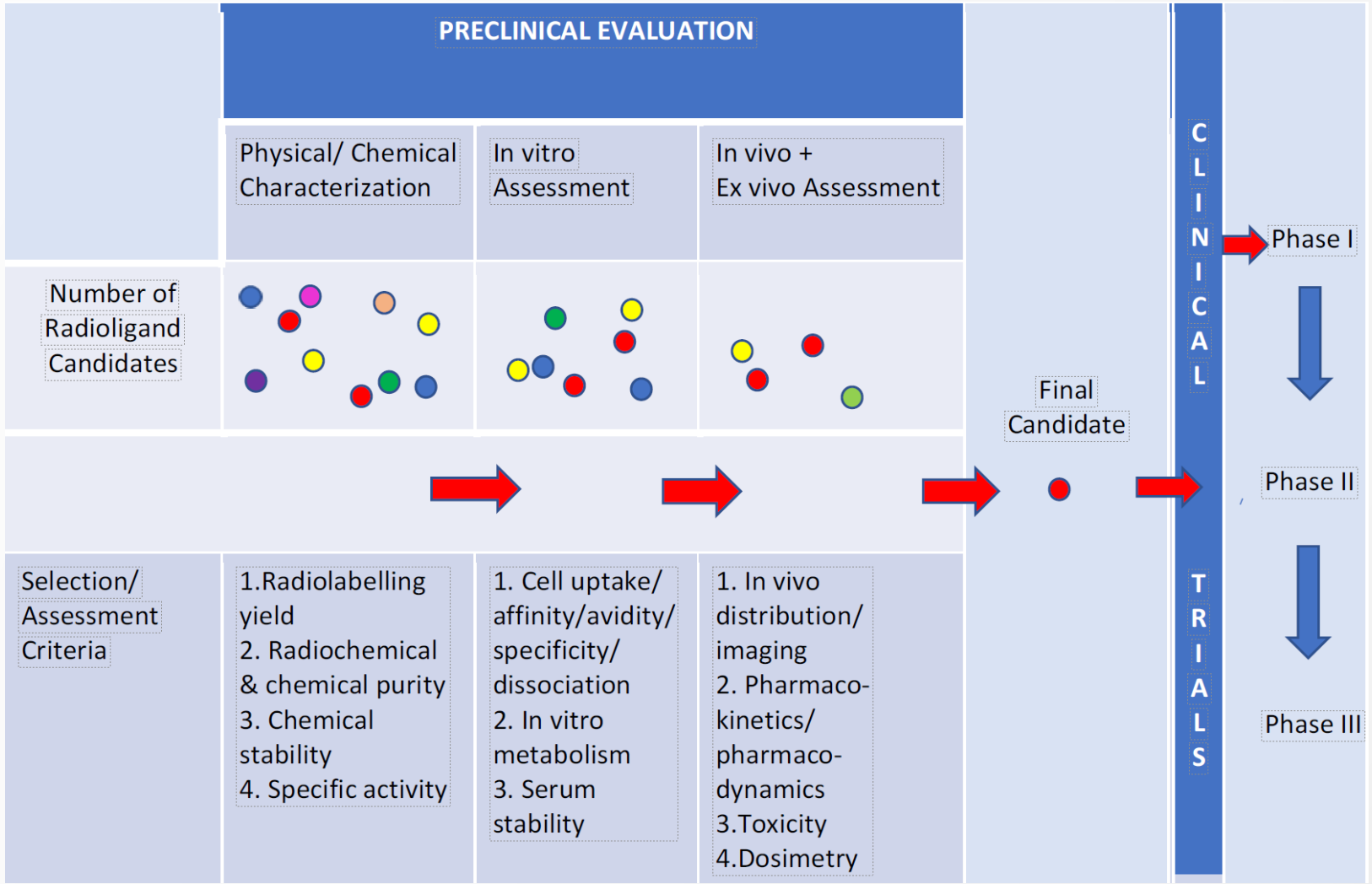
Overview of Preclinical Radiopharmaceutical Development



Important - In **therapeutic radiopharmaceuticals** the **biodistribution** and **dosimetry** play a much more significant role to assess **radiation induced toxicity** and at the same time give information on **efficacy**

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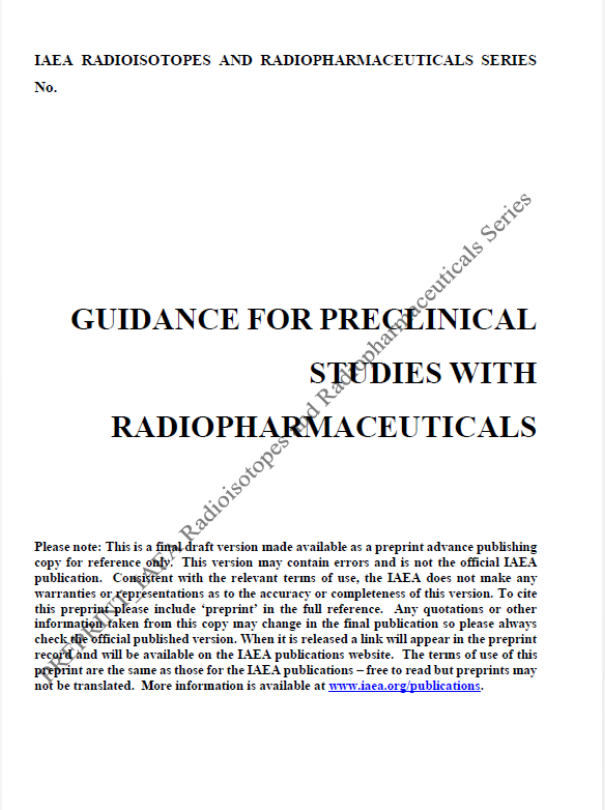
Radiopharmaceuticals: Preclinical Research and Development Phase

- Target identification and validation
- Selection of targeting molecule
- Chemical synthesis of targeting molecule
- Radiolabeling and characterization
- (Radio)Chemical and metabolic stability

- *In vitro* cell binding and cellular internalization
- *In vivo* biodistribution and tumor targeting ability
- *In vitro* Imaging and/or therapeutic effects
- Toxicology
- Dosimetry



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Data Reporting and Management /Facilities Requirement /Safety Accreditation/ Quality Assurance and Quality Control / Protocols

Radiopharmaceuticals

Radionuclide (type of emission, energy, half-life) determine its potential diagnostic, theranostic or therapeutic application,

Carrier molecule - chemical and/or biological behaviour govern its affinity, selectivity and broadly its pharmacological profile

Radiopharmaceuticals (RPs) - should accumulate specifically within the target region and possess low binding to any other tissue

The design of any radiopharmaceutical must ensure:

– Rapid uptake and sufficient period of retention in the region of interest;
– Minimal uptake or quick washout from non-target regions;
– Minimal non-selective retention in the target region;
– Adequate *in vitro* and *in vivo* stability, with minimal presence of redistributing radio metabolites;
– Minimal unintended toxic or pharmacologic effects;
– Viable economics and logistics for preparation and administration.

Peptide Receptor Radionuclide Therapy (PRRT) and imaging

Regulatory peptide receptors are overexpressed in numerous human cancers. These receptors have been used as **molecular targets** by which radiolabeled peptides can localize cancers in vivo and to treat cancers with peptide receptor radiation therapy

Neuroendocrine tumors - Somatostatin receptor- 2017/18
^{177}Lu- dotatate Lutathera (+ diagnostic partner ^{68}Ga-dotatate)

Metastatic castration-resistant prostate cancer - PSMA targeted with peptidomimetic PSMA617
^{177}Lu-PSMA 617 Pluvicto (+ diagnostic partner ^{68}GaPSMA 11) 2022

In pre-clinical or clinical trials
Other peptide receptors, such as cholecystokinin-2 (CCK2) gastrin-releasing peptide (GRPR), neurotensin-, substance P-, glucagon-like peptide 1-, neuropeptide Y-, or corticotropin-releasing factor-receptors

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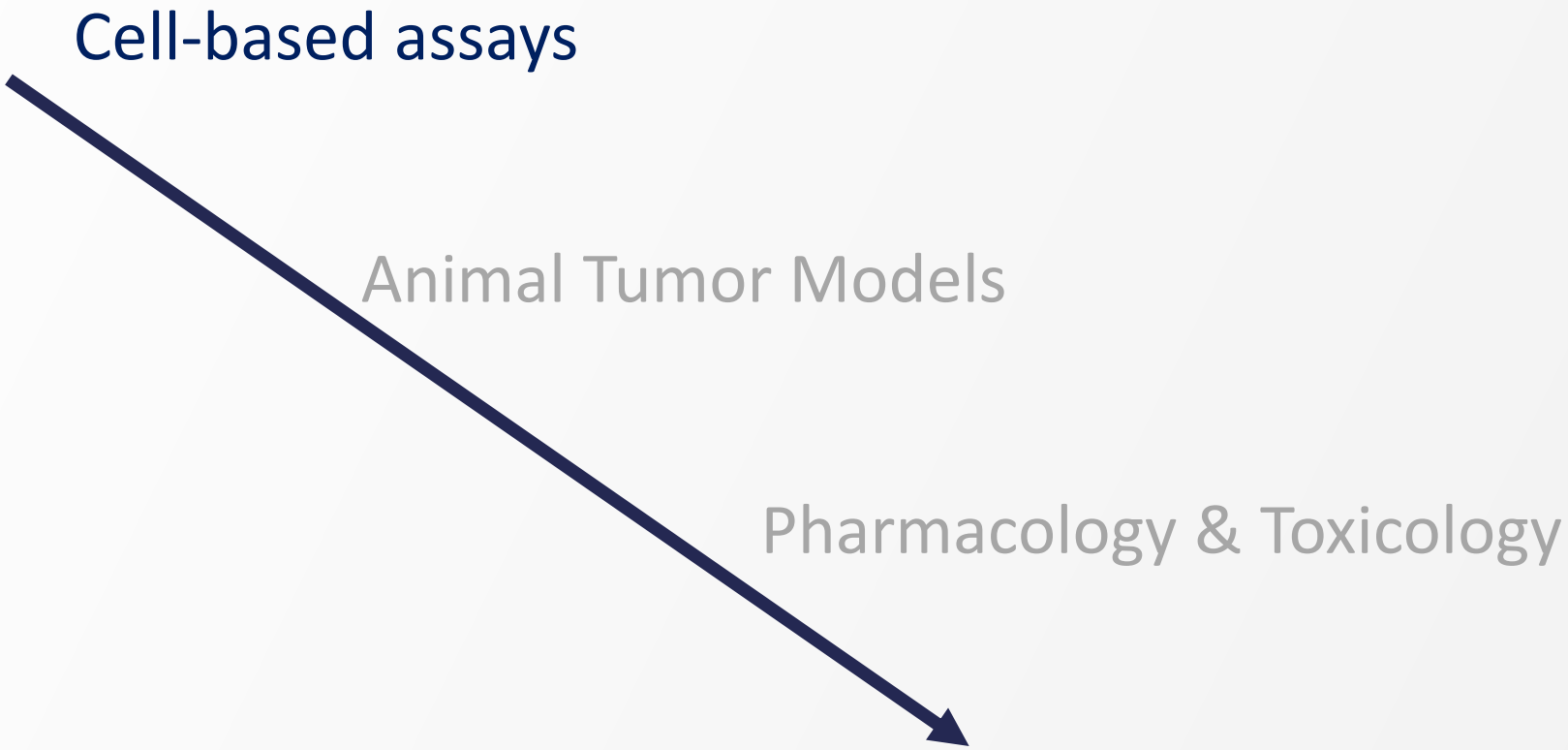
Overview of Preclinical (Radio)pharmaceutical Development



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In vitro preclinical evaluation of radiopharmaceuticals

- Studies conducted in a controlled environment outside of a living organism
- Cultured cells or fractionated cell extracts (isolated nuclei, membranes etc.), plasma, or tissue samples (whole or dispersed/homogenized) derived from humans or animals
- The main purpose is to eliminate or reduce the need for animal testing by allowing selection of lead compounds using specific tests.

Important: Selection of adequate cell lines and tissues (e.g. transfected cell lines are not representative; always use negative control cell lines. Animal tissues have limited validity)

In vitro preclinical evaluation of radiopharmaceuticals

Examples:

- Binding studies affinity and specificity
- Cell uptake and efflux studies
- Internalization and subcellular distribution studies
- Blocking studies specificity and saturability
- Efficacy / functional assays functional - test whether tracer launches or blocks a certain biochemical pathway after binding
  efficacy – ability to exert therapeutic action on the target expressing cells
- Efflux pump assays and blood brain barrier permeability tests
- In vitro autoradiography (to assess affinity and binding selectivity to the target)
- Metabolite analysis (in organ, mainly the liver or cell or tissues homogenates)
- Stability during storage
- Serum stability

Cell-Based Assays

Cell culture

Tissue from an explant is dispersed, 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, …

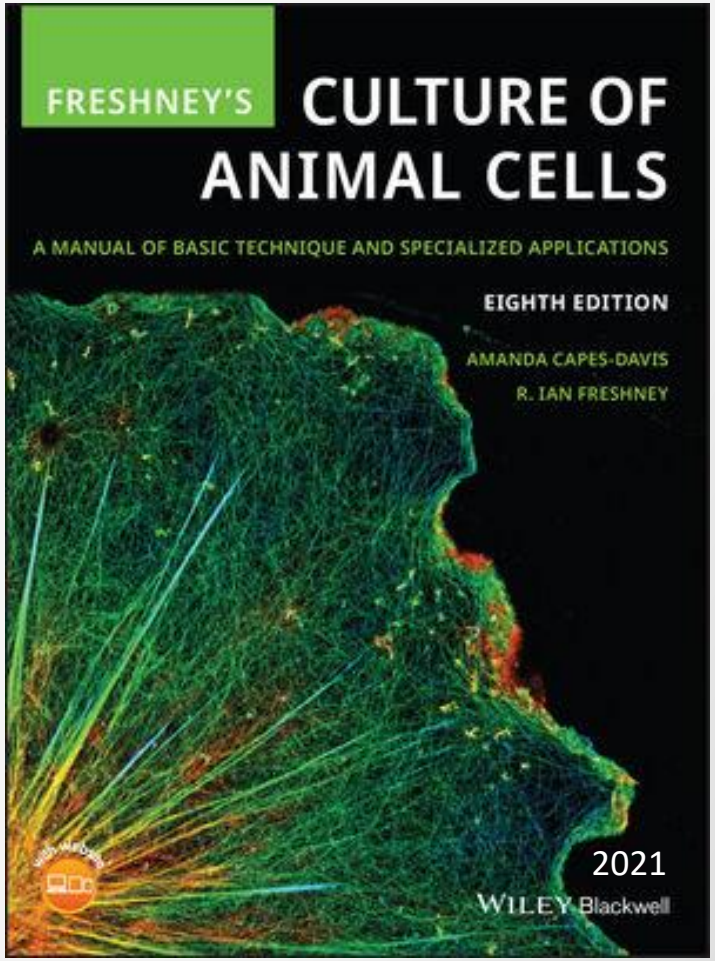
Cell Lines - Classification

Primary Cultures

- derived directly from excised, normal animal tissue
- cultures either as an explant culture or following dissociation into a single cell suspension by enzyme digestion
- labour intensive, maintained in vitro only for a limited period of time
- relatively limited lifespan primary cells - usually retain many of the differentiated characteristics of the cell in vivo

Continuous Cultures

- single cell type that can be serially propagated in culture indefinitely
- tumour cell lines are often derived from clinical tumours
- transformation may also be induced using viral oncogenes, chemical treatments
- immortalization with hTERT – without cancer-associated changes or altering phenotypic properties
- almost limitless availability, but the retained little of the original in vivo characteristics



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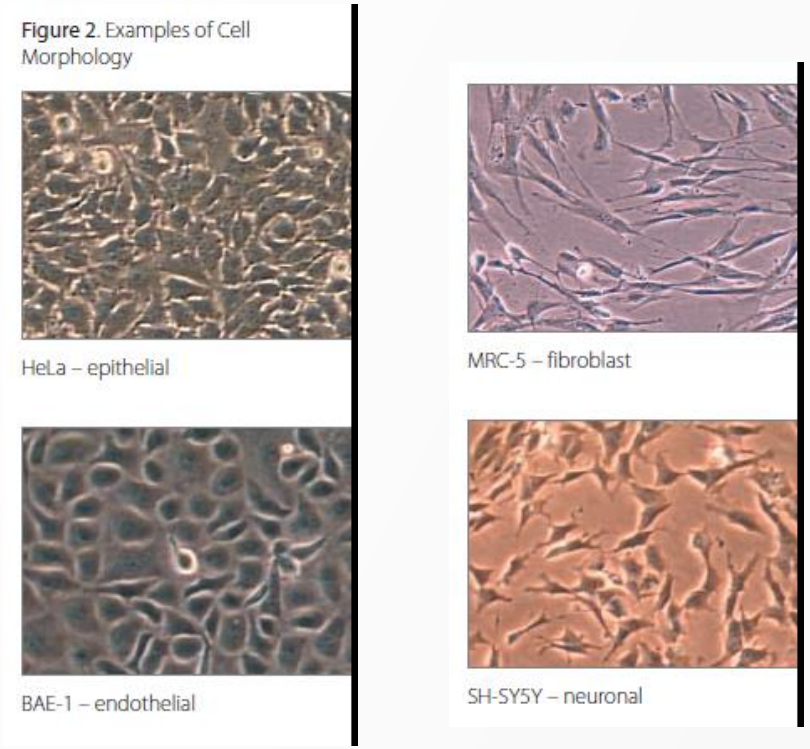
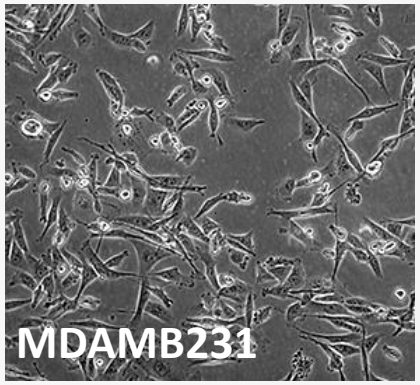
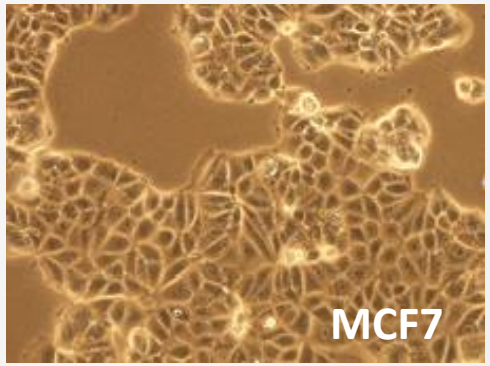
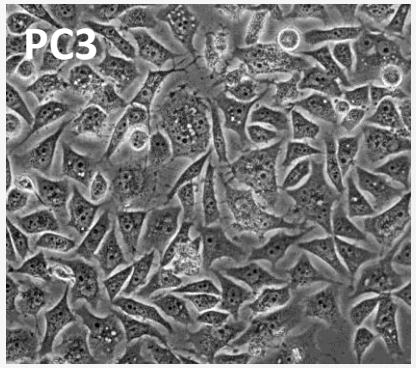
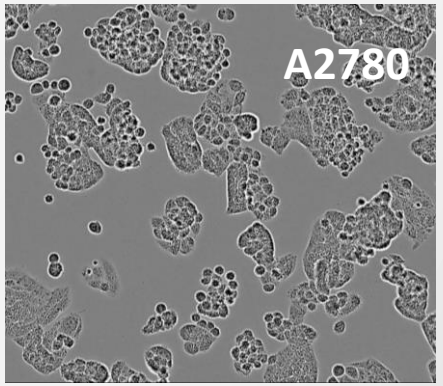
Cell Lines - Morphology











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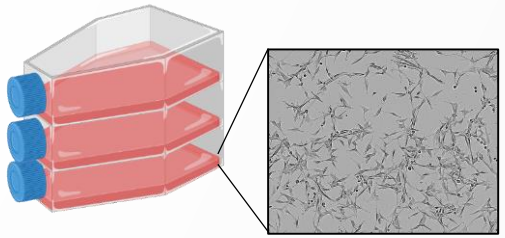
FUNDAMENTAL TECHNIQUES IN CELL CULTURE - LABORATORY HANDBOOK, Sigma

Preclinical evaluation of radiopharmaceuticals

2D culture models | 3D culture models | *In vivo* models

Monolayer-cultured cells | Tumourspheres | Organoids



- ✓ Information on the overall biology and specific molecular mechanisms of tumour cells
- ✓ Scale-up
- ✓ Some cell lines are tumorigenic
- ✓ Cost effective
- ✓ Simple

- × Suffer genotypic mutations
- × Lack 3D structure

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In vitro biological evaluation - cell-based assays

Biomolecule-target binding

- Binding affinity - Receptor Saturation Assay / Competitive Binding Assay

Biomolecule-target interaction

- Cell uptake and internalization studies

Therapeutic effects – efficacy

- Radiocytotoxicity
- DNA damage

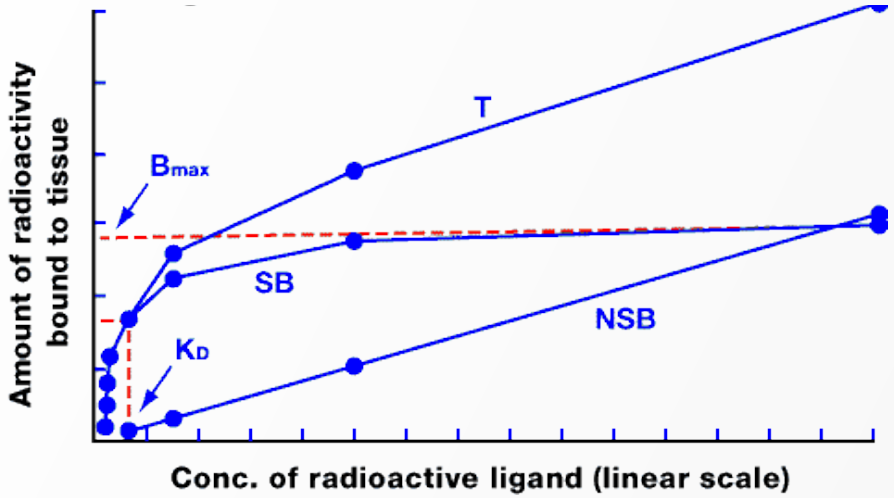
Affinity: Radioligand binding assays

Saturation binding assay

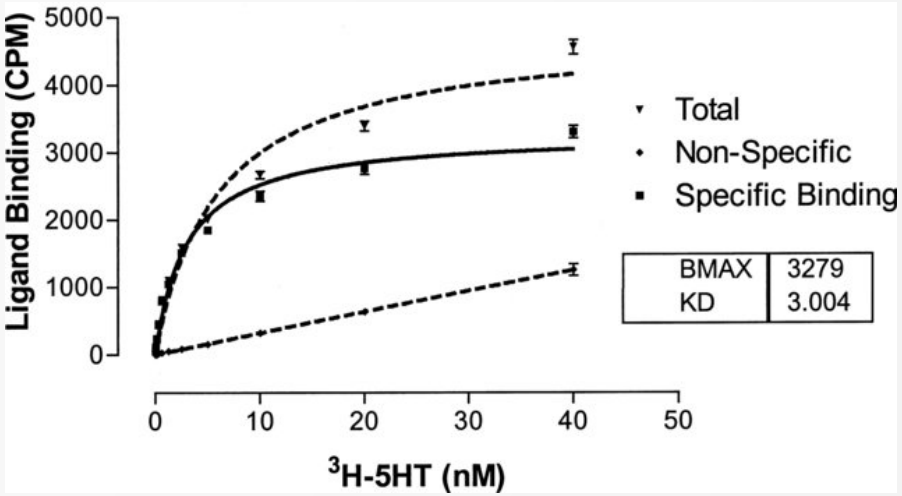
Competitive binding assay

Radioligand-receptor binding assays - Saturation assay

Measure equilibrium binding of various concentrations of the radioligand (+/- excess unlabeled ligand) - to determine the number of binding sites, Bmax, and the ligand affinity, Kd ([L] for 50% of R occupied)



Glaxo Wellcome Pharmacology Guide



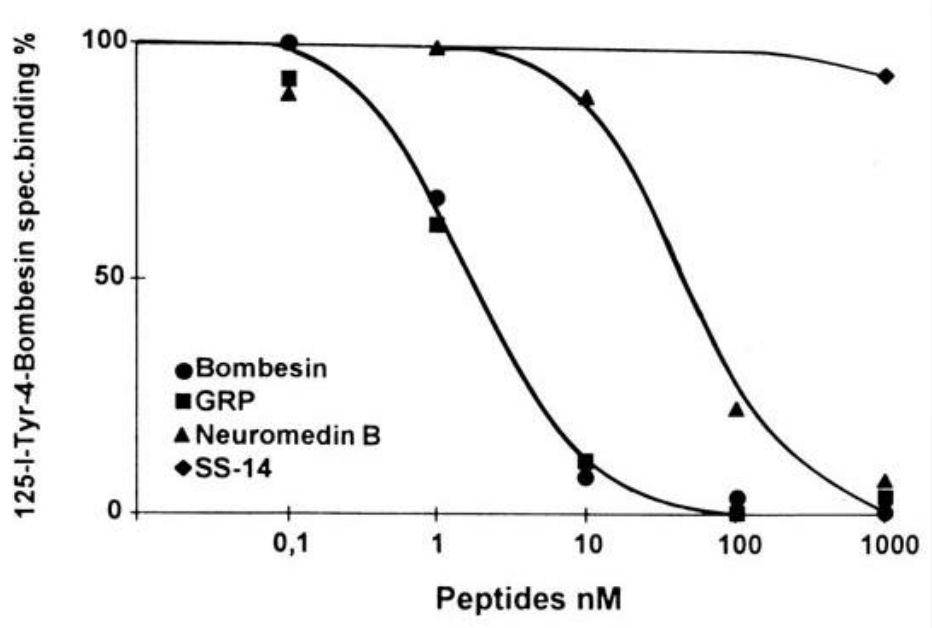
Saturation binding of 3 H-5HT to 5HT 2C membranes: SPA format. Unlabeled 5-HT (10 µM), assay buffer and the indicated amounts of [3 H]-5HT ligand were added to 20 µg of 5HT 2C membranes, and the plates were incubated for 30min rt. 0.5 mg of WGA-PVT SPA beads were added, plates were mixed by shaking every 30 min for 2 h, and then counted

Methods mol biol, 2002, 190:31

As a rule of thumb, the ratio between expected target density (Bmax) and the ligand affinity (KD or Ki) should be >5. Radiopharmaceuticals currently in use mostly satisfy this rule IAEA 2021 Draft

Radioligand-receptor binding assays - Competitive binding assay

Measure equilibrium binding of a single concentration of a high affinity radioligand at various concentrations of an unlabeled competitor - to obtain the affinity of the receptor for the competitor **IC50**, [L] 50% of bound



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

^{125}I-[Tyr^{4}]-bombesin + increasing concentrations of unlabeled:
Bombesin / GRP / Neuromedin B / Somatostatin-14

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Lab Invest 2000, 80:1807

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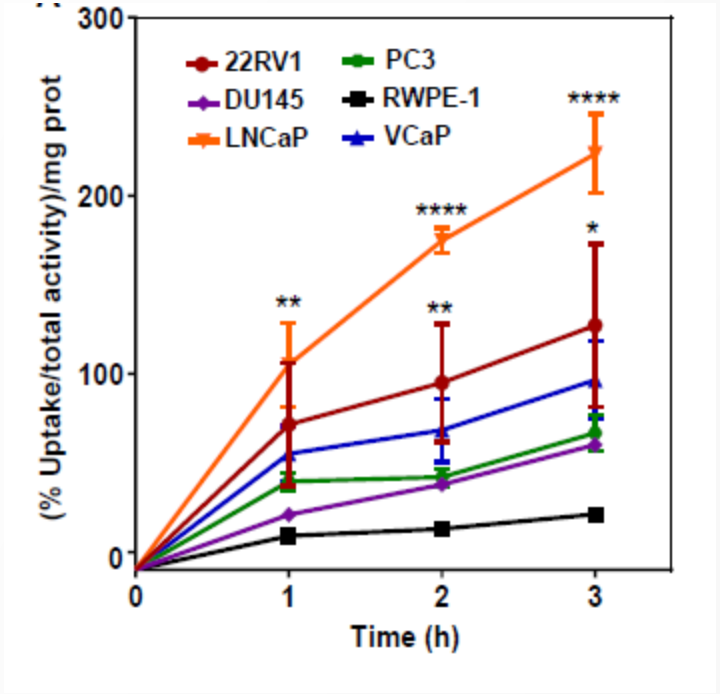
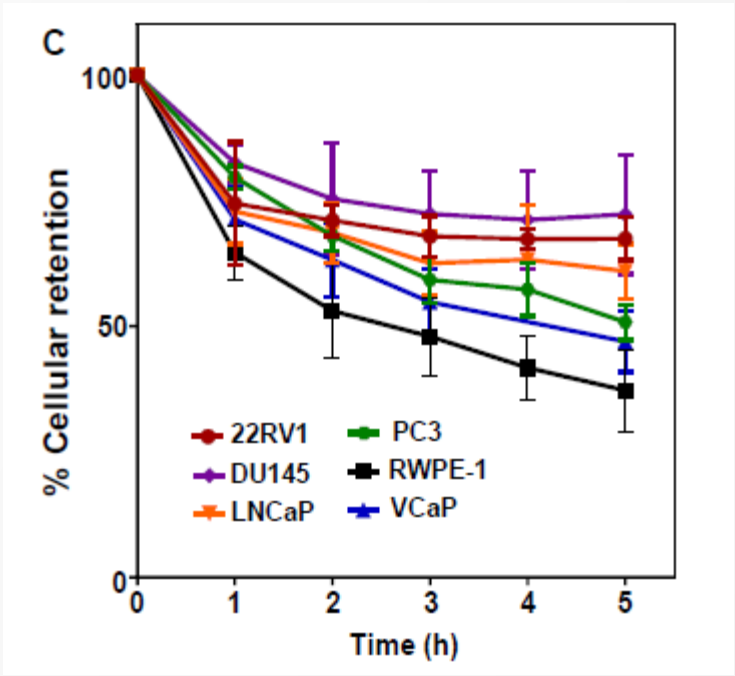
Cell uptake and retention studies





$^{64}CuCl_2$ uptake and retention in Prostate cancer cell lines

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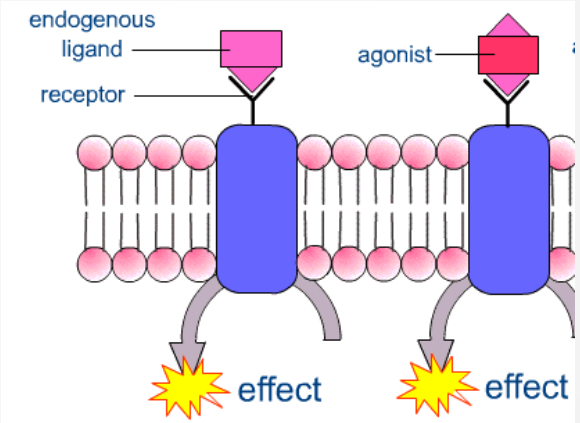
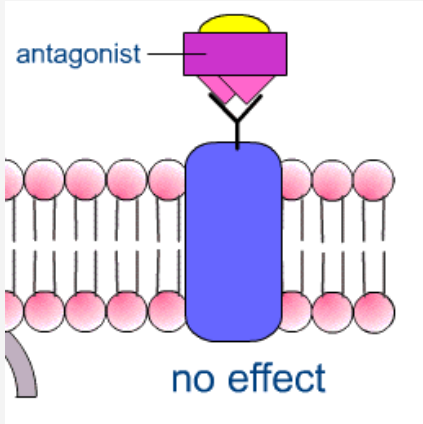
Molecules, 2018, 23:2944

RadioPeptide-receptor binding - Agonist vs Antagonist

- **Radiopeptide Agonist**
  - Mimetizes the endogenous peptide
  - Binds, stimulates and activates the receptor
    (producing an intracellular biological response, internalization for some types of receptors)
  - **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**



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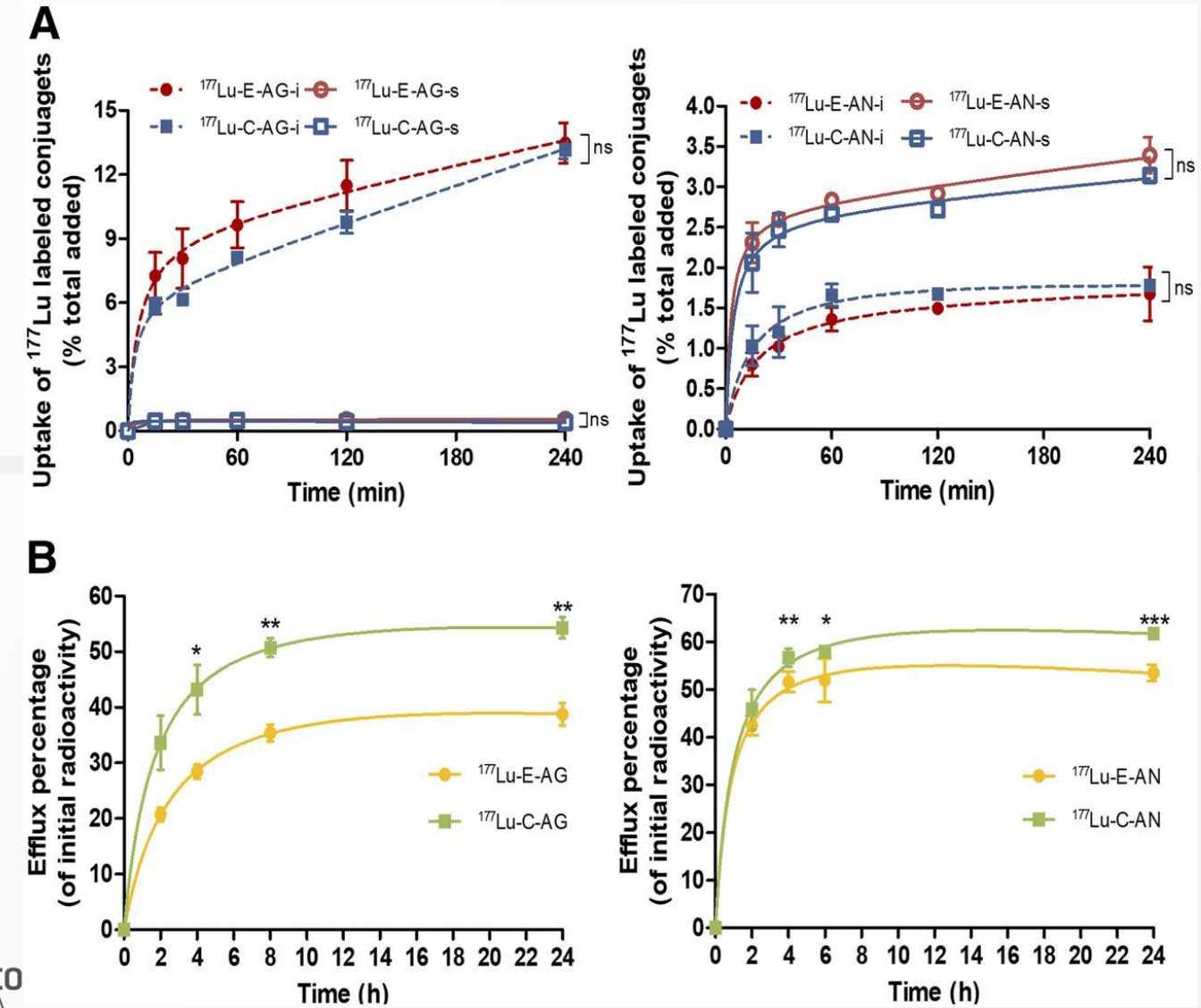
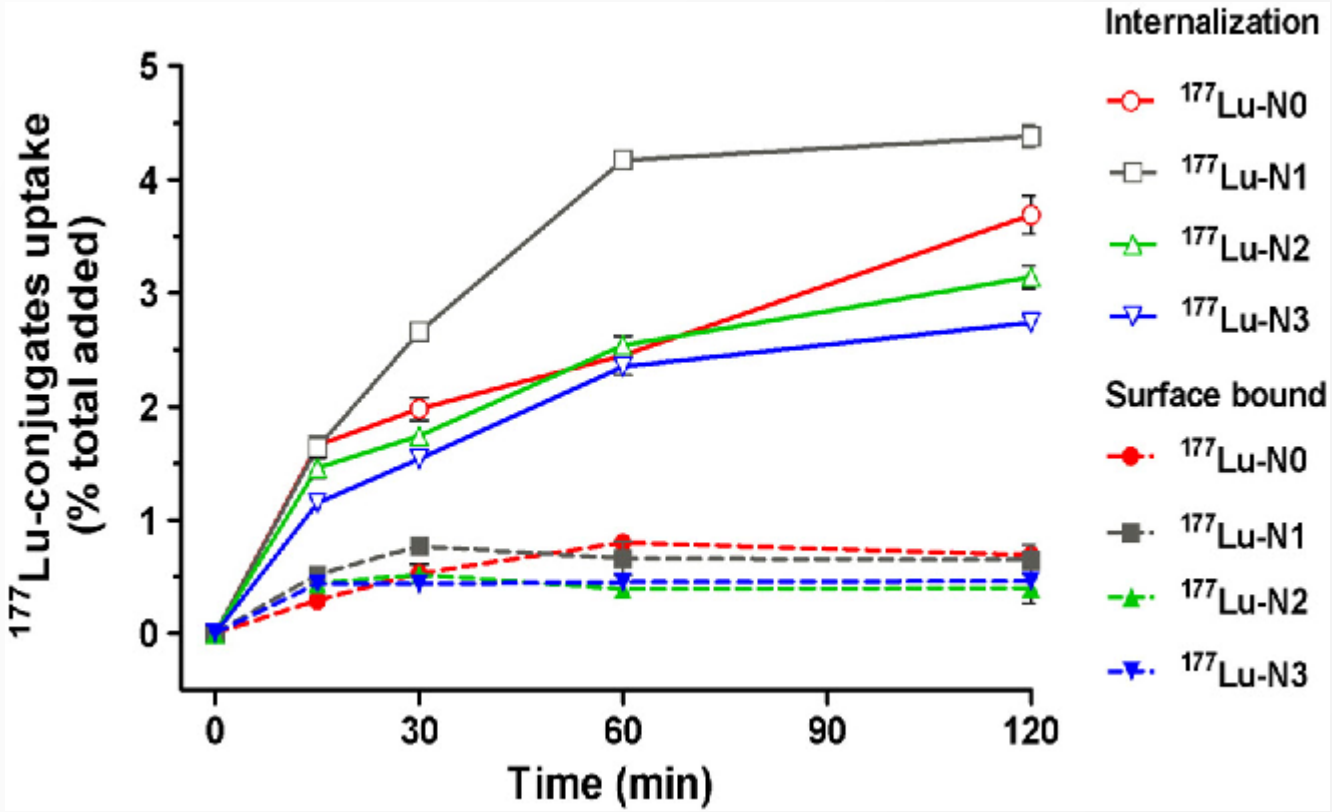
Cell uptake and internalization studies



J Nucl Med, 2020, 61:443

Cell uptake and internalization studies



Spacer-enhanced biological performance

NucMedBiol, 2015, 42:816

In vitro biological evaluation - cell-based assays

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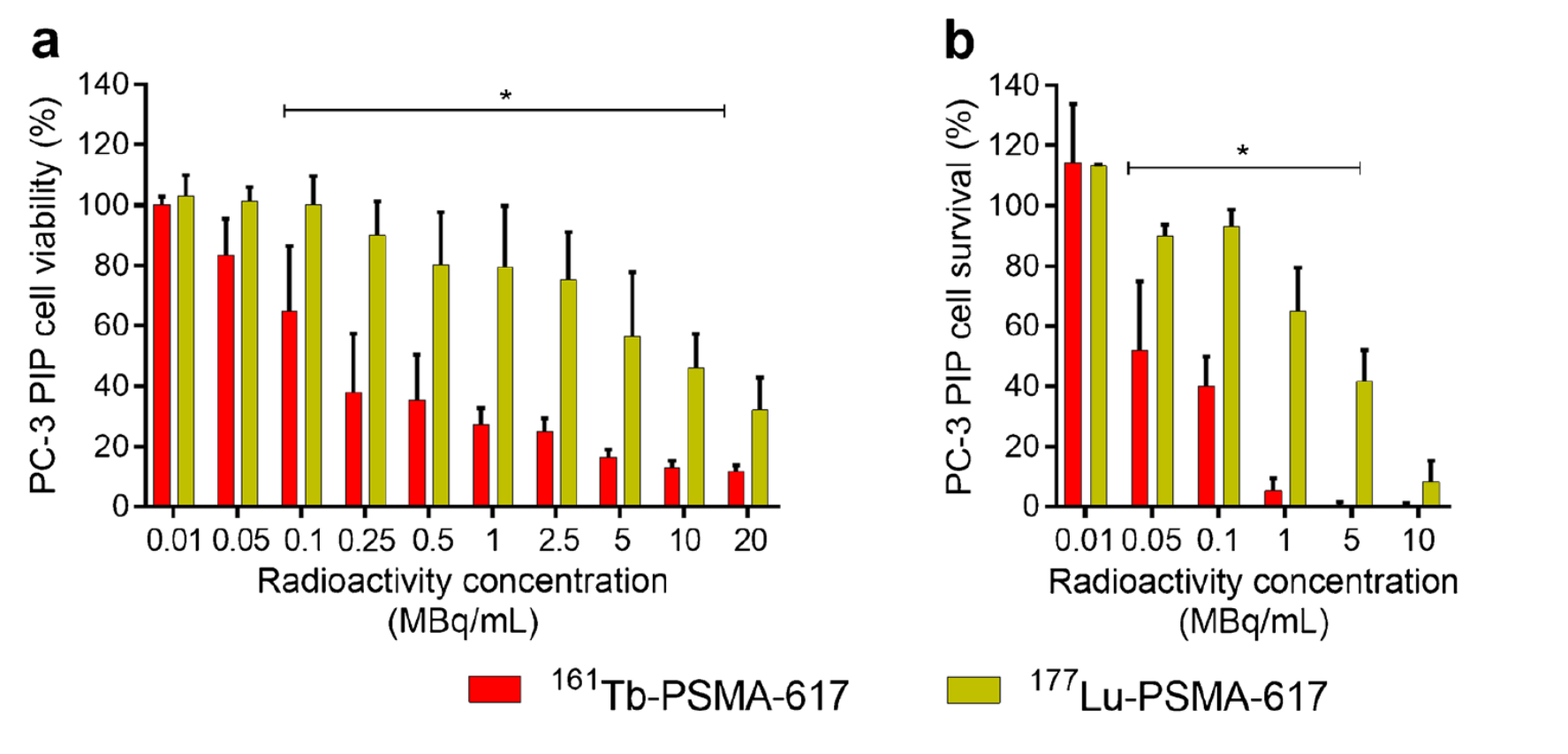
Therapeutic effects – efficacy

- Radiocytotoxicity
- DNA damage

Radiocytotoxicity



Eur J Nucl Med Mol Imaging, 2019, 46:1919

Clonogenic Assays

Reflect the ability of cells to divide and form colonies

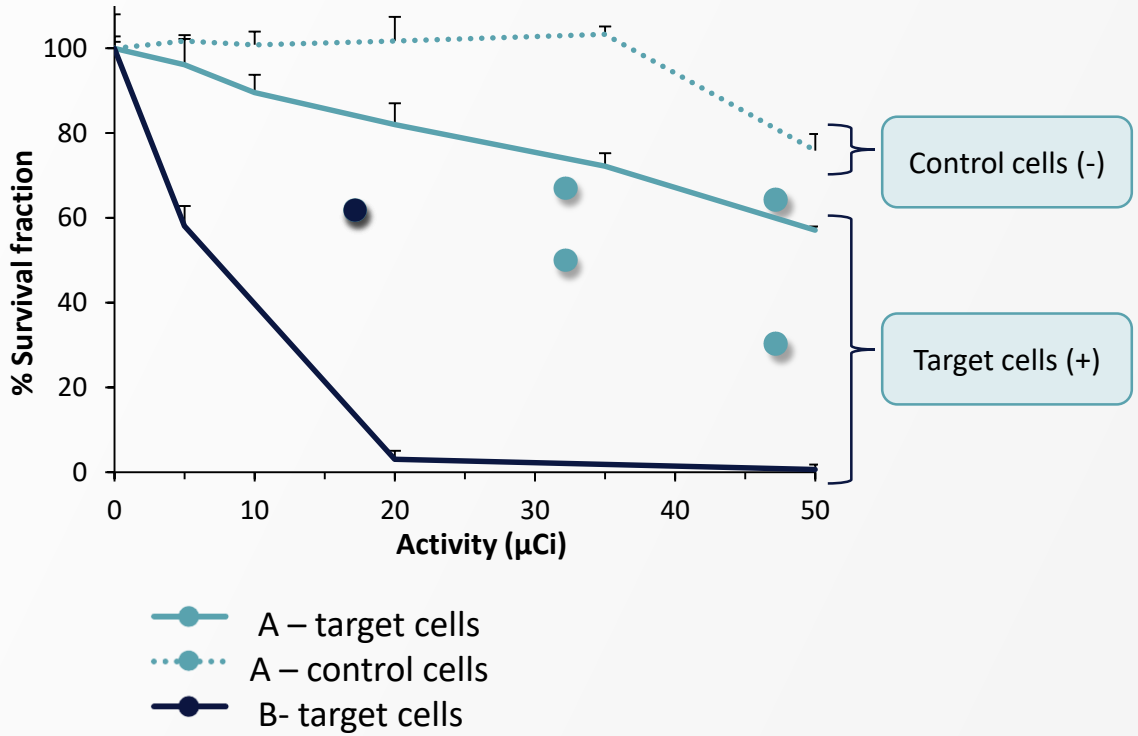
Plate cells

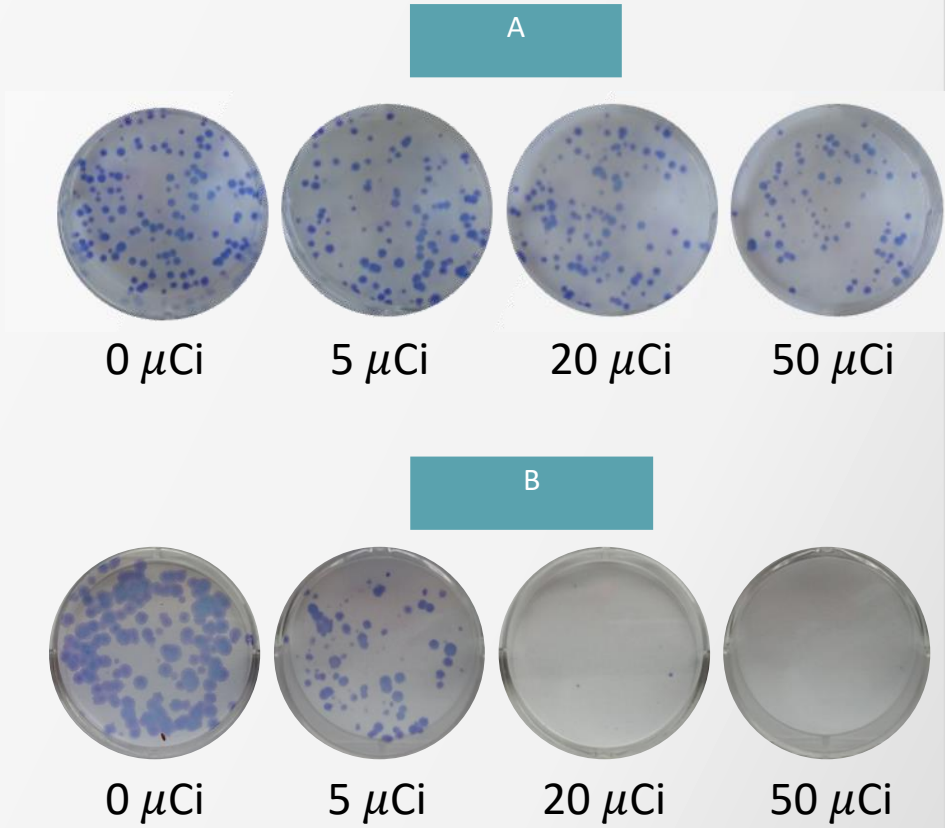
24 h

Incubation Radiopharmaceutical

10 d

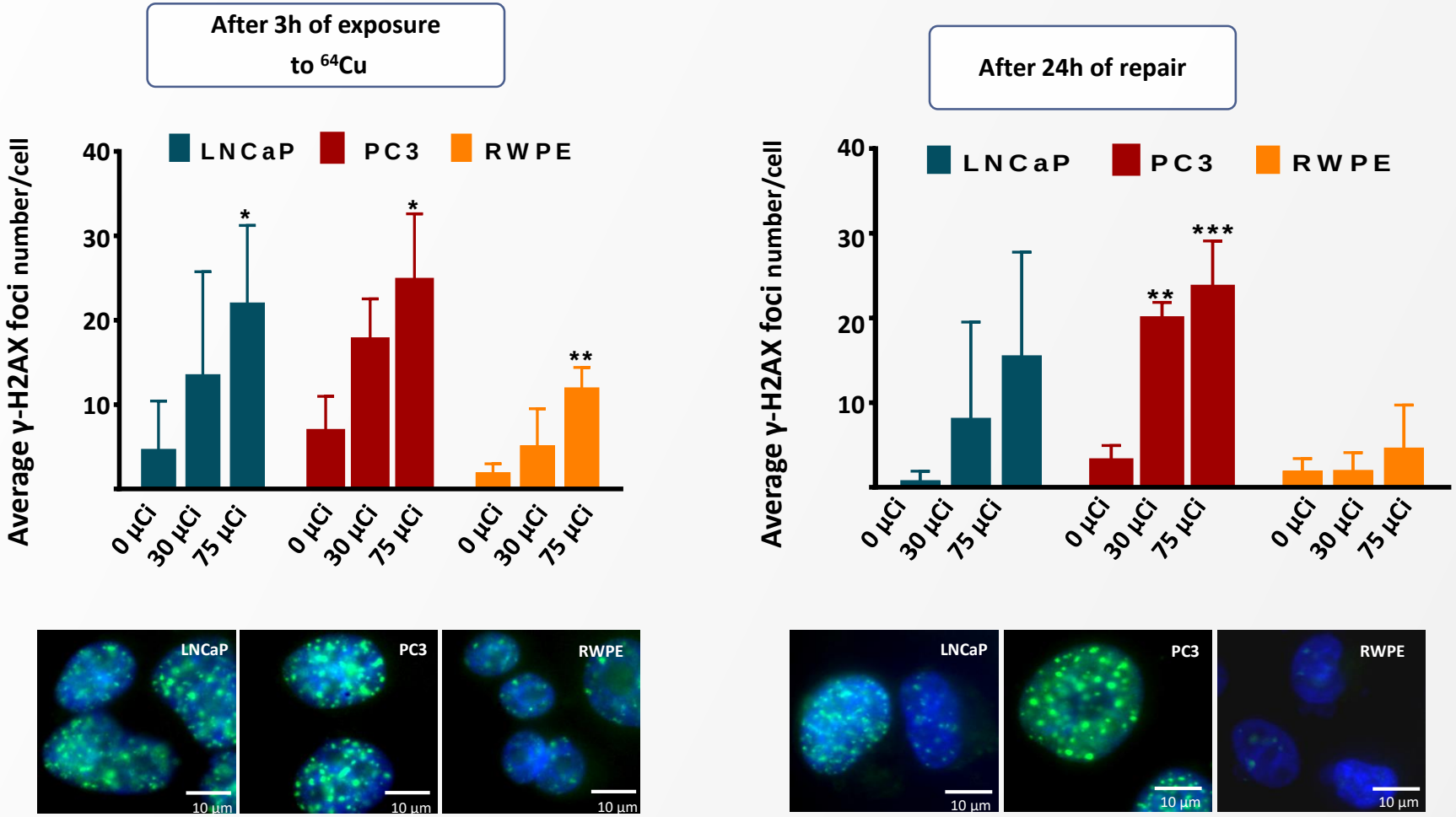
Clonogenic assay





unpublished, C²TN

Genotoxicity/DNA damage - γ-H2AX assay



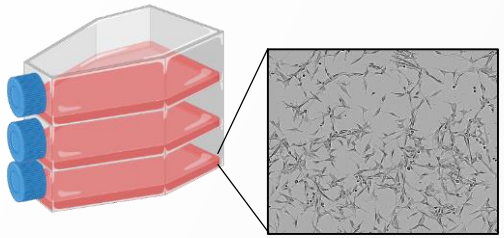
$^{64}CuCl_2$ was able to efficiently induce DNA damage in PCa cells, which exceeded the cellular capacity repair.

Molecules, 2018, 23:2944

Preclinical evaluation of radiopharmaceuticals

2D culture models

Monolayer-cultured cells



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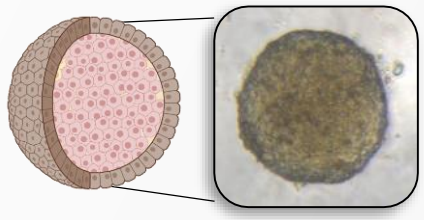
- ✓ Scale-up
- ✓ Some cell lines are tumorigenic

- × Suffer genotypic mutations
- × Lack 3D structure

3D culture models

Tumourspheres



- ✓ Cell-cell and cell-ECM interactions
- ✓ Gradients of oxygen, nutrients, and metabolites
- ✓ Presence of CSCs

- × Lack representativity of tumors' complexity

Organoids

In vivo models

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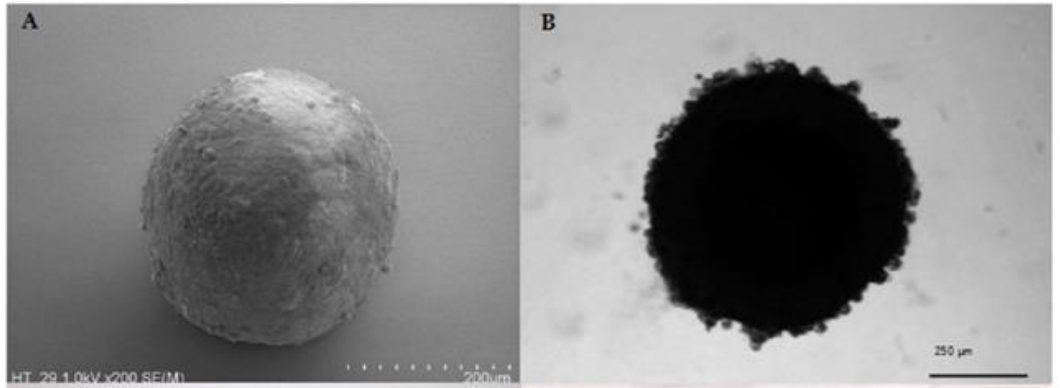
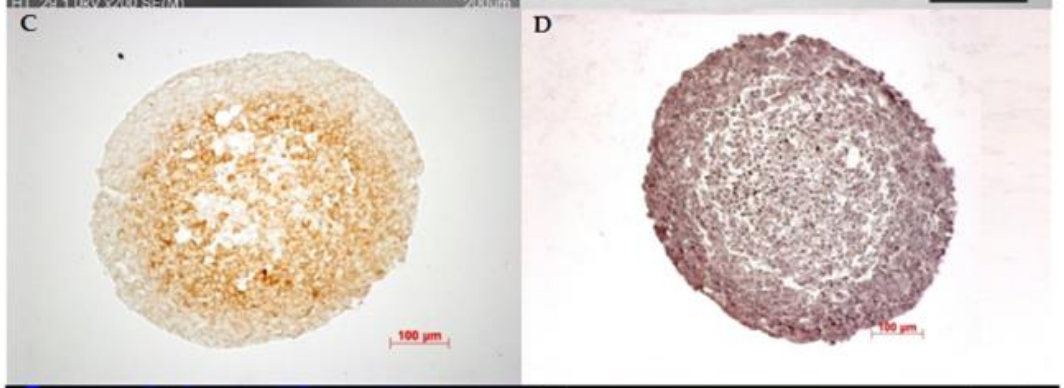
Characteristics of spheroids from human cancer cell lines

SEM image of
a colorectal cancer cell line
(HT29) spheroid



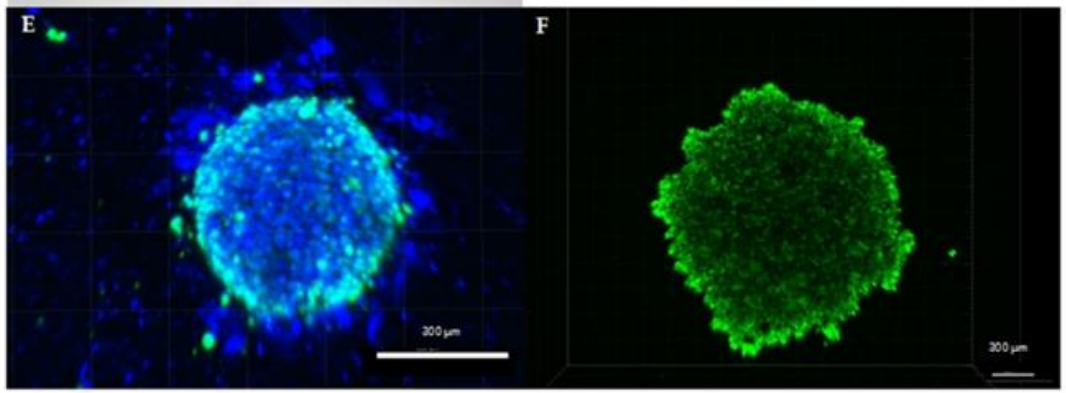
Bright-field microscopy image of a pancreatic cancer cell line (HPAF II) spheroid

Physiological zoning - hypoxic core of a melanoma cell line (A2508) spheroid with a diameter of ~ 0.55 mm is shown by pimonidazole staining



Hematoxylin and eosin staining of a melanoma cell line (A2508) spheroid showing the heterogeneous composition that can vary in cell and extracellular matrix arrangement depending on cell line and treatment

Active migration of cells - fluorescence staining with DAPI (blue)
and calcein (green) in a 10-day-old co-cultivated spheroid of pancreatic carcinoma cell line (SW1990) and pancreatic stellate cells (HPaSteC) (ratio 1:3).



formation of gradients - nutrients, metabolites, and drugs similar to in vivo
- passive transport of the fluorescent marker calcein in a 10-day-old pancreatic cancer cell line (PanC-1) spheroid

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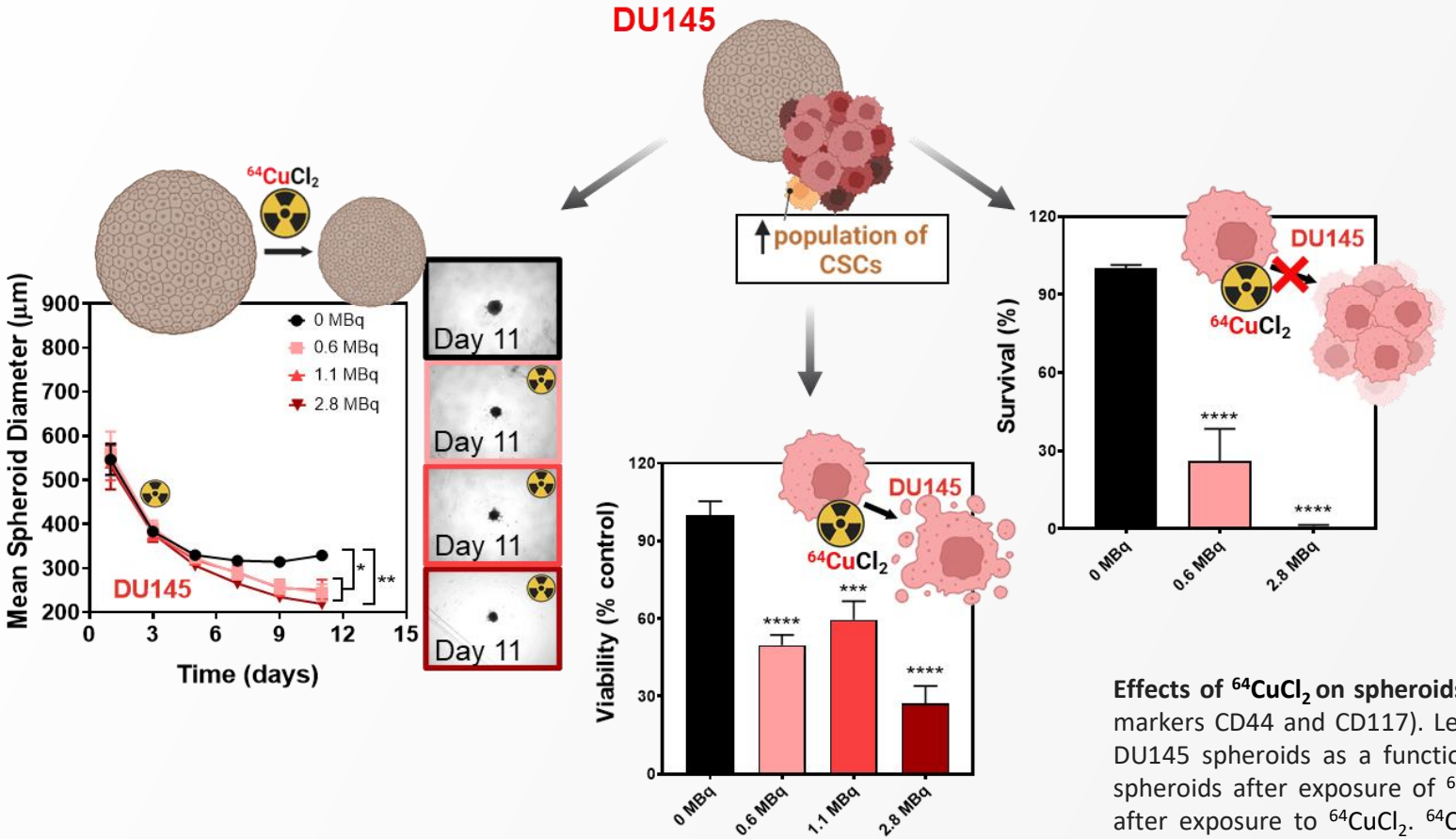
Cancers 2020, 12:2765

Radiocitoxicity in 3D spheroids



Effects of $^{64}CuCl_2$ on spheroids derived from DU145 cell line (positive for cancer stem-like cells markers CD44 and CD117). Left - Growth curves represented by the mean diameter (in µm) of DU145 spheroids as a function of the number of days in culture. Center -Viability of DU145 spheroids after exposure of $^{64}CuCl_2$. Right- Survival fractions of DU145 spheroids-derived cells after exposure to $^{64}CuCl_2$. $^{64}CuCl_2$ significantly reduced the growth and impaired viability and reproductive ability of DU145 spheroids.

Front Mol Biosci, 2020, 7:609172

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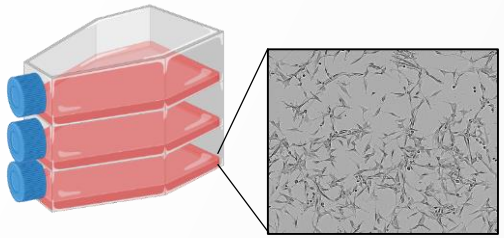
2D culture models

Monolayer-cultured cells

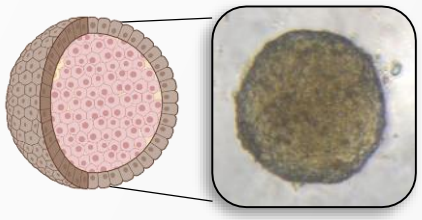


- ✓ Information on the overall biology and specific molecular mechanisms of tumour cells
- ✓ Scale-up
- ✓ Some cell lines are tumorigenic

- × Suffer genotypic mutations
- × Lack 3D structure

3D culture models

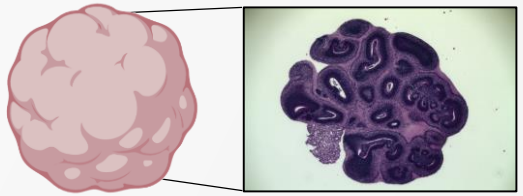
Tumourspheres



- ✓ Cell-cell and cell-ECM interactions
- ✓ Gradients of oxygen, nutrients, and metabolites
- ✓ Presence of GSCs

- × Lack representativity of tumors' complexity

Organoids



- ✓ Recapitulate tumors' histological features
- ✓ Intra- and inter-cellular heterogeneity
- ✓ Genetic profile of the primary tumor
- ✓ Allow for the development of PDOXs

In vivo models

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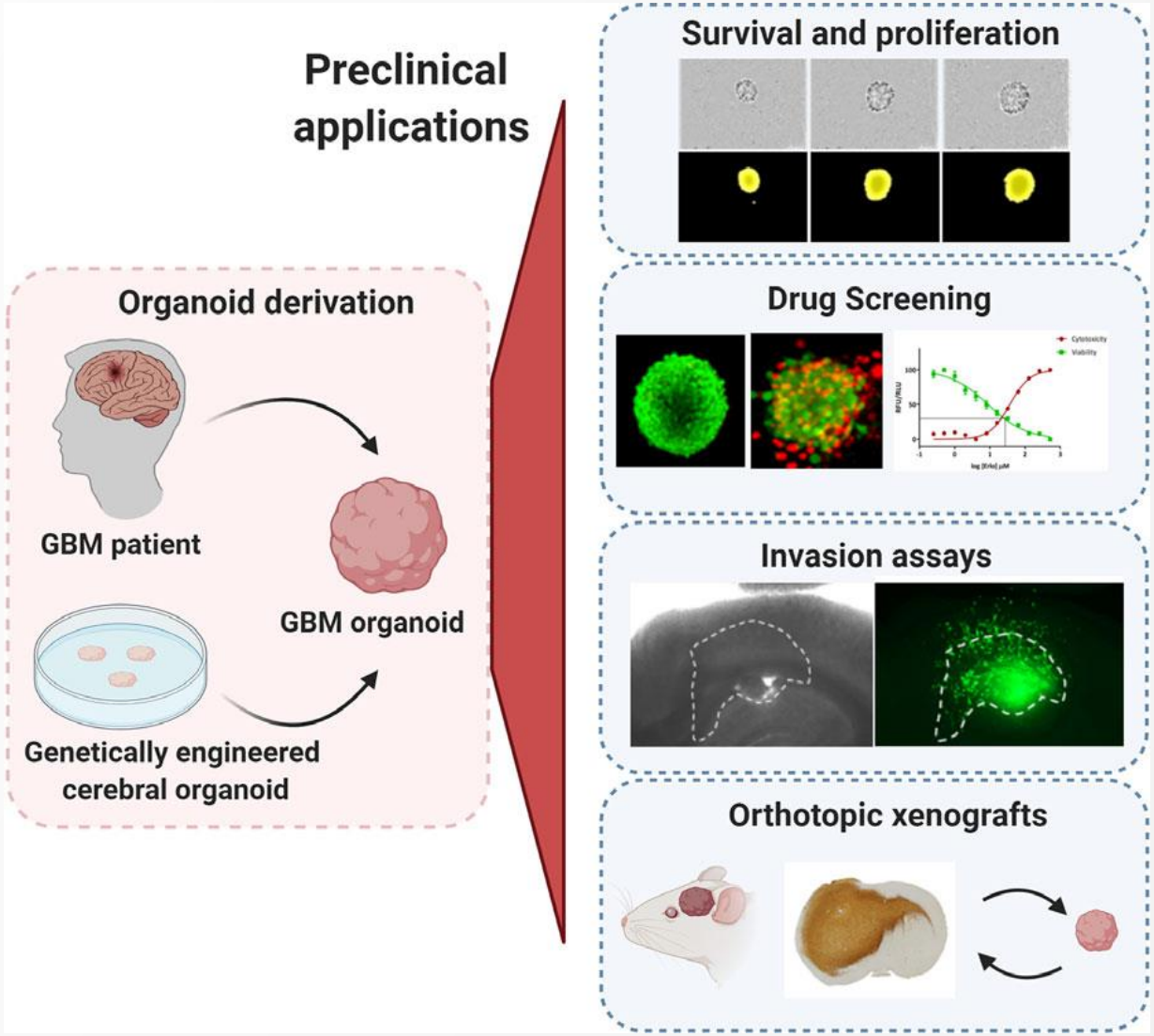
Preclinical applications of organoids

GBM organoids
derived from patient tumors
or
from genetically engineered cerebral organoids

can be applied for numerous functional assays such as:

- tumor cell survival
- proliferation and self-renewal
- drug screening
- ex vivo invasion assays
- derivation of orthotopic xenografts



Front. Oncol. 2020, 10:604121

cancers

MDPI

Review

Three-Dimensional Cell Culture Systems in Radiopharmaceutical Cancer Research

Alina Doctor [1,2], Verena Seifert [1,2], Martin Ullrich [1], Sandra Hauser [1,†] and Jens Pietzsch [1,2,*,†]

[1] Department of Radiopharmaceutical and Chemical Biology, Institute of Radiopharmaceutical Cancer Research, Helmholtz-Zentrum Dresden-Rossendorf, 01328 Dresden, Germany; a.doctor@hzdr.de (A.D.); v.seifert@hzdr.de (V.S.); m.ullrich@hzdr.de (M.U.); s.hauser@hzdr.de (S.H.)

[2] School of Science, Faculty of Chemistry and Food Chemistry, Technische Universität Dresden, 01069 Dresden, Germany

* Correspondence: j.pietzsch@hzdr.de; Tel.: +49-351-260-2622

† S.H. and J.P. share senior authorship.

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check for updates

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Cell-based studies

Binding, internalization, efflux, radiocitotoxity studies

are necessary

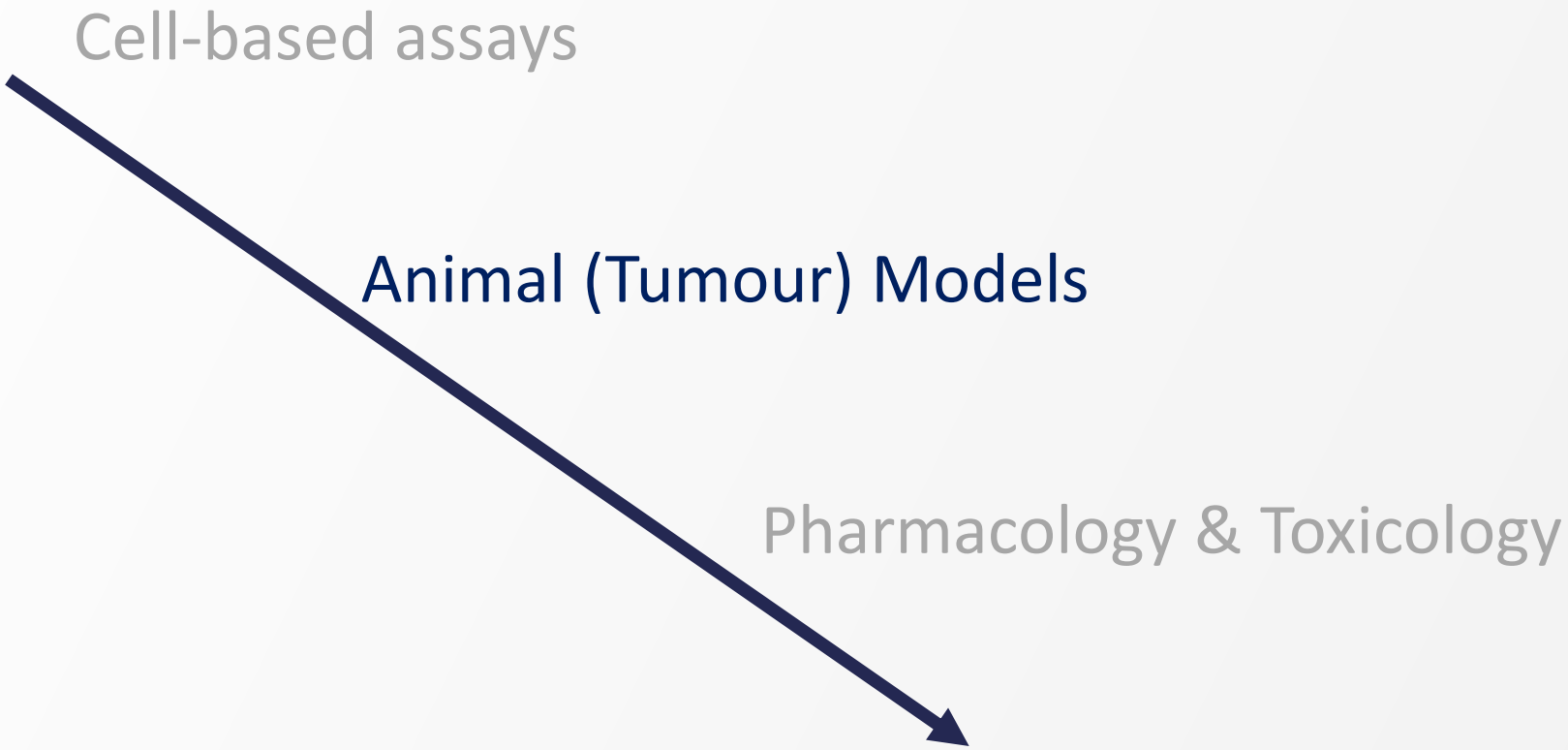
for an initial characterization of the behavior of a new radiopharmaceutical

but

will give a only preliminary picture of the real *in vivo* situation:

- metabolism
- specific uptake in target tissues
- unspecific uptake /uptake by blood cells
- clearance route and kinetics
- retention in excretion organs, plasma protein binding
- penetration of the blood brain barrier...

Overview of Preclinical (Radio)pharmaceutical Development



In vivo studies

Radiolabelled Biomolecules/Compounds with suitable Radiochemical and *In Vitro* Biological Profile

In vivo biological evaluation in animal models

- Small animal models are important tools in bridging the gap between *in vitro* concepts and translation to the clinic.
- Clinical translation is often viewed as a 2-step process: the translation of *in vitro* data to preclinical animal models, and the transfer of knowledge gained from preclinical animal models to clinical practice
- However, an important question is how well preclinical models reflect human disease phenotypes and responses to treatment
- Rodents are mostly used due to their small size, short generation times, known genetic background and relative ease of procurement, handling and housing.

In vivo biological evaluation

Biodistribution and pharmacokinetics in Animal Model

In Vivo Stability / Metabolic Studies & *Ex vivo*

Molecular Imaging

Assessment of Therapeutic Potential (*e.g.* Tumor regression)

Usefulness for clinical application as molecular imaging or radiotherapeutic agent

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In vivo and *Ex vivo* testing – important parameters

- **Number of animals and randomization** (numbers too small – underpowered, too large – no reduction principle; randomization- no bias, especially for treatment)
- **Administration of a radiopharmaceutical -** reliable delivery of the radiopharmaceutical into systemic circulation with minimal discomfort to the animal (iv, ip, catheter)
- **Circadian rhythm**
- **Physiological monitoring -** body temperature, respiratory and heart rate, blood glucose level
- **Anaesthesia -** method of anaesthesia has to be carefully chosen (injectable agents and more commonly, by inhalation agents)
  - normally for preclinical in vivo PET/SPECT imaging, both to decrease animal discomfort and to keep the animal motionless
  - decreases the heart rate, the respiratory frequency and the body temperature
  - can change the PK and tissue accumulation patterns of tracers - [18F]FDG - brain and heart uptake affected (awake animals show increased uptake in the brain, whereas the use of isoflurane increases the heart uptake)
- **Euthanasia**

  Cervical dislocation: fast procedure recommended for mice but not for bigger animals;
  Anaesthetic overdose/ CO_2 saturation: preferred protocol since is non-invasive - can influence the results of ex vivo analysis
  Heart extirpation: requires surgery under deep anaesthesia - when organs will be used for ex vivo biodistribution and/or autoradiography;
  Decapitation: carried out with the help of a guillotine - under anaesthesia. Not recommended for radiopharmaceuticals;

Animal Models - Ethical considerations

3-Rs Principle

All animal experiments must follow applicable laws according the well defined national structure to assure judicious use of animals in experimentation as well as for licencing facilities for animal handling.
Any animal experiment must take into account the ethical principles of laboratory animal science, known as the **3Rs principles: replacement, reduction and refinement**

Replacement

In vitro techniques
Microorganisms
computer modelling

Reduction

Study design – minimum animal number
Improve statistics
Use “lower” *vs* “higher” animals

Refinement

Reduce pain and stress
Non-invasive techniques
Improve conditions

Animal Models

Principles for animal experiments

- Essential for significant relevant information
- Obligation to treat animals with respect
- Investigator has ultimate responsability
- Balance between effects on animals and benefit for health

- Appropriate species
- Bred in captivity
- Scientifically valid using minimum number
- Well trained and competent staff
- Brief experiments
- No unnecessary repeats

Preclinical evaluation of radiopharmaceuticals

2D culture models

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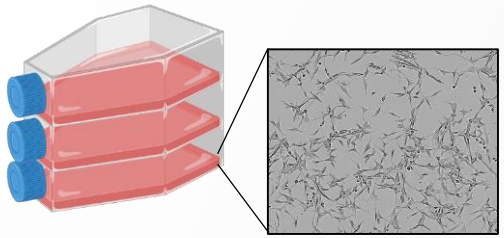

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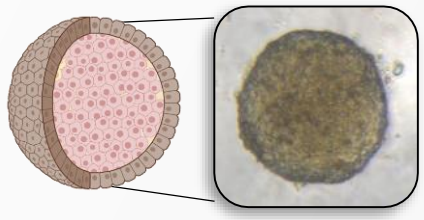

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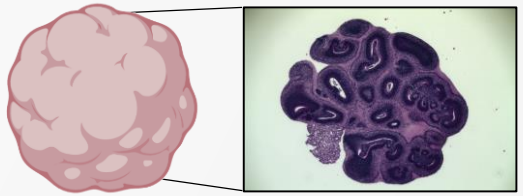

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In vivo models

Xenograft models

- ➡ Immunocompromised host
- ➡ Subcutaneous or orthotopic engraftment

Genetically engineered models

- ➡ Immunocompetent host
- ➡ Follow up of tumor initiation and evolution

Syngeneic models

- ➡ Immunocompetent host
- ➡ To study compounds with immuno-therapeutic potential

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Animal Models / disease models

Infection/ Inflamation Animal Models

Tumour-Bearing Animals

Transgenic Animals

Biomolecules specifically bind *in vivo* to infection sites, antigens, overexpressed receptors,….

Animal Models / disease models

Table 2 | **Mouse tumour models that are useful in imaging**

Model	Origin of tumour	Species	Host	Tumour site
Syngeneic mouse model	Spontaneous	Mouse	Immunocompetent	Organ-specific
	Hormonally or chemically induced	Mouse	Immunocompetent	Variable and not necessarily organ-specific
	Allograft or isograft	Mouse	Immunocompetent	Subcutaneous
GEMM	Globally expressed mutation	Mouse	Immunocompetent	Various tissues
	Conditionally expressed mutation	Mouse	Immunocompetent	Organ-specific
	Chimerically expressed (humanized) mutation	Mouse and human	Immunocompetent	Organ-specific
Xenograft	Cell line-based	Human	Immunodeficient	Subcutaneous, subrenal or orthotopic
	Patient-derived	Human	Immunodeficient	Subcutaneous, subrenal or orthotopic

GEMM, genetically engineered mouse model.

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NatRevCancer, 2014, 14:481

Biodistribution and Pharmacokinetics

Distribution of radiolabelled biomolecule in main organs

Uptake and retention time in receptor-negative tissues *vs* receptor –positive tissues

Blocking experiments by co-administration of unlabelled biomolecule

Rate of blood clearance

Rate and route of excretion

In vivo stability of radiolabeled biomolecules

Biodistribution and Pharmacokinetics

- Determine biodistribution over time (depends on the application)
- Determine % Radioactivity Excretion;
- Determine % I.A. per organ; % I.A. per gram;

  Dissection and counting

  Quantification by PET or SPECT camera

  Autoradiography
- Target-to-non target ratio
- Clearance of radiolabelled biomolecule and its radioactive metabolites

Biodistribution

There are some limitations in extrapolating data from animal models due to:

- Different genotypes between mice and men
- Size difference – specially dosimetric calculations
- Faster sequestring and metabolizing

<u>Major variations between species</u>:

Uptake by specific organs + Clearance

Mouse heart beats much faster than human hearts

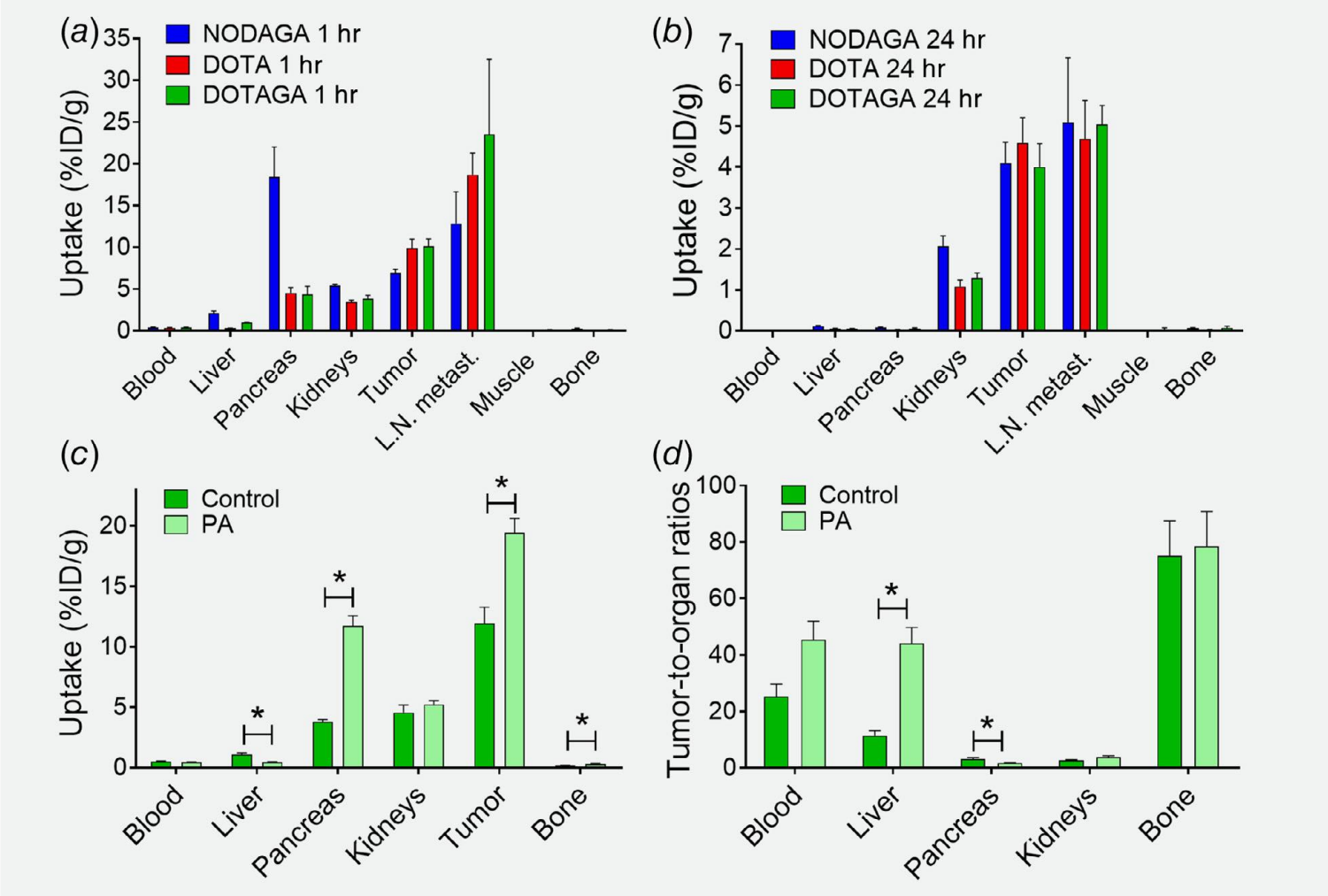
Mice breathe much faster

- Shorter tissue perfusion times
- Shorter gastrointestinal transit time
- More rapid pharmacokinetics

Biodistribution

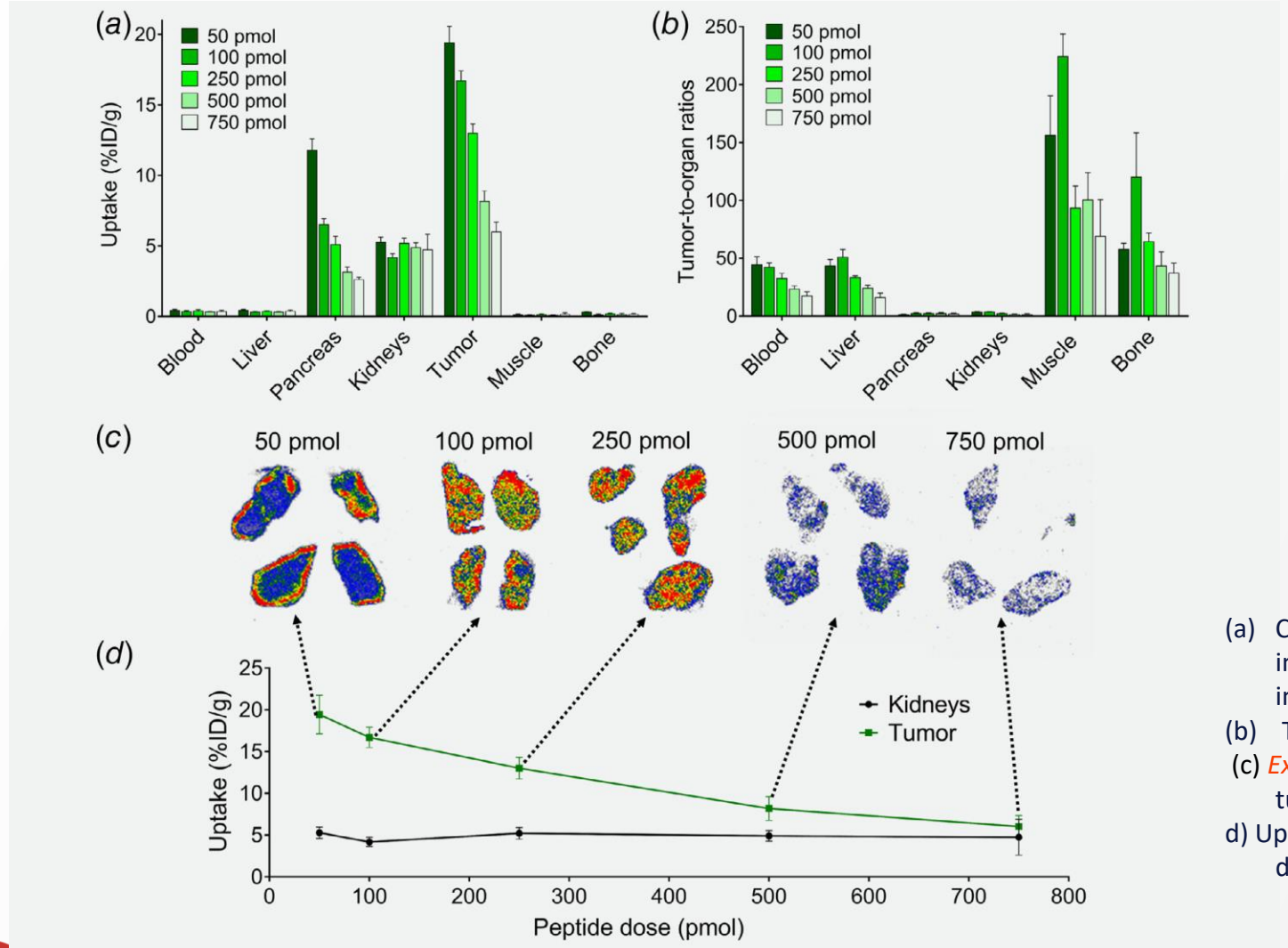
PC-3 prostate cancer xenografts treated with the GRPR antagonist ^{177}Lu-DOTAGA-PEG2-RM26

PA stabilization of radiopeptides *in vivo*



Int J Cancer, 2019,145:3347

Biodistribution and *ex vivo* autoradiography



(a) Comparative biodistribution of different injected peptide doses (80 kBq in 50, 100, 250, 500 and 750 pmol/animal) of ^{177}Lu-DOTAGA-PEG2-RM26 in BALB/C nu/nu mice bearing PC-3 tumors at 1 hr pi

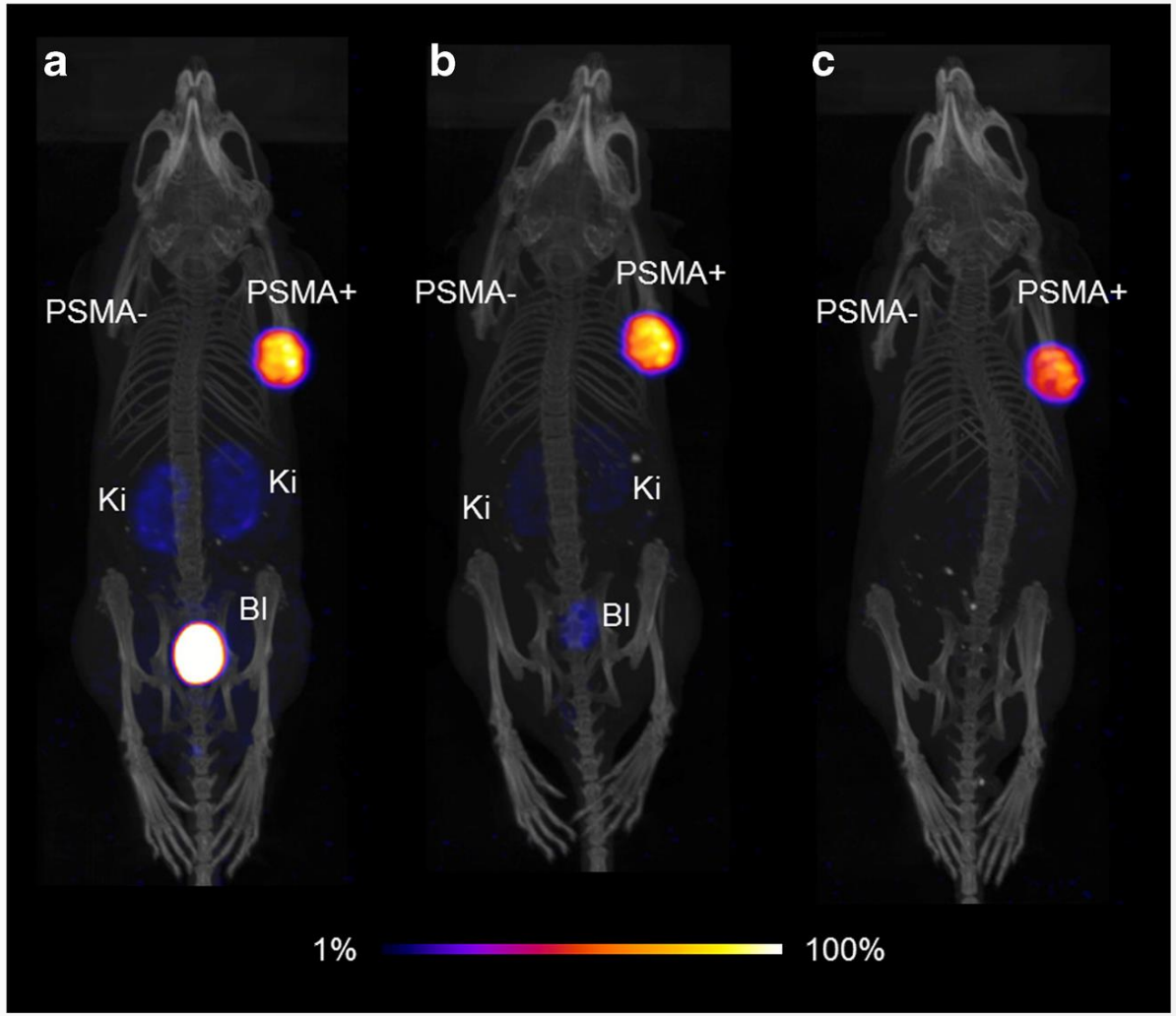
(b) Tumor/to-normal-tissue ratios at 1 hr pi

(c) *Ex vivo* GRPR-autoradiography of tumor slices of mice bearing PC-3 tumors after the injection of ^{177}Lu-DOTAGA-PEG2-RM26

d) Uptake of activity in tumor and kidneys as a function of injected peptide dose.

Int J Cancer 2019,145:3347

Small animal imaging - specificity



SPECT/CT images of
mice after injection of ~25 MBq
^{161}Tb-PSMA-617 shown as
maximum intensity projections.
a - 1 h p.i.
b – 4 h p.i.
c - 24 h p.i.

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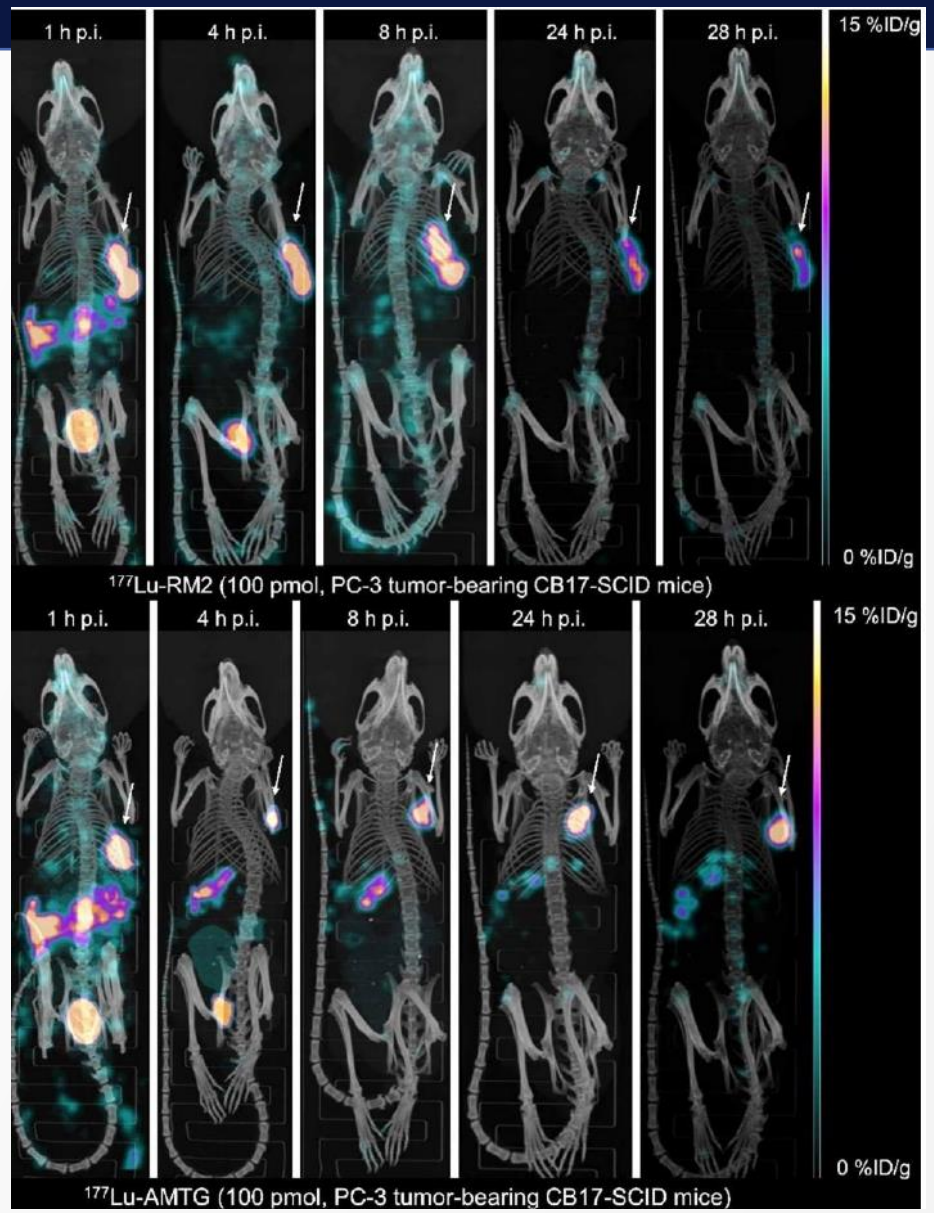
Eur J Nucl Med Mol Imaging, 2019, 46:1919

Small animal imaging - longitudinal studies



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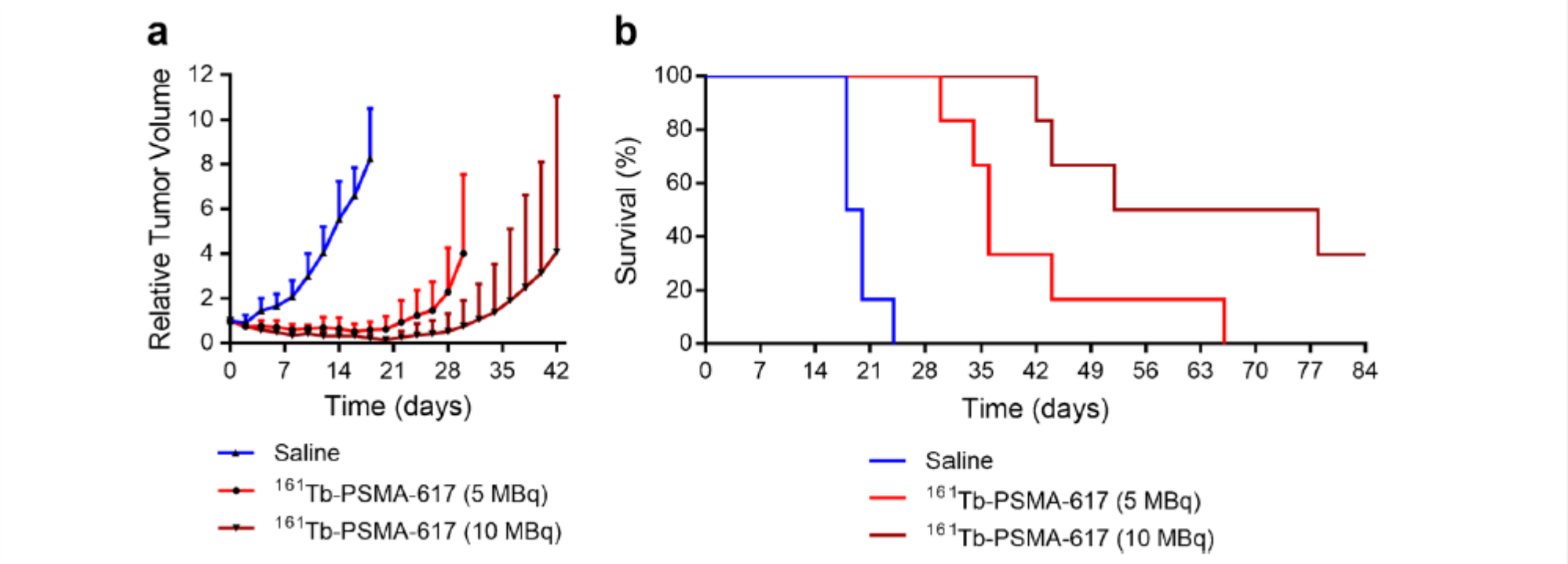
J Nuclear Medicine, 2022, 63:12

In vivo biological evaluation – therapeutic effects



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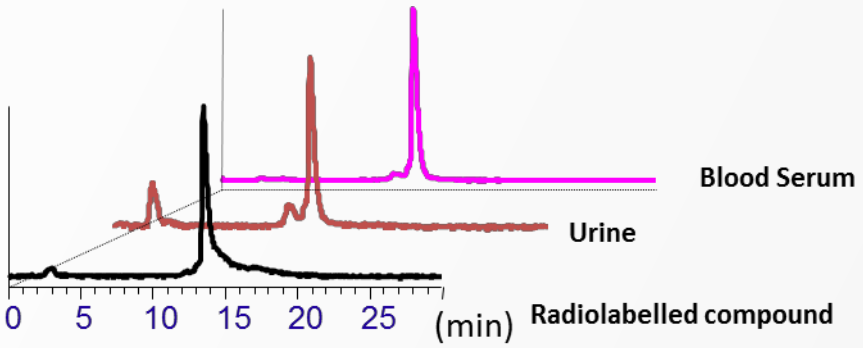
Eur J Nucl Med Mol Imaging, 2019, 46:1919

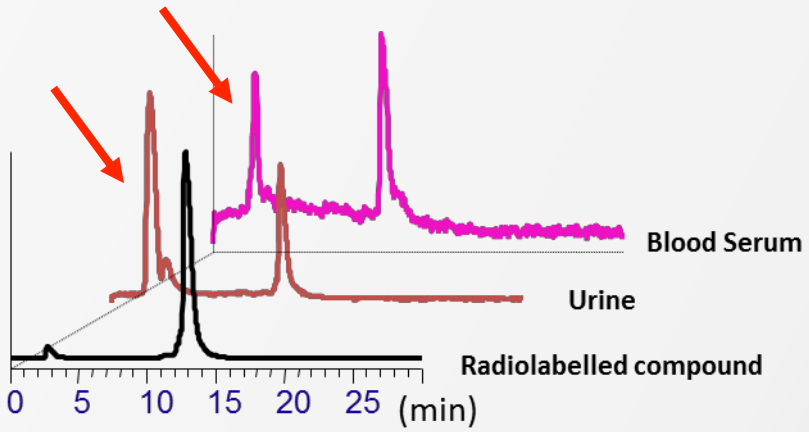
In vivo stability

HPLC analysis of samples of blood serum; collected urine and organ homogenates (liver, kidney, brain,…)



Treatment of biological samples (protein precipitation)

Chromatographic analysis (HPLC)



Peptide Radionuclide Receptor Therapy

- Specific
- Rapid tumor uptake
- Long residence time in tumor
- Rapid clearance from non-target organs

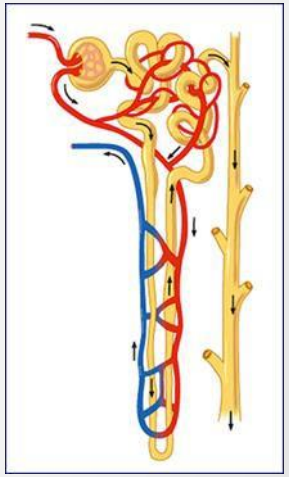
- Improvement of patients´ quality of life
- Pain relief
- Tumor regression
- Decreased level of tumoral markers

Nefrotoxicity limits the dose

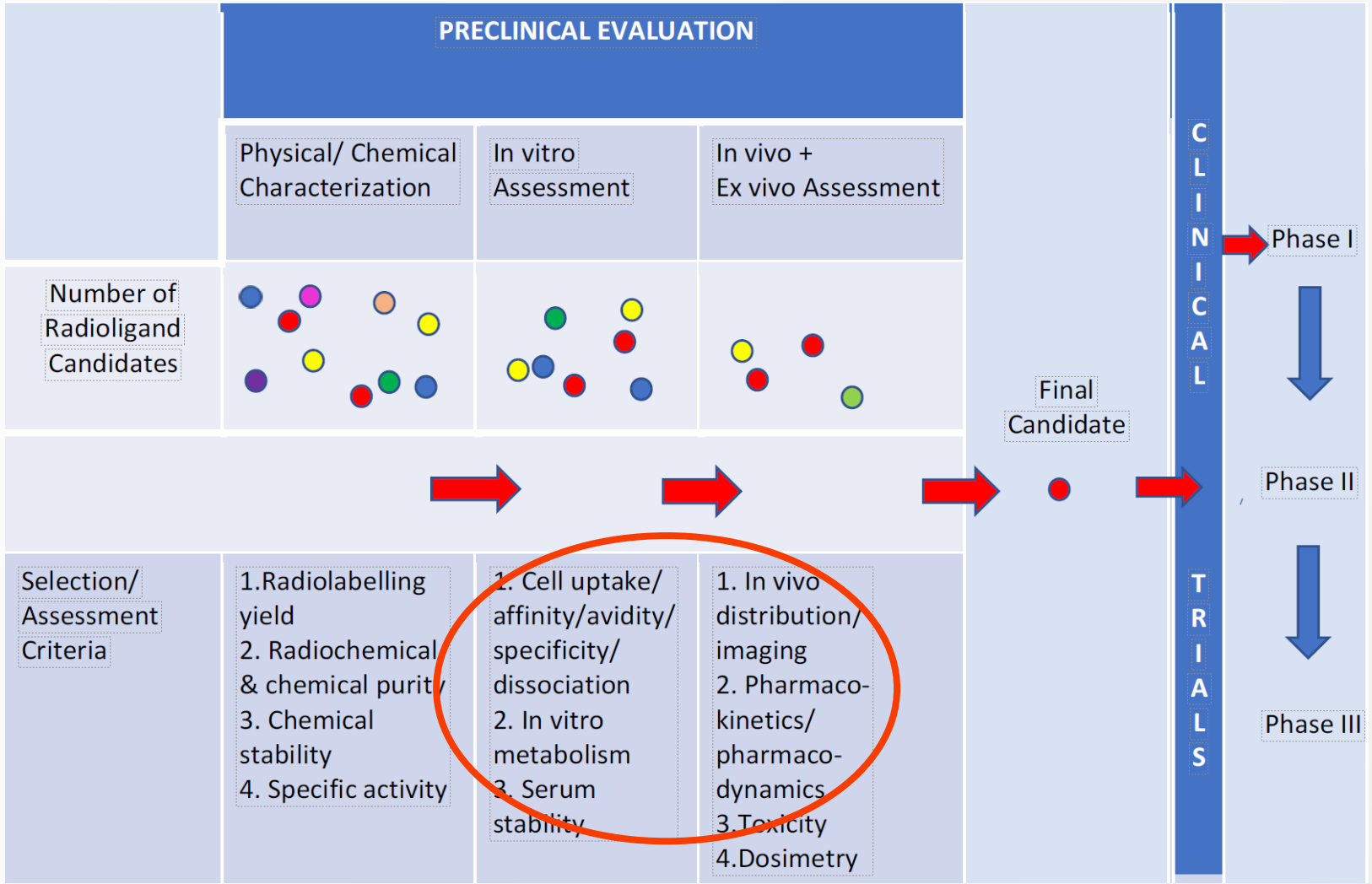
Peptides

- **Predominant renal excretion**
- **Glomerular filtration**
- **Resorption in proximal tubule**
- **Retention in lisossomes**

High radioactivity concentration in the kidney



Overview of Preclinical Radiopharmaceutical Development



Important - In **therapeutic radiopharmaceuticals** the **biodistribution** and **dosimetry** play a much more significant role to assess **radiation induced toxicity** and at the same time give information on **efficacy**

Thank you

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CENTRO DE CIÊNCIAS
E TECNOLOGIAS NUCLEARES

Filipa Mendes

fmendes@ctn.tecnico.ulisboa.pt