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Julia Martina Raitanen, BSc MSc

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Univ.-Prof. Mag. Dr. Markus Mitterhauser
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**„If we knew what it was we were doing, it would not be called
research, would it?” – *Albert Einstein***

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Abstract

In the field of nuclear medicine, the development of new therapeutic radiopharmaceuticals against cancer plays an important role. After chemical synthesis, firstly *in vitro* studies need to be conducted. Only after successful evaluation, *in vivo* animal studies can follow. In the phase of *in vitro* studies, different binding studies and radiobiological assays are performed. Despite the limited comparability with *in vivo* tumors, they have so far mostly been performed using two-dimensional (2D) cell culture.

The aim of this thesis is to establish a set of radiobiological assays for the in-house evaluation of new therapeutic radiopharmaceuticals for cancer treatment and to assess the usability of the more *in vivo* tumor resembling three-dimensional (3D) cell culture in this context.

In two of the presented publications, we described the differences in radiobiological response observed between monolayers (2D) and multicellular tumor spheroids (3D) of different cancer cell lines, after treatment with either external beam radiation (EBR) or targeted radioligand therapy (TRT) with [^{177}Lu]Lu-PSMA-I&T.

Regarding external beam, differences in radiosensitivity between the different cell lines were observed, and spheroids showed to be more radioresistant, compared to the monolayers. The latter was particularly noticeable in the PC-3 (bone metastasis of human prostate cancer) cell line, where spheroids were radioresistant up to 8 Gy, while the surviving fraction in monolayers, after 8 Gy irradiation, was only about 10%.

TRT with [^{177}Lu]Lu-PSMA-I&T showed specificity for the target expressing cell line LNCaP (lymph node metastasis of human prostate cancer) in both models (2D & 3D), but higher treatment effect in spheroids, compared to monolayers. However, the latter can be explained by dosimetry simulation using the Monte Carlo method, showing higher absorbed dose in the spheroids compared to the monolayer.

Moreover, external beam radiation was used as a tool for the characterization of impurities during the upscaling of [^{177}Lu]Lu-PSMA-I&T production. EBR, followed by HPLC-MS analysis, lead to the conclusion that during the upscaled production, radiolysis generated the deiodinated product. Hence, the production method was optimized.

Lastly, the usability of spheroids in combination with the *in ovo* model was investigated. Although different sizes of spheroids were applied, the resulting xenografts were too small in size to enable sufficient further processing. Nevertheless, progress in the field of 3D cell culture may lead to better results in the future.

In summary, this thesis provides insights into the differences between 2D and 3D cell culture systems in radiopharmaceutical development, demonstrates the usability of spheroids and highlights the differences in experimental design and result interpretations that are necessary when using external irradiation or targeted radionuclide therapy.

Zusammenfassung

Auf dem Gebiet der Nuklearmedizin spielt die Entwicklung neuer therapeutischer Radiopharmaka gegen Krebs eine wichtige Rolle. Nach der chemischen Synthese müssen zunächst *in-vitro* Studien durchgeführt werden. Erst nach erfolgreicher Evaluierung können *in-vivo* Tierstudien folgen. In der Phase der *in-vitro* Studien werden verschiedene Bindungsstudien und radiobiologische Tests durchgeführt. Trotz der eingeschränkten Vergleichbarkeit mit *in-vivo* Tumoren wurden diese jedoch bisher meist an zweidimensionalen (2D) Zellkulturen durchgeführt.

Ziel dieser Arbeit ist es, eine Reihe strahlenbiologischer Tests für die Evaluierung neuer therapeutischer Radiopharmaka für die Krebstherapie zu etablieren, sowie die Verwendbarkeit der *in-vivo* Tumor ähnlicheren dreidimensionalen (3D) Zellkultur in diesem Zusammenhang zu bewerten.

In zwei der vorgestellten Publikationen wurden die Unterschiede in der radiobiologischen Wirkung beschrieben, die zwischen Einzelschichtkultur (2D) und multizellulären Tumorsphäroiden (3D) verschiedener Krebszelllinien nach einer Behandlung entweder mit externer Strahlenbestrahlung oder gezielter Radioligandentherapie mit [^{177}Lu]Lu-PSMA-I&T beobachtet wurden.

Bei der externen Bestrahlung wurden Unterschiede in der Strahlenempfindlichkeit zwischen den verschiedenen Zelllinien festgestellt, wobei sich die Sphäroide im Vergleich zu den Einzelschichtkultur als strahlenresistenter erwiesen. Letzteres war besonders bei der Zelllinie PC-3 (Knochenmetastase des menschlichen Prostatakrebs) zu beobachten, bei welcher die Sphäroide bis zu 8 Gy strahlenresistent waren, während der Anteil der überlebenden Zellen in der Einzelschichtkultur nach 8 Gy Bestrahlung nur etwa 10 % betrug.

Die gezielte Radioligandentherapie mit [^{177}Lu]Lu-PSMA-I&T zeigte in beiden Modellen (2D und 3D) Spezifität für die Target-exprimierende Zelllinie LNCaP (Lymphknotenmetastase des menschlichen Prostatakrebs), aber einen höheren Behandlungseffekt in Sphäroiden im Vergleich zu Einzelschichtkultur. Letzteres lässt sich jedoch anhand einer Dosimetriesimulation unter Verwendung der Monte-Carlo-Methode

erklären, die eine höhere absorbierte Dosis in den Sphäroiden im Vergleich zu der Einzellschichtkultur ergab.

Darüber hinaus wurde externe Bestrahlung zur Charakterisierung von Verunreinigungen bei der Hochskalierung der Produktion von [^{177}Lu]Lu-PSMA-I&T eingesetzt. Die externe Bestrahlung, gefolgt von einer HPLC-MS-Analyse, führte zu der Schlussfolgerung, dass bei höheren Mengen an Radioaktivität die Radiolyse des Radiopharmakons zu einem deiodierten Produkt führt. Anschließend wurde die Produktionsmethode optimiert.

Zusätzlich wurde die Verwendbarkeit von Sphäroiden in einem etablierten Modell des befruchteten Hühnereies untersucht. Obwohl verschiedene Größen von Sphäroiden verwendet wurden, waren die resultierenden Xenotransplantate zu klein, um sinnvoll weiterverarbeitet werden zu können. Fortschritte auf dem Gebiet der 3D-Zellkultur könnten jedoch in Zukunft zu besseren Ergebnissen führen.

Zusammenfassend lässt sich sagen, dass diese Arbeit Unterschiede zwischen 2D- und 3D-Zellkultursystemen für die Entwicklung von Radiopharmazeutika belegt und die Verwendbarkeit von Sphäroiden nachweist. Die daraus resultierenden Unterschiede in der Versuchsplanung und der Interpretation der Ergebnisse sind bei der Anwendung von externer Bestrahlung oder gezielter Radioligandentherapie zu berücksichtigen.

TABLE OF CONTENTS

Acknowledgements	i
Abstract	iii
Zusammenfassung	v
1. INTRODUCTION	3
1.1 Cancer: Incidence and Hallmarks	3
1.2 Cancer therapies.....	4
1.2.1 Radiotherapy	5
1.3 Development of radiopharmaceuticals for targeted therapy	7
1.3.1 Selection of suitable target and ligand	7
1.3.2 Selection of radionuclide.....	8
1.3.3 Examples of TRT in clinical use	9
1.4 Principles of Molecular Radiobiology.....	11
1.4.1 Damage and radiobiological effectiveness	11
1.4.2 DNA repair	11
1.4.3 Cell death mechanisms.....	12
1.4.4 Senescence	13
1.4.5 Radiobiological assays	14
1.5 Three-dimensional (3D) cell culture.....	17
1.5.1 Multicellular tumor spheroids	17
1.5.2 Different techniques for spheroid generation	18
1.5.3 Spheroids in literature	19
1.6 In ovo model	20
2. AIM & RATIONALE FOR SELECTION OF MANUSCRIPTS.....	23
3. SCIENTIFIC PART	25
3.1 Author contributions	25
3.1.1 Manuscript 1	27
3.1.2 Manuscript 2	50
3.1.2 Manuscript 3	65
3.2 Unpublished results	81
4. SUMMARY AND DISCUSSION	89
5. CONCLUSIONS AND FUTURE CONSIDERATIONS	92

Bibliography 93

List of abbreviations 101

List of figures 103

List of tables 104

List of equations 104

List of peer-reviewed publications..... 105

1. INTRODUCTION

1.1 Cancer: Incidence and Hallmarks

After cardiovascular diseases, cancer is the second leading cause of death in the world. According to the Global Cancer Observatory of the World Health Organization, nearly 10 million people died in 2020 worldwide due to cancer. The most common types of cancer in women are breast, colorectal and lung cancer, whereas lung, prostate and colorectal cancer are most common among men. [1]

To define cancer and the underlying characteristics, Hanahan and Weinberg first introduced the hallmarks of cancer in the year 2000. Originally, six hallmarks were stated, describing functional assets, which distinguish a cancer cell from a normal cell. The six hallmarks were the following:

- (1) Sustaining proliferative signaling
- (2) Evading growth suppressors
- (3) Activating invasion and metastasis
- (4) Enabling replicative immortality
- (5) Inducing angiogenesis
- (6) Resisting cell death

Over the years, this list was extended. In 2011, Hanahan and Weinberg introduced the next generation by adding two emerging hallmarks: (7) Deregulating cellular energetics, and (8) Avoiding immune destruction, as well as two enabling characteristics: (9) Genome instability and mutation, and (10) Tumor-promoting inflammation. [2]

In 2022, Hanahan then added new dimensions, resulting in the latest version of the hallmarks of cancer (depicted in Figure 1). He included (11) Unlocking phenotypic plasticity, (12) Non-mutational epigenetic programming, (13) Polymorphic microbiomes and (14) Senescent cells. [3]

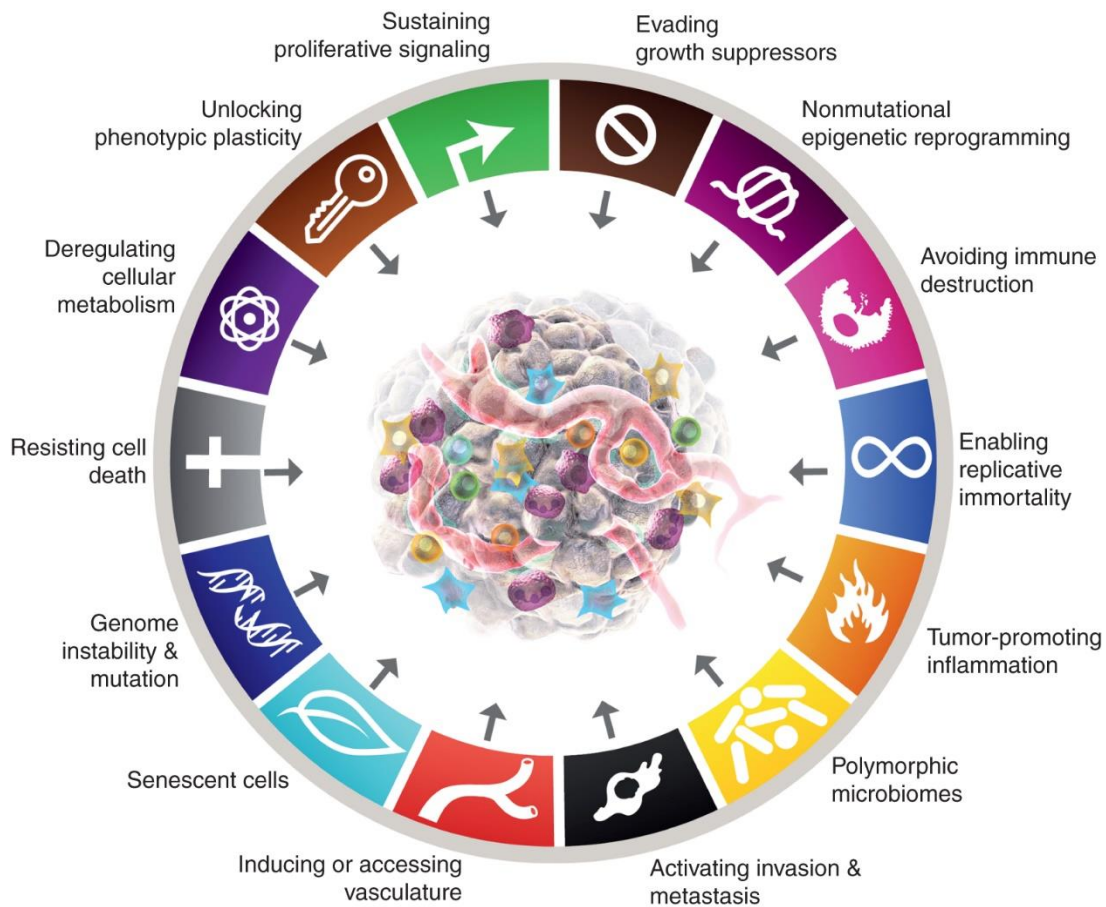


Figure 1: Latest version of the hallmarks of cancer. Graphic reproduced with permission from Cancer Discovery [3].

1.2 Cancer therapies

Over the decades, a variety of cancer therapies has been investigated and introduced, in order to personalize patient treatment. Figure 2 depicts the different types of cancer treatment regimens available, ranging from surgery, over chemotherapy up to immunotherapy.

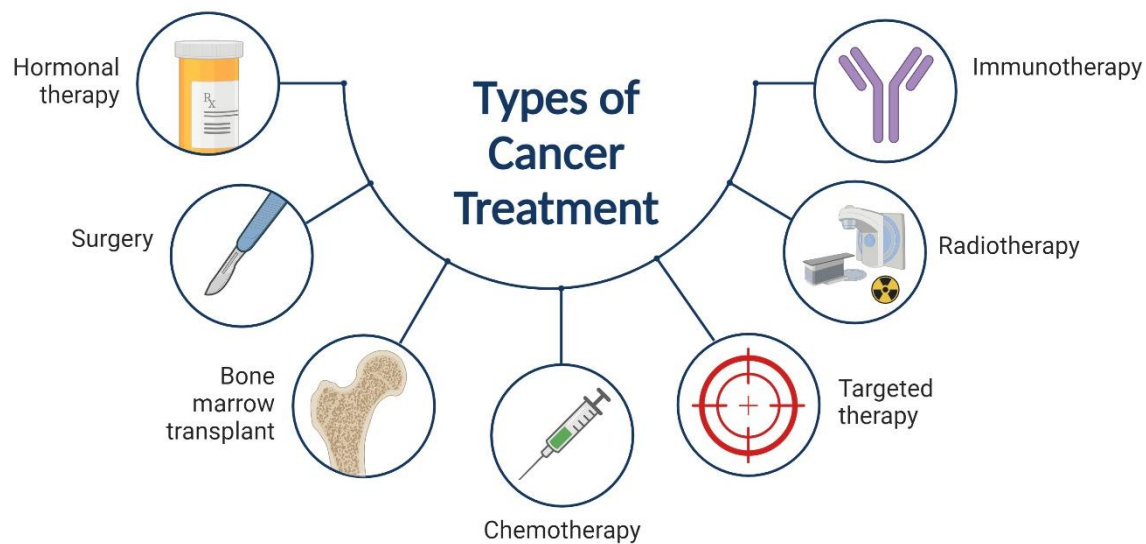


Figure 2: Types of Cancer Treatment. Created with BioRender.com (adapted template).

After surgical removal of the tumor, chemotherapy and radiotherapy are still the most common treatment options. Besides those, immunotherapy and gene therapy, among others, are emerging fields. [4] Herein, the focus lies on radiotherapy and its various application forms.

1.2.1 Radiotherapy

In general, there are two main types of radiotherapy to be distinguished:

- 1) external beam radiation therapy (EBRT) and
- 2) internal radiation therapy (IRT).

They can both be used pre-surgically for downsizing the tumor, post-surgically for the avoidance of recurrence, or to improve quality of life in palliative treatment [5].

In EBRT, a high-energy collimated beam of ionizing radiation is applied from a source outside of the patient's body. Different particles can be used, such as photons, protons or electrons, depending on the type and location of the tumor. [6]

In contrast, IRT is performed by transporting the radiation source directly into the body of the patient. The radiation source can either be solid (Brachytherapy) or liquid (systemic therapy). Regarding Brachytherapy, radioactive seeds, wires or capsules are brought to the tumor site, leading to local irradiation and therapy while sparing the rest of the body. Is the radioactive substance a radiopharmaceutical in a parenteral or peroral formulation, systemic radiation therapy is applied. [5,6] This kind of treatment is mostly utilized in the case of disseminated diseases, metastases, or when the tumors are located in close proximity to sensitive organs. A commonly applied form of systemic treatment is targeted radionuclide therapy (TRT). [7]

Targeted radionuclide therapy TRT is described by using molecules that specifically target cancer cells which are equipped (radiolabeled) with a therapeutic radionuclide. The vector can be for example, a peptide or monoclonal antibodies. The selective accumulation within the tumor and its close environment leads to cytotoxic radiation primarily in the tumor, causing only limited damage to healthy tissue. [7,8]

In Table 1, the respective characteristics of EBRT and TRT are compared.

Table 1: Characteristics of EBRT vs TRT

	EBRT	TRT
Particle type	photons, electrons, protons	alpha, beta
Radiation dose	high	low to medium
Exposure	short	long
Dose rate	high	low
Linear energy transfer	low	low and high
Dose distribution	homogenous	heterogenous
Treatment regimen	localized disease	metastatic disease

Another difference to consider is tumor vascularization, which is highly relevant for systemic therapy in contrast to EBRT.

1.3 Development of radiopharmaceuticals for targeted therapy

In order to achieve a therapeutic effect and simultaneously sparing healthy tissue, several aspects need to be taken into account. First of all, a suitable target and ligand need to be determined, before a suitable radionuclide is chosen. The respective considerations are described below.

1.3.1 Selection of suitable target and ligand

In general, a radiopharmaceutical consists of two parts, the radionuclide (radioactive label), and the ligand, targeting a specific protein. Once, a target with high abundance, accessibility and specificity is found, the following aspects, regarding the ligand, are of high importance and therefore need to be addressed:

- 1) biologic specificity,
- 2) affinity to target,
- 3) selectivity for the target,
- 4) chemical stability,
- 5) possibility to radiolabel without impeding the characteristics, and
- 6) biologic half-life of carrier molecule $\leq$ physical half-life of the radionuclide. [9]

There is a large variety of assays to evaluate target, radioligand as well as target-ligand interaction. As we described previously [10], *in vitro* binding studies can serve to provide information about association and dissociation rates between ligand and target. Further, there is a variety of assays to assess the subsequent subcellular localization of the radiopharmaceutical [11].

1.3.2 Selection of radionuclide

For appropriate therapeutic efficacy, alpha particle, beta particle or Auger electron emitters can be utilized. The intensity of energy transfer (in keV) thereby depends on energy, charge and mass of the particle and is described by the term linear energy transfer (LET). [9] The characteristics of the different modes of decay are given in Table 2.

Table 2: Characteristics and examples of therapeutic radionuclides. Adapted from [9,12].

Mode of decay	Particles	Range	LET	Radionuclide examples
Alpha (α)	helium nuclei	50-100 μ m	High (50-230 keV/ μ m)	Ac-225, Ra-223
Beta (β)	electrons	1-12 mm	Low (0.2 keV/ μ m)	Lu-177, I-131
EC/IC*	Auger electrons	Few nm	Medium to high (4-26 keV/ μ m)	In-111, I-125

* EC= electron capture, IC= internal conversion

Since beta radiation is sparingly ionizing and not very effectively damaging the DNA, their use in therapy requires high radionuclide concentrations within the target tissue. However, due to their long range, cells that are not expressing the target but are near a target cell are simultaneously irradiated. This so-called cross-fire effect thus allows for a certain degree of heterogeneity in target expression within the tumor. Cumulative cross-fire effects can then even lead to cytotoxic doses. [9] Furthermore, radiation induced bystander effects and abscopal effects can lead to responses in non-irradiated neighboring cells or remote lesions, respectively. The effects rely on signals, which are received from irradiated cells. [13]

While approximately 20.000 beta particles need to traverse the nucleus to kill a cell, the high LET of alphas leads to the same effect by only a single traverse [14–16], by efficiently

inducing chromosomal aberrations and double strand breaks (DSBs). The high LET and short range of alphas further aids to high energy deposition at the targeted tissue, and only minimal irradiation of the surrounding healthy tissue.

In the case of Auger electron emitters, their amount and position are determining the extent of radiotoxicity. It is believed that highest cytocidal effects are reached, when the emitting radionuclide decays in the cell nucleus, near the DNA, whereas decays in the cytoplasm or membrane are less effective. [9] However, the cell membrane and mitochondria are recognized as potent extra-nuclear targets for radiotherapy due to their central roles for cellular functioning [7].

When utilizing isotopes with multiple decays, as Ac-225, decay spectrum of the daughter radionuclides needs to be considered and whether there might be a change in affinity for the chelator with possible detachment. This is especially relevant, if the radioactivity is not trapped inside the cell but rather extracellular. [7,13] Another important factor to consider is also the physical half-life of the radionuclide as higher dose rates delivered over a short treatment time are usually more effective. [9]

Taking all these considerations into account, chemical synthesis, stability tests and further *in vitro* assays can follow. Providing successful *in vitro* studies, *in vivo* assessments of the radiopharmaceutical are conducted, with possible clinical studies and approval by the authorities in the long run. In the following, examples of successful radiopharmaceuticals in TRT are given.

1.3.3 Examples of TRT in clinical use

One of the simplest and earliest used targeting agents is Iodine-131 administered as sodium iodide in case of differentiated thyroid cancer. [9,17] Similarly chemically simple, Ra-223 dichloride (Xofigo®) is an approved drug against bone metastasis in metastatic castrate resistant prostate cancer (mCRPC). [18,19]

A more advanced type of TRT is the Peptide Receptor Radionuclide Therapy (PRRT). The target is usually a peptide receptor and therefore peptides are used as targeting ligands. In neuroendocrine tumors (NETs), the somatostatin receptor (SSTR) is targeted. For the

treatment of well differentiated gastrointestinal NETs, [^{177}Lu]Lu-DOTATATE, an SSTR agonist, was approved by the European Medicines Agency (EMA) in 2017 and the US Food and Drug Agency (FDA) in 2018, where it is considered to be a first-in-class medication (Lutathera® = lutetium (^{177}Lu) oxodotreotide) [20–22].

In mCRPC, a specific protein, the prostate-specific membrane antigen (PSMA; type II transmembrane glycoprotein) is highly overexpressed, making it a suitable target for PRRT. [^{177}Lu]Lu-PSMA-617 recently got approved by EMA and the FDA (Pluvicto® = lutetium (^{177}Lu) vipivotide tetraxetan; 2022) [23,24]. However, the better available [^{177}Lu]Lu-PSMA-I&T has also proven to be highly effective and safe for the treatment of advanced prostate cancer patients. [25,26] In the further development, other radionuclides, such as Ac-225, are being investigated, showing promising results [27].

The first commercially available targeted radioimmunotherapy with monoclonal antibodies was Zevalin® (ibritumomab tiuxetan), which targets the CD20 antigen and is used in the treatment of Non-Hodgkin lymphomas. [28,29]

In clinical practice, often a theranostic (=therapeutic + diagnostic) approach is used. For diagnostic purposes, positron emission tomography (PET) together with computed tomography (CT) or magnetic resonance tomography (MRT) is performed, either using the same element or the diagnostic pair. In the case of therapy with Lu-177, often Ga-68 is used for the diagnostic PET-scan. With this approach, prior imaging can give insights into the tumor topography as well as spatial dosimetry in order to develop the optimal treatment regimen following the principle: you see what you treat, you treat what you see. [8]

However, the development path from the first idea to the clinically available drug is long. An important early step in the development of specifically therapeutic radiopharmaceuticals is the justification of the treatment and the justification of the side effects. Radiobiological assays investigating cell survival and replicability of target tumor cells, are a prerequisite for subsequent preclinical and clinical investigations. The following chapter will focus on the principles of molecular radiobiology and the corresponding assays used in this field.

1.4 Principles of Molecular Radiobiology

1.4.1 Damage and radiobiological effectiveness

When ionizing radiation (IR) interacts with matter, either direct or indirect effects can lead to biological damage. Direct effects are caused by interaction with critical molecules such as DNA, proteins or lipids, leading to alterations of the respective biomolecule. In contrast, indirect effects originate from the radiolysis of water. The resulting reactive oxygen species (ROS) are highly reactive and produce biological damage when interacting with the above-mentioned critical targets. In the case of high LET radiation, direct effects are the main mode of action, while low LET radiation foremost leads to indirect effects. [17]

The relative biological effectiveness (RBE) thereby increases with increasing LET until about 100 keV/μm, whereafter the so-called overkilling effect comes into place and RBE decreases. Moreover, RBE is also dependent on radiation dose, dose rate, radiation quality, fractionation, biological system, and the measured endpoint. [17]

In general, the most effective damages are damages to the genome, such as DNA single strand breaks (SSBs), double strand breaks (DSBs), damages of sugar and bases or DNA cross-links between two bases of the same strand or with surrounding molecules. [9,30] Especially in terms of particle radiation, clustered DNA lesions are found.

However, during cell division, processes of a regulated network of proteins, known as the DNA damage response (DDR), get active.

1.4.2 DNA repair

The most common fundamental repair process is base excision repair (BER). During BER, single-nucleotide base lesions are removed from the genome. [31] With regard to radiotherapy, also nucleotide excision repair (NER) is relevant. Thereby, helix-distorting DNA lesion, such as bulky adducts are removed from the DNA. The Mismatch Repair (MMR) pathway detects and repairs incorrect deletions, insertions or base substitutions. For the repair of the most lethal damages, DNA double strand breaks, two mechanisms can be employed: homologous recombination (HR) and non-homologous end joining

(NHEJ). While HR uses the undamaged sister chromatid as a template, NHEJ eliminates the damaged part and performs direct ligation. [30] However, the error-free HR can only be performed in the late S and G2 stage of the cell cycle, since an undamaged template is needed, whereas the more error-prone NHEJ has no phase restriction but shows increased activity when cells progress from G1 phase [7,30,32].

Despite many different repair systems, sometimes not all the damage can be removed, which can lead to mutations or induction of cell death. This happens more frequently in cancer cells, as they have a higher proliferation rate as well as defects in the DDR. [33]

1.4.3 Cell death mechanisms

The way of cell death is influenced by different factors:

- 1) radiation properties (radiation quality, dose and regimen),
- 2) cellular properties (cell type, proliferation, metabolism, etc.) and
- 3) cellular microenvironment.

A variety of different mechanisms leading to cell death are known.

Mitotic cell death/ Mitotic catastrophe

The mitotic cell death is one of the most common mechanisms of IR induced cell death. It can be triggered by misrepair or failed repair of DNA damages and is described as cell death prior or during mitosis. It is characterized by altered nuclear morphology, micronucleation and formation of multinucleated cells. The cell cycle cannot be completed, and cells either die via apoptosis or necrosis, or become senescent. [30,34]

Apoptosis

Apoptosis is a highly controlled programmed cell death, which can be triggered upon exogenous or endogenous signals and is executed via intrinsic, extrinsic or membrane stress pathways. [30] The morphological changes during apoptosis include cellular

shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation. [34] While low doses of IR are suggested to induce apoptosis, higher doses rather induce necrosis. Further, apoptosis is considered as less immunogenic compared to necrosis [33].

Necrosis

Necrotic cell death, in contrast, can be categorized into either accidental cell death or regulated necrotic cell death, which can further be differentiated into the following subtypes: ferroptosis, necroptosis, pyroptosis, among others. However, all types involve immunogenic cell death. Necrosis can further be a secondary process after apoptosis, if the apoptotic cells are not properly cleared by other cells, resulting in plasma membrane disintegration. [30,34]

Autophagy

Autophagy is a cytoprotective mechanism, that can be triggered by irradiation. The complex mechanism results in self-digestion, providing nutrients for vital cellular functions and promoting radioresistance. However, attempts to inhibit autophagy for radiosensitization were not successful. [30,35]

1.4.4 Senescence

An interesting process, where the cell does not die, remains metabolically active, but stops dividing due to irreversible cell cycle arrest, is known as senescence. [33] It is characterized by an enlarged and flattened cellular morphology, as well as increased granularity. [34,36] Since 2022, senescence is also a hallmark of cancers [3], as previously shown in chapter 1. Regarding TRT using Lutetium-177, O'Neill et al. suggested that senescence also plays a detrimental role in the treatment efficacy and therefore might be a desirable outcome of TRT. [37]

1.4.5 Radiobiological assays

In order to specifically assess the effects of ionizing radiation on cells, different biomarkers can be utilized. In the following part, some of the most important assays are described.

Micronucleus assay

Mis- or unrepaired DNA damages can result in asymmetrical chromosome rearrangements and thereby lead to the formation of extranuclear bodies, containing chromosome fragments or whole chromosomes. These structures are commonly known as micronuclei (MN). For evaluations of cytotoxicity as well as genotoxicity, micronuclei assays can be performed. Depending on the type of cell, a diverse range of assays can be utilized. In general, MN are scored either by eye or automatically using microscopy or flow cytometry. [30]

Comet assay

For the detection of DNA strand breaks, a comet assay can be performed. Specifically, a single cell gel-electrophoresis is conducted. In short, treated single cells are embedded in a thin agarose layer on a microscope slide. Then the cell is lysed in neutral or alkaline conditions, DNA is unwound, followed by electrophoresis and staining. Damaged DNA (tail) is thereby separated from the intact DNA (head), resulting in a comet. Scoring can be performed manually or automatically with the use of imaging software. [38]

Foci assays

Ionizing radiation-induced foci are specific biomarkers of DSBs and can therefore be used for the assessment of DNA damage induction and repair. A commonly used method is the visualization of γ H2AX. [39] By generation of DSBs, the histone H2A variant H2AX is phosphorylated at Ser-139, providing an abundant, fast and sensitive marker for radiation damage. [40] Foci start to form a few minutes after radiation exposure and reach the highest level at around 0.5 to 1 h after irradiation. DSBs can be quantified and used for

dosimetry. Similarly to γ H2AX, foci of the p53-binding protein 1 (53BP1) can be utilized [41,42]. The respective foci colocalize and can be used simultaneously, if DSBs cluster extensively, at high background signal or low radiation dose. However, due to their specific signaling, not necessarily all foci colocalize. [30]

Oxidative stress assays

IR can induce oxidative stress by generation of high amounts of ROS or reactive nitrogen species (RNS) in the cell, which cannot be scavenged by the available amount of antioxidants. This further leads to different kinds of damages, such as mitochondrial respiratory chain impairment and consequently cell death. To assess the amount of oxidative stress, different biomarkers can be used. An example is the measurement of S-glutathionylation of proteins. [30] By now, also diverse oxidative stress detection kits are existing, utilizing dyes that exhibit for example fluorescence upon oxidation [43].

Dose-response assays: Viability vs. Survival assays

In order to assess dose-response, different *in vitro* assays can be performed. However, it is important to distinguish between cell viability assays and cell survival assays. [30] As described before, senescence is a cellular response, where the cell does not replicate but still stays alive. These kinds of cells are detected as viable in cell viability assays but will be detected as not replicating in survival assays. Hence, both assays are needed to distinguish between proliferating viable cells and senescent cells.

Viability assays can be performed in many different ways. The most common type are probably dye exclusion assays [44], where only dead cells are stained, due to lack of membrane integrity. Furthermore, there are diverse kits on the market, utilizing for example adenosine triphosphate (ATP) as a surrogate for viability (e.g., CellTiter-Glo® [45]) .

The gold standard in survival assays is the clonogenic assay, also known as colony survival assay. [30,46] In short, cells are evenly and sparsely seeded into plates, treated, and afterwards incubated to let them grow for several weeks. After sufficient colony growth

time (depending on cell proliferation rate), colonies are washed, fixated, and stained. Stained colonies are counted by eye or automatically and the surviving fractions (SF) to the corresponding doses (D) are calculated, yielding a cell survival curve. The survival curve is generated by fitting a linear quadratic (LQ) model (see Equation 1).

$$SF = \exp(-\alpha D - \beta D^2) \quad (1)$$

The LQ model is used to predict the response using the surviving fraction. While the linear component (αD) describes lethal (single hit needed to kill the cell) damages, the quadratic component (βD^2) describes sublethal (two hits needed to kill the cell) damages. Therefore, alpha is the slope at low doses and beta the slope of high doses. The ratio (α/β) determines the position of the shoulder, giving information on the capacity of the cell to repair the damage. The higher the LET, the straighter the resulting curve. [7,9,17,30] Extensions of the LQ model also include corrections for protracted exposures: the repair between tracks (G) and repopulation (γ), see Equation 2.

$$SF = \exp(-\alpha D - G\beta D^2) \exp(\gamma t) \quad (2)$$

However, this model can only be used for the application of uniform absorbed doses in the cells and the assumption of identical radiosensitivity, repair and repopulation for all cells. [47]

Dosimetry

In contrast to *in vitro* dose-response assays, computational dosimetry assessments can also predict the radiobiological effects of IR by tracing back the microscopic patterns of energy deposition. [48] The special field of microdosimetry “systematically studies the spatial, temporal and spectral aspects of the stochastic nature of the energy deposition processes in microscopic structures” [49]. Cell-specific morphologies are considered, as

well as realistic activity distributions and cross-dose from clusters of proliferating cells. It enables the prediction of the resulting biological effects as surrogate for DSB induction.

In addition, mathematical models like Monte Carlo (MC) simulations, have gained increasing interest in radiotherapy dosimetry. [50] In the MC method, numerical calculations are utilized for dose estimations. The stochastic events through which energy is deposited by radiation, are simulated.

However, the use of empirical tools also inherits limitations, making knowledge of the underlying radiobiological pathways of TRT essential to move towards mechanistic modeling in the future.

1.5 Three-dimensional (3D) cell culture

Up until now, most of the assays used in the development and assessment of new radiopharmaceuticals are performed using 2D cell culture in form of monolayers, which are well-established and have proven to be simple, fast and feasible. However, 2D cell culture also has some limitations. Specifically, cultivation of cells in monolayers leads to unnatural cell shapes and exposure to nutrients or drugs is equally distributed, not complying with *in vivo* tumors. Further, there is a lack of cell differentiation and cell-to-cell communication [2]. 3D cell culture can overcome some of the limitations. The term 3D cell culture comprises a whole set of different models, from simple multicellular tumor spheroids, generated from one cancer cell line, over to co-culturing methods using different cell types up until patient-derived organoids. Depending on the complexity of the model, expenses in terms of time and money are hugely different. The presented thesis focuses on the most basic form of multicellular tumor spheroids, described in the following section.

1.5.1 Multicellular tumor spheroids

In 1943, Holtfreter first introduced three-dimensional multicellular tumor spheroids [51]. They showed to be superior to monolayers not only in terms of mimicking *in vivo* tumor

structure (necrotic core, quiescent zone, proliferating outer layer) but also growth behavior, among others. [52–54] The necrotic core consists mostly of dead cells, due to the lack of nutrients and hypoxic conditions. Cells in the quiescent zone are viable but not proliferating, which is the case in the outermost zone, the proliferating zone. In Figure 3, an exemplary LNCaP (lymph node metastasis of human prostate cancer) spheroid, stained with propidium iodide (necrotic core) and calcein acetoxymethylester (calcein-AM; viable cells), and the respective gradients of nutrients, metabolic waste, etc. are depicted. Lack of oxygen in the core of the spheroid, also known as hypoxia, is one of the main drivers of necrosis and is also commonly observed in *in vivo* tumors [55].

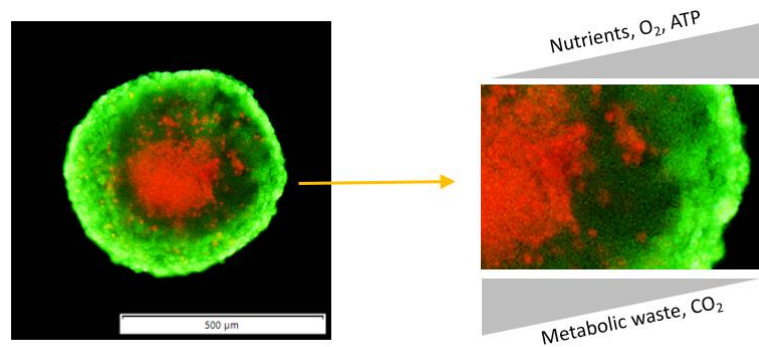


Figure 3: Structure of a multicellular LNCaP tumor spheroid, stained with propidium iodide (red; necrotic cells) and calcein-AM (green; viable cells). From the outermost layer (proliferating zone, green) to the center (necrotic core, red): decreasing gradient of nutrients, oxygen (O_2), and adenosine triphosphate (ATP); increasing gradient of metabolic waste and carbon dioxide (CO_2)

1.5.2 Different techniques for spheroid generation

In order to generate this type of 3D model, different techniques can be utilized. In general, they can be divided into:

- 1) Non-scaffold-based, and
- 2) Scaffold-based techniques.

Among the non-scaffold-based techniques are hanging drop [56], liquid overlay [57], magnetic levitation [58], 3D bioprinting, rotatory methods and microgravity bioreactors [59]. For the scaffold-based techniques, hydrogels made from collagen or Matrigel™, among others can be used. [11]

In the presented thesis, the liquid overlay technique was utilized. Using this rather simple, and comparably low-cost method, spheroids are produced highly reproducibly and are easily accessible. [60] Cells are seeded into non-adhesive, round bottom 96-well plates, which promote fast self-aggregation and spheroid formation. This method can be used for many different cell lines, however, not all cell lines are able to self-aggregate up to the extent of spheroid formation. In this case scaffolds need to be employed. For PC-3 (bone metastasis of prostate cancer), for example, we found that 3% Geltrex™ needed to be added to the medium to yield proper spheroids instead of loose aggregates. [61]

1.5.3 Spheroids in literature

Spheroids have been used in science for decades and there is a plethora of literature reviews on this method. [52,55,62–68] However, the use of spheroids in radiopharmaceutical development is limited until now. Previously, radiobiological assessments of radiopharmaceuticals in spheroids were performed with Ac-225 [69], Cu-64 [70], Pb-212 [71] and Ra-223 [72]. Most of the provided data result from the application of spheroids in drug testing of non-radioactive drugs or EBRT. Regarding EBRT, monolayers and spheroids of different cell lines were compared on a dose-response level, resulting into coining the term ‘contact effect’. [55,73–78] The contact effect describes the observed increased radioresistance of spheroids compared to the respective monolayer culture, after EBRT. [79] This was also reflected in our study, utilizing prostate and breast cancer cell lines [61].

1.6 *In ovo* model

One step further in the pipeline of radiopharmaceutical development is *in vivo* testing. In this context, the *in ovo* model has gained attention in nuclear imaging a decade ago. [80] Being an intermediate model between cell and animal experiments the *in ovo* model aims at the reduction of animal experiments in accordance with the 3Rs (Replacement, Reduction and Refinement) of animal testing [81]. In Austria and some other countries, ethical approval by the authorities is currently not required for the use of non-mammalian embryos if sacrifice takes place before hatching[82].

Specifically in the CAM-xenograft model, tumor cells are inoculated on the highly vascularized and not innervated chorioallantoic membrane (CAM), leading to vascularized xenografts. The characteristics of tumor growth, angiogenesis, metastasis as well as therapeutic efficacy can be investigated. [83] In general, the experimental procedure involves eggshell puncturing, cell inoculation, eventually treatment and sacrifice. [84–86] An exemplary procedure is depicted in Figure 4.

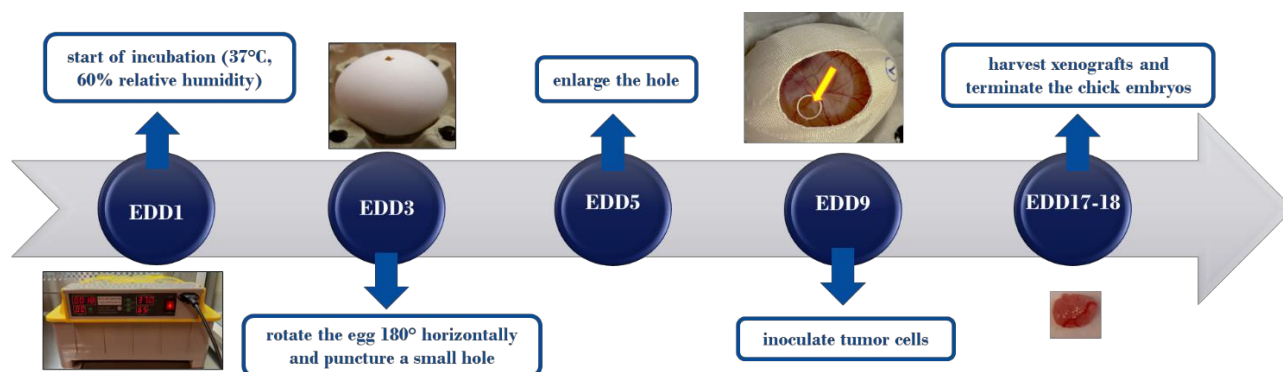


Figure 4: Exemplary experimental procedure of the CAM-xenograft model (adapted from Katarína Benčurová); EDD=embryo development day

Due to the easy accessibility of the CAM and low rejection rate of tumor grafts, it was already previously suggested to increase the complexity of the model by engrafting spheroids or organoids. [84] Focusing on spheroids, there is only a very limited number of publications existing. Dupertuis et al. described an *in ovo* method for the evaluation of

nutritional therapies utilizing engrafted spheroids, concluding that spheroid models *in ovo* provide a simple and reliable method for the assessment of dose-response analysis. [87] Further, Elberskirch et al. reported that the HET-CAM based tumor model leads to more possibilities for 2D and 3D cell culture models, also as a screening platform for photosensitizers. [88]

Regarding prostate cancer, the inoculation of cell suspensions was successful and is used, for example, with multi-modal PET/MR imaging for *in vivo* testing of target-specific radiopharmaceuticals. However, tumor cell density in the xenografts varied largely between the cell lines LNCaP and PC-3. [89] To improve tumor cell density in LNCaP xenograft, possibly the inoculation of 3D tumor spheroids could be of help.

2. AIM & RATIONALE FOR SELECTION OF MANUSCRIPTS

Researchers are constantly working on the development of new cancer therapies. In the field of nuclear medicine, the focus is currently shifting to targeted radiopharmaceuticals. To assess the effectiveness of new therapeutic radiopharmaceuticals, a radiobiological assessment is compulsory for further evaluation steps in a preclinical and clinical setting. In this cause, different assays are used to investigate cell viability, reproductive ability, and growth inhibition.

The aim of this thesis is to establish the methodology for radiobiological assessments of new therapeutic radiopharmaceuticals in-house and to further assess the usability, advantages, and limitations of monolayers (2D cell culture) and spheroids (3D cell culture) in the context of radiopharmaceutical development.

Therefore, the methodology was firstly set up using external beam to provide the ground truth with an established and validated system. In this course, the differences in radiation effects on 2D and 3D models of different human cancer cell lines were investigated. The corresponding work is presented in **manuscript 1** “Comparison of Radiation Response between 2D and 3D Cell Culture Models of Different Human Cancer Cell Lines”.

After contributing to the investigations of impurity formation in the upscaling process of [^{177}Lu]Lu-PSMA-I&T (**manuscript 2** “Quality Assurance Investigations and Impurity Characterization during Upscaling of [^{177}Lu]Lu-PSMA-I&T”), [^{177}Lu]Lu-PSMA-I&T was used to further develop the established methods for the use of radiopharmaceuticals and to assess the difference in radiation response between 2D and 3D cell culture models of prostate cancer. The latter is described in **manuscript 3** “Radiobiological Assessment of Targeted Radionuclide Therapy with [^{177}Lu]Lu-PSMA-I&T in 2D vs. 3D Cell Culture Models”.

In order to move towards *in vivo*, the use of 3D models was lastly also investigated in the *in ovo* model. The results are presented in the section **unpublished results**.

3. SCIENTIFIC PART

3.1 Author contributions

I hereby declare having significantly contributed to all peer-reviewed articles included in the presented thesis. My specific contributions for each publication are stated below.

*Concerning **manuscript 1**, I designed the experiments, established the methodology, supervised, and partly produced, analyzed and interpreted the data. Furthermore, I wrote and revised the manuscript:*

Raitanen J, Barta B, Hacker M, Georg D, Balber T, Mitterhauser M. Comparison of Radiation Response between 2D and 3D Cell Culture Models of Different Human Cancer Cell Lines. *Cells*. 2023; 12(3):360. <https://doi.org/10.3390/cells12030360>

*In terms of **manuscript 2**, I designed and performed the irradiation experiments, wrote the corresponding parts, and revised the manuscript:*

Schmitl S, **Raitanen J**, Witoszynskyj S, Patronas E-M, Nics L, Ozenil M, Weissenböck V, Mindt TL, Hacker M, Wadsak W, Brandt MR, Mitterhauser M. Quality Assurance Investigations and Impurity Characterization during Upscaling of [¹⁷⁷Lu]Lu-PSMA^{I&T}. *Molecules*. 2023; 28(23):7696. <https://doi.org/10.3390/molecules28237696>

*Regarding **manuscript 3**, I designed the study, supervised, acquired funding, partially conducted the experiments, analyzed and visualized the data. Furthermore, I interpreted the data, wrote, and revised the manuscript:*

Raitanen J, Barta B, Fuchs H, Hacker M, Balber T, Georg D, Mitterhauser M. Radiobiological Assessment of Targeted Radionuclide Therapy with [¹⁷⁷Lu]Lu-PSMA-I&T in 2D vs. 3D Cell Culture Models. *International Journal of Molecular Sciences*. 2023; 24(23):17015. <https://doi.org/10.3390/ijms242317015>

3.1.1 Manuscript 1

Raitanen J, Barta B, Hacker M, Georg D, Balber T, Mitterhauser M. Comparison of Radiation Response between 2D and 3D Cell Culture Models of Different Human Cancer Cell Lines. *Cells*. 2023; 12(3):360. <https://doi.org/10.3390/cells12030360>

Article

Comparison of Radiation Response between 2D and 3D Cell Culture Models of Different Human Cancer Cell Lines

Julia Raitanen ^{1,2,3,4} , Bernadette Barta ¹, Marcus Hacker ² , Dietmar Georg ⁵ , Theresa Balber ^{1,2}  and Markus Mitterhauser ^{1,2,4,*}

¹ Ludwig Boltzmann Institute Applied Diagnostics, 1090 Vienna, Austria

² Department of Biomedical Imaging and Image-Guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, 1090 Vienna, Austria

³ Vienna Doctoral School of Chemistry (DoSChem), University of Vienna, 1090 Vienna, Austria

⁴ Institute of Inorganic Chemistry, Faculty of Chemistry, University of Vienna, 1090 Vienna, Austria

⁵ Department of Radiation Oncology, Division of Medical Radiation Physics, Medical University of Vienna, 1090 Vienna, Austria

* Correspondence: markus.mitterhauser@univie.ac.at

Abstract: Radiation therapy is one of the most effective tools in cancer therapy. However, success varies individually, necessitating improved understanding of radiobiology. Three-dimensional (3D) tumor spheroids are increasingly gaining attention, being a superior in vitro cancer model compared to 2D cell cultures. This in vitro study aimed at comparing radiation responses in 2D and 3D cell culture models of different human cancer cell lines (PC-3, LNCaP and T-47D) irradiated with varying doses (1, 2, 4, 6, 8 or 20 Gy) of X-ray beams. Radiation response was analyzed by growth analysis, various cell viability assays (e.g., clonogenic assay, resazurin assay) and amount of DNA damage (γ H2AX Western Blot). Results showed decreasing cell proliferation with the increase of radiation doses for all cell lines in monolayers and spheroids of LNCaP and T-47D. However, significantly lower radiosensitivity was detected in spheroids, most pronounced in PC-3, evincing radiation resistance of PC-3 spheroids up to 8 Gy and significant growth inhibition only by a dose escalation of 20 Gy. Cell line comparison showed highest radiosensitivity in LNCaP, followed by T-47D and PC-3 in 2D, whereas, in 3D, T-47D showed highest sensitivity. The results substantiate the significant differences in radiobiological response to X-rays between 2D and 3D cell culture models.

Keywords: radiobiology; radiation oncology; radiosensitivity; external photon beam (X-ray); therapy; human cancer cell lines (PC-3; LNCaP; T-47D); multicellular tumor spheroids (3D); clonogenic assay; resazurin assay; γ H2AX



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1. Introduction

Radiation biology often provides the rationale for the implementation of new treatment strategies in clinical practice; hence, it is important to foster the basic understanding of radiation effects. Up until now, mostly (two-dimensional) 2D cell monolayers were used to assess therapeutic efficacy of radiation treatments in basic research. Although this technique has many advantages, being simple, cheap, and highly reproducible, it also comprises limitations when trying to compare obtained results to in vivo tumors. These limitations include unnatural cell shapes, the lack of cell differentiation and cell-to-cell communication, as well as the unnaturally equal exposure of cells to nutrients or drugs [1]. To overcome these limitations, and to revolutionize disease modeling and treatment testing, three-dimensional multicellular tumor spheroids were introduced in the early 1940s by Holtfreter [2,3]. In contrast to monolayer culture, multicellular tumor spheroids mimic in vivo tumors more accurately, due to similar growth behaviors as well as cellular heterogeneity within the spheroid and formation of molecular gradients, among others. In this context, promising results utilizing higher dimensionality models for

disease simulations and tumor-targeted therapies have been obtained [4]. Their specific characteristics mainly result from their layered structure, consisting of an inner necrotic core, which is surrounded by a quiescent zone and an outer layer of proliferating cells [3,5,6]. Over the years, different techniques for generating spheroids were investigated [7]. The most common methods are: liquid overlay [8], hanging drop [9], spinner flask [10] and magnetic levitation [11]. Due to simplicity, low costs and homogeneity in spheroid size and shape, the liquid overlay technique was used in this study [12].

In the early 1970s, Durand and Sutherland introduced the so-called contact effect when firstly noting the increased radioresistance of spheroids compared to monolayers after irradiation using V79 Chinese hamster lung cells [13]. Since then, this effect has been demonstrated for a number of other cell lines [14–19], with the exception of E.E. melanoma spheroids [20]. In this study, the widely used prostate cancer cell lines PC-3 and LNCaP, as well as the not-yet well described breast cancer cell line T-47D, were assessed. Cells were cultivated in 2D and 3D, and irradiated with varying doses (1, 2, 4, 6, 8 and 20 Gy) of X-ray irradiation. Since the existing radiobiological studies use only a limited number of assays, and generally lack data on long term effects, we utilized a variety of standardized assays to analyze the radiation response not only through the short term, but also through the long term (up to 29 days) effects. The aim of this study was to assess the differences in radiation response between monolayers and spheroids, and thereby determine the radiosensitivity of the different human cancer cell lines. To this, growth analysis, including necrotic core monitoring, and the commonly used clonogenic assay were performed. Cell viability was assessed using trypan blue exclusion assay, Cell Titer Glo® 3D and resazurin assay to complement the data. To further investigate molecular mechanisms, γ H2AX was investigated by Western Blot analysis.

2. Materials and Methods

2.1. Cell Cultures

Cell lines were obtained from the American Type Culture Collection (ATCC). PC-3 and LNCaP cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 2 mM Glutamine and 1% penicillin (10,000 U/mL)/streptomycin (10,000 μ g/mL). T-47D cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum and 4 mM Glutamine. All reagents were obtained from Gibco Life Technologies Ltd. (Paisley, UK). Cells were cultured at 37 °C with 5% CO₂ and tested routinely for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland), according to the manufacturer's instructions [21].

2.2. Generation of Spheroids

Spheroids were generated using the liquid overlay technique. 2000 cells/well (in 100 μ L medium) were seeded into U-bottom 96-well plates, precoated with a thin layer of 1.5% low gelling agarose (Sigma Aldrich, St. Louis, MO, USA). LNCaP and T-47D were cultured scaffold-free, whereas 3% Geltrex™ (LDEV-Free Reduced Growth Factor Basement Membrane Matrix, Gibco, Paisley, UK) was added to the PC-3 cell suspension, followed by centrifugation at $161 \times g$ for 10 min at 4 °C. During spheroid establishment, any influence of Geltrex™ on radiosensitivity was excluded (see Supplementary Figure S1).

2.3. Irradiation Procedure

X-ray irradiation (1, 2, 4, 6, 8 or 20 Gy absorbed dose) was performed at 200 kV, 20 mA with a focus size of 5.5 mm using an YXLON reference irradiator (Maxishot, YXLON International GmbH, Hamburg, Germany) with a 0.5 mm copper filter and a 3 mm aluminum filter. Before exposure, dose rate was measured with a cross calibrated ionization chamber (Semiflex TM31012; PTW, Freiburg, Germany). The average dose rate at the level of the respective tumor model systems was about 1.2 Gy/min [22].

2.4. Microscopic Growth Analysis

Spheroid growth was observed by both visual inspection and size measurements using an Olympus IMT-2 Inverted Microscope with an XC50 Color Camera (Olympus, Tokyo, Japan) and the cellSens Entry (Version 2.3, Olympus, Tokyo, Japan) software on days 3, 5, 8, 9, 11, 13, 15, 19, 23 and 29 after seeding. Media were renewed once per week. Per experiment, one representative spheroid per cell line and radiation dose was chosen, and a mean value of three diameter measurements was calculated and plotted with Prism Version 8 (GraphPad, San Diego, CA, USA).

2.5. Live/Dead Staining—Necrotic Core Examination

Spheroids were washed with 100 μ L phosphate buffered saline (PBS), and afterwards incubated with propidium iodide (Sigma-Aldrich, St. Louis, USA; 1:500 in medium) for 5 h 45 min followed by the addition of calcein acetoxymethyl ester (Sigma-Aldrich; 4 mM in dimethyl sulfoxide, 1:800 in medium) with further incubation for 15 min. Spheroids were then transferred to flat-bottom 96-well plates, washed with PBS and imaged under an IMT-2 Inverted fluorescence Microscope (Olympus) equipped with a 100 W Mercury Power Supply (Model BH2-RFL-T3, Olympus) for fluorescence detection. Diameters of total spheroid and necrotic core were measured using the Digimizer software version 5.7.2 (MedCalc Software Ltd, Ostend, Belgium).

2.6. Clonogenic Assay

Cell survival and reproductive ability after irradiation were determined by performing standard clonogenic assays [23]. 250 PC-3/T-47D or 1000 LNCaP cells were seeded in 6-well plates prefilled with 2 mL of respective medium. On the following day, plates were irradiated with 1, 2, 4, 6 or 8 Gy. After irradiation, PC-3 cells were further cultured for 7 days, T-47D for 14 days and LNCaP for 21 days, with their media being changed once per week. After the respective culturing periods, cells were washed with PBS, fixed with methanol for 30 min at 4 °C and, after another washing step, stained with crystal violet (Sigma-Aldrich; 1% in water) for 3 min. To assess the reproductive ability, stained colonies were counted using a Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany). Three independent experiments were performed in triplicate for each cell line. Plating efficiency was calculated by dividing the average number of colonies in the control by the number of seeded cells. Plating efficiency was then multiplied with the number of colonies after treatment, resulting in the Surviving fraction (SF). Coefficients (α , β) were determined by linear quadratic curve fitting using Equation (1). D corresponds to radiation dose in Gray (Gy).

$$SF(D) = \exp(-\alpha D - \beta D^2) \quad (1)$$

2.7. Trypan Blue Exclusion Assay

Cells were seeded in 6-well plates: 500,000 for evaluation after 0 and 24 h, 100,000 PC-3 or 250,000 LNCaP/T-47D for evaluation after 72 h. After overnight incubation, cells were irradiated with single doses of 2, 4, 8 and 20 Gy. After the aforementioned time points, supernatants, wash fraction and harvested cells were combined and centrifuged for 4 min at $161 \times g$ at room temperature. The supernatant was discarded, and the cell pellet was resuspended in medium. Single cell suspensions were mixed with Trypan blue (1:1; Gibco) to determine cell viability and cell counts, using the LUNATM automated cell counter (Logos Biosystems, Anyang, Republic of Korea).

2.8. Resazurin Assay

Cells were seeded as monolayers in 6-well plates with respect to their growth rate (2000 PC-3, 60,000 LNCaP and 10,000 T-47D), incubated for 2 days and irradiated with 0, 2, 4 or 8 Gy. Cell monolayers were further cultivated under standardized conditions (see Section 2.1) for up to 19 days. On days 5, 13 and 19, the supernatant and detached cells were combined and the liquid volume to seed a total of 5000 cells was calculated from

the control. This volume was seeded for all treatment conditions (in total 200 μL /well) in 96-well plates. The next day, cells were incubated with 20 μL resazurin (110 $\mu\text{g}/\text{mL}$ in PBS; TCI, Tokyo, Japan) for 17 h. Fluorescence detection was performed using a Synergy HTX multimode reader (Ex590/Em645; Biotek, Winooski, VT, USA).

In 3D, irradiated spheroids were transferred into flat-bottom 96-well plates and incubated with resazurin (17 h) one day prior to fluorescence detection on days 7, 15 and 21, as described before.

2.9. Cell Titer Glo[®] 3D

The assay was performed according to the manufacturer's protocol [24]. On days 6, 14 and 20, control and irradiated spheroids were transferred to white-walled 96-well plates (100 μL medium/well). Cell Titer Glo[®] 3D reagent (Promega, Madison, WI, USA) was thawed in the fridge overnight before use. The next day, the plate and reagent were equilibrated to room temperature before the addition of 100 μL reagent per well. Afterwards, the plate was vigorously shaken for 5 min using the Synergy HTX multimode reader (Biotek, Winooski, VT, USA) and incubated at room temperature for another 25 min before measurement of luminescence.

2.10. Western Blot

The amount of DNA double-strand breaks in 2D and 3D cultures was assessed by determining γH2AX as surrogate. Whole cell lysates of monolayers and spheroids were prepared directly after irradiation. For monolayers, 1 million cells were seeded in petri dishes one day prior to irradiation. Lysates were obtained by mechanical scraping using 300 μL radioimmunoprecipitation assay (RIPA) lysis buffer (Gibco) together with a protease inhibitor (P2714 Sigma Aldrich, 1:10 after reconstitution in 10 mL water), shaking for 30 min on ice before centrifugation at $13,523\times g$, 4 $^{\circ}\text{C}$ for 20 min. For 3D lysates, 20 spheroids were pooled and centrifuged at $135\times g$ for 4 min at room temperature. After washing with phosphate buffered saline, 60 μL RIPA lysis buffer and a protease inhibitor were added and further treated as described for monolayers.

Protein concentrations were determined using the Pierce[™] BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated on Mini-PROTEAN TGX precast Gels (Bio-Rad Laboratories Inc., Berkeley, CA, USA) with a running buffer consisting of 3 g Tris(hydroxymethyl)aminomethane (TRIS), 14.5 g Glycine and 1 g Sodium dodecyl sulfate (SDS), dissolved in distilled water. Semi-dry blotting was performed on a Trans-Blot[®] Turbo[™] Transfer System (Bio-Rad) using a Trans-Blot Turbo 5 $\times$ Transfer Buffer (Bio-Rad) and a nitrocellulose membrane (Amersham[™] Protran[™] Premium 0.2 μm ; GE Healthcare, Chicago, IL, USA).

Membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) in TRIS buffered saline with Tween (TBS-T; Sigma-Aldrich) on a shaker for 1 h and at room temperature. Primary antibody incubation (anti-phospho-Histone H2A.X (Ser139), clone JBW301 (Merck, 1:1000 in 5% BSA in TBS-T), β -Tubulin antibody (Cell Signaling Technology; 1:1000 in 5% BSA in TBS-T) was performed overnight at 4 $^{\circ}\text{C}$. Incubation with the respective horseradish peroxidase (HRP) conjugated secondary antibodies was performed for 1 h and at room temperature before detection of chemiluminescence (Pierce[™] ECL Western Blotting Substrate Kit; Thermo Fisher Scientific, Waltham, MA, USA) and a ChemiDoc Imaging System (Bio-Rad), according to the manufacturer.

Band intensities were determined using ImageJ, ratios were calculated and plotted using the Prism software version 8 (GraphPad).

2.11. Statistical Analysis

Data was analyzed using Prism (GraphPad). Data comparison was performed by multiple unpaired *t*-tests. *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Cell Viability: Trypan Blue Exclusion Assay

Cell viability in monolayers, evaluated by Trypan Blue Exclusion Assay, showed no significant change in viability when measured immediately (0 h) and 72 h after irradiation up to 8 Gy (Figure 1A,B). However, a decrease in cell numbers with increasing radiation doses was observed 72 h after irradiation (Figure 1C).

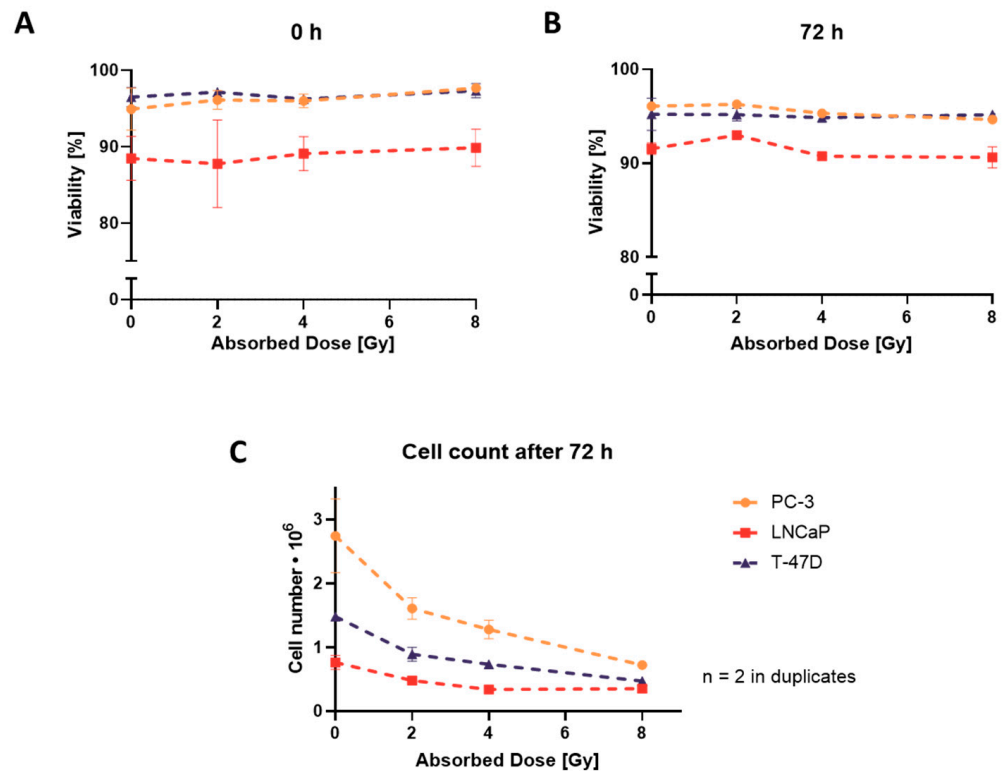


Figure 1. Trypan Blue Exclusion Assay: evaluation of cell viability and proliferation in monolayers. Cell viability immediately (0 h; (A)) and 72 h (B) after irradiation. (C) Cell count 72 h after irradiation. (A–C) Graphs are plotted as mean $\pm$ standard deviation from two independent experiments ($n = 2$) performed in duplicates.

3.2. Evaluation of Reproductive Potential by Clonogenic Assay

To further investigate this effect in monolayers, clonogenic assays for evaluation of reproductive potential were performed. All cell lines showed a decreased number of colonies with increasing radiation dose, albeit to a different extent (Figure 2). Values for 6 and 8 Gy for LNCaP were excluded, since there were no colonies visible.

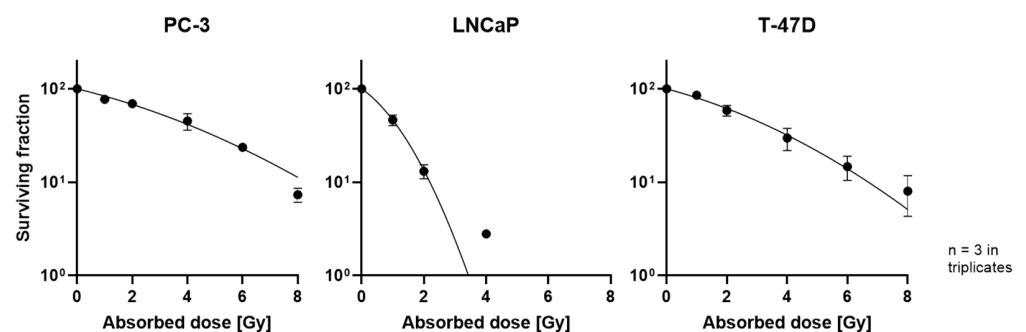


Figure 2. Survival curves of PC-3 (left), LNCaP (middle) and T-47D (right) cells; plotted as mean $\pm$ standard deviation from three independent experiments ($n = 3$) performed in triplicate.

For better comparability, the coefficients alpha and beta of the survival curve were determined. Higher values correspond to higher radiosensitivity (Table 1), showing highest radiosensitivity in LNCaP, followed by T-47D and PC-3. Representative photographs of assay plates are depicted in the (Supplementary Figure S2).

Table 1. Coefficients obtained from the linear quadratic curve fitting to measured data.

Cell Line	α (Alpha)	β (Beta)
PC-3	0.165	0.013
LNCaP	0.532	0.236
T-47D	0.199	0.017

3.3. Growth Analysis

3.3.1. Monolayers

PC-3 monolayers grew with a doubling time of 24 h, whereas LNCaP took 60 h and T-47D 32 h. After irradiation of the LNCaP and T-47D monolayers, further culturing was possible solely for the cells irradiated with 2 or 4 Gy. Irradiation with 8 Gy completely hampered cell proliferation in the two cell lines, as already shown with the clonogenic assay, making further culturing impossible. However, regarding PC-3, even the monolayers irradiated with 8 Gy recovered and regrew.

3.3.2. Spheroids

Spheroid growth was cell line dependent and is shown in Figure 3A. Non irradiated PC-3 spheroids showed exponential growth until day 15, whereas LNCaP and T-47D controls grew rather linearly, LNCaP until day 15 and T-47D until the end of the measured time period. LNCaP and PC-3 reached a plateau at a diameter of around 0.8 and 1 mm, respectively.

Irradiating PC-3 spheroids with 2 Gy showed no significant growth difference to control. However, 4 or 8 Gy radiation initially resulted in a decrease in growth rate, followed by a period of faster growth rate, ultimately leading to no significant size difference to control after 29 days of growth. Only the highest single fraction doses of 20 Gy resulted in a sustained growth inhibition. In contrast, LNCaP and T-47D showed significant size differences to control on day 29 after irradiation with 4 Gy or 2 Gy, respectively. Dose dependent growth inhibition was also shown for higher doses, without regrowth in the observed period after 8 Gy irradiation. Dose escalation to 20 Gy did not result in a significantly stronger growth inhibition compared to 8 Gy, neither for LNCaP nor T-47D spheroids, suggesting 8 Gy already being a highly effective treatment. Disintegration of the spheroids was shown for PC-3 and T-47D after irradiation with 20 Gy. In contrast, LNCaP retained their dense structure and only deformed slightly. Photographs of representative spheroids on day 29 are shown in Figure 3B. Time-lapse images of all types of spheroids described are given in the (Supplementary Figure S3).

3.4. Live/Dead Staining

To test whether irradiation directly induces necrosis, live fluorescent imaging was performed. Therefore, spheroids were stained for viable (Acetoxymethyl ester positive; green) and necrotic (PI positive; red) cells.

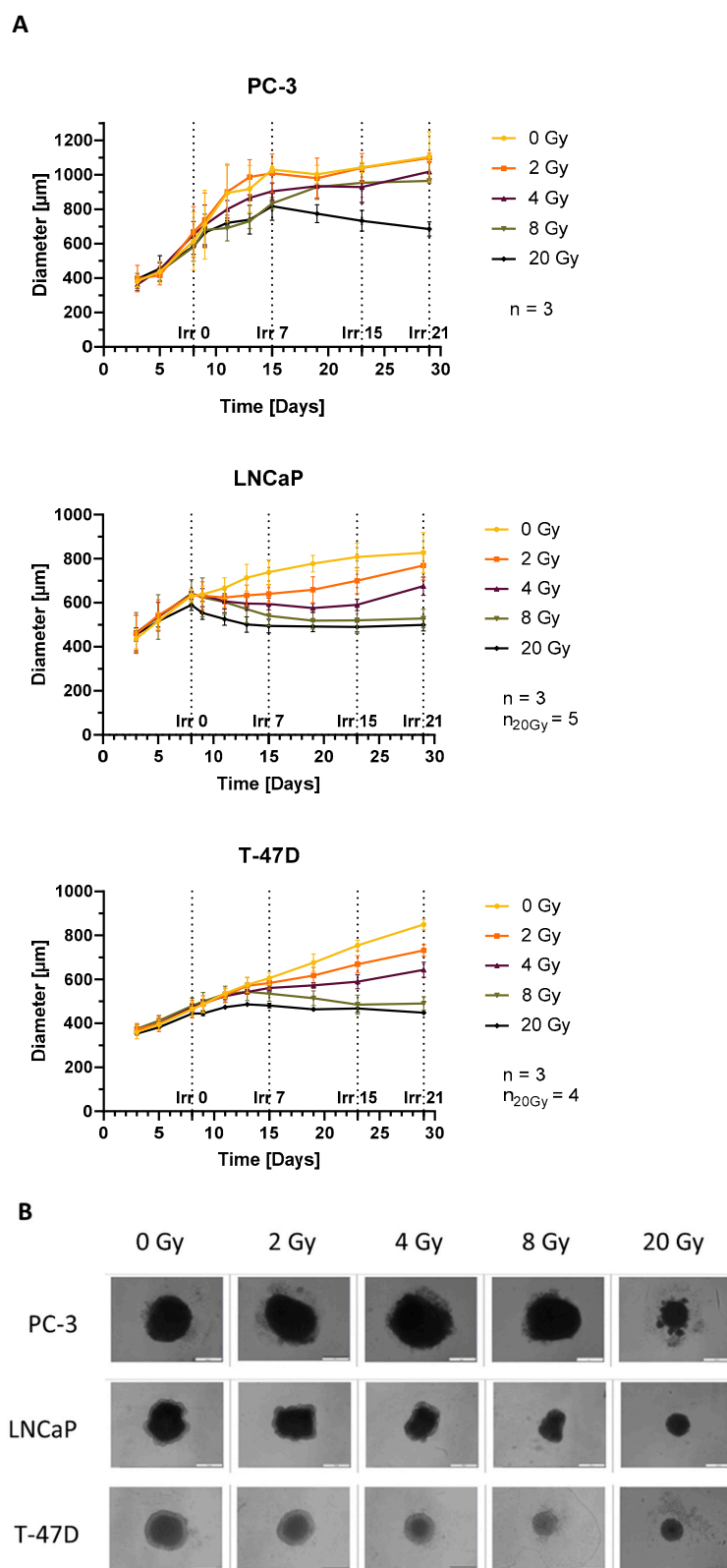


Figure 3. Growth observation of spheroids after single irradiation with 0, 2, 4, 8 or 20 Gy on day 8 (Irr 0); (A) Diameter of spheroids; average over three diameter measurements per spheroid and time point plotted as mean $\pm$ standard deviation from three to five independent experiments ($n = 3$ –5); Irr 0, 7, 15 and 21 indicate the time after irradiation in days (B) Growth analysis of spheroids on day 29 (Irr 21); white scale indicates 500 μm .

All spheroid models consisted of a viable outer layer and central necrosis on day 8, except T-47D whose necrotic core was first observed on day 15 of growth. This is in line with previous studies, stating that the spheroid diameter needs to be bigger than 500 μm diameter to develop a necrotic core [8]. However, sizes of necrotic core varied between the cell lines. The ratio of necrotic core to total spheroid size was determined and indicated an increase in the necrotic core ratio with spheroid size, but not with dose, in terms of LNCaP and T-47D up to 8 Gy. Only irradiation with the highest single dose (20 Gy) resulted in higher necrotic core ratios compared to the control in T-47D and PC-3 suggesting induction of necrosis. PC-3 spheroids show neither an increase in the necrotic core ratio with increasing radiation dose, nor with spheroid size up to 8 Gy. These results suggest that there is no additional necrosis induced by irradiation up to 8 Gy. Live staining and semiquantitative assessment are demonstrated in the (Supplementary Figure S4).

3.5. Cell Viability Determination Using Cell Titer Glo® 3D

Viability measurements correlated well with diameter measurements of spheroids. As expected, cell viability decreases with increasing radiation doses (Figure 4). Radiation shows dose-dependent growth inhibition in LNCaP and T-47D at all time points, and in PC-3 at 7 and 15 days after irradiation. However, 21 days after irradiation, there is no significant difference between the luminescence signal (viability) of control and irradiated spheroids up to 8 Gy in PC-3. Only after single fraction doses of 20 Gy, viability in PC-3 spheroids is significantly reduced.

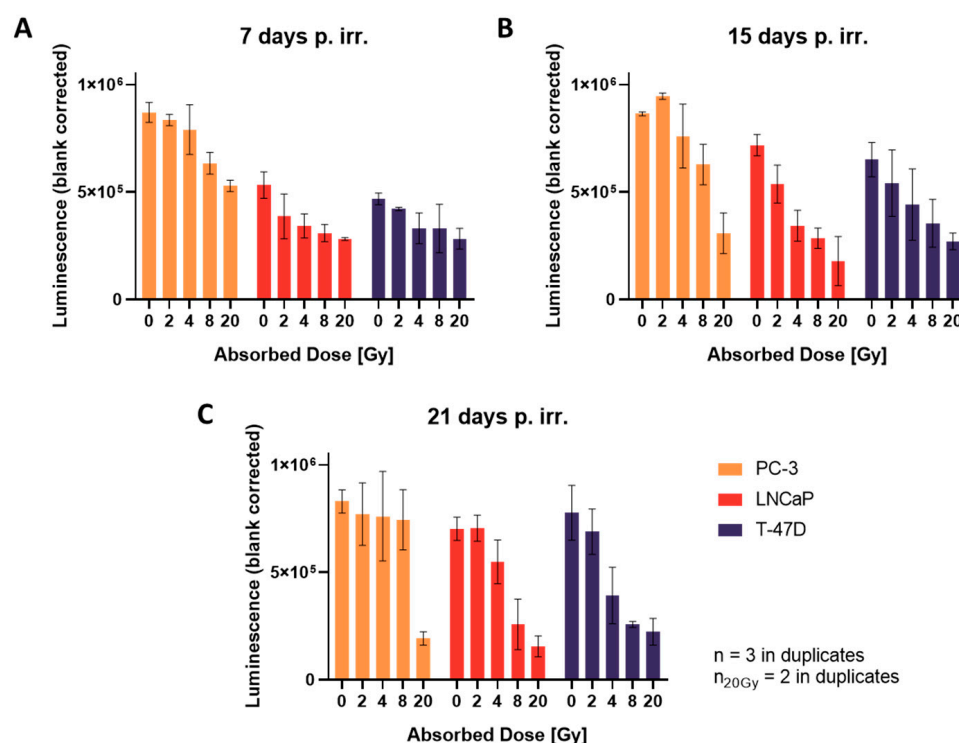


Figure 4. Cell viability of spheroids, (A) 7, (B) 15 and (C) 21 days after irradiation; plotted as mean $\pm$ standard deviation from three independent experiments ($n = 3$), each performed in duplicates; p. irr. = post irradiation.

3.6. Differences in Metabolic Activity between 2D and 3D (Resazurin Assay)

To compare the radiation response in 2D and 3D using exactly the same method, the resazurin assay was performed. Differences in metabolic activity (measured as fluorescence intensity) to control were higher in 2D than in 3D for all cell lines and all radiation doses. This difference further increased with dose and time (Figure 5A,B).

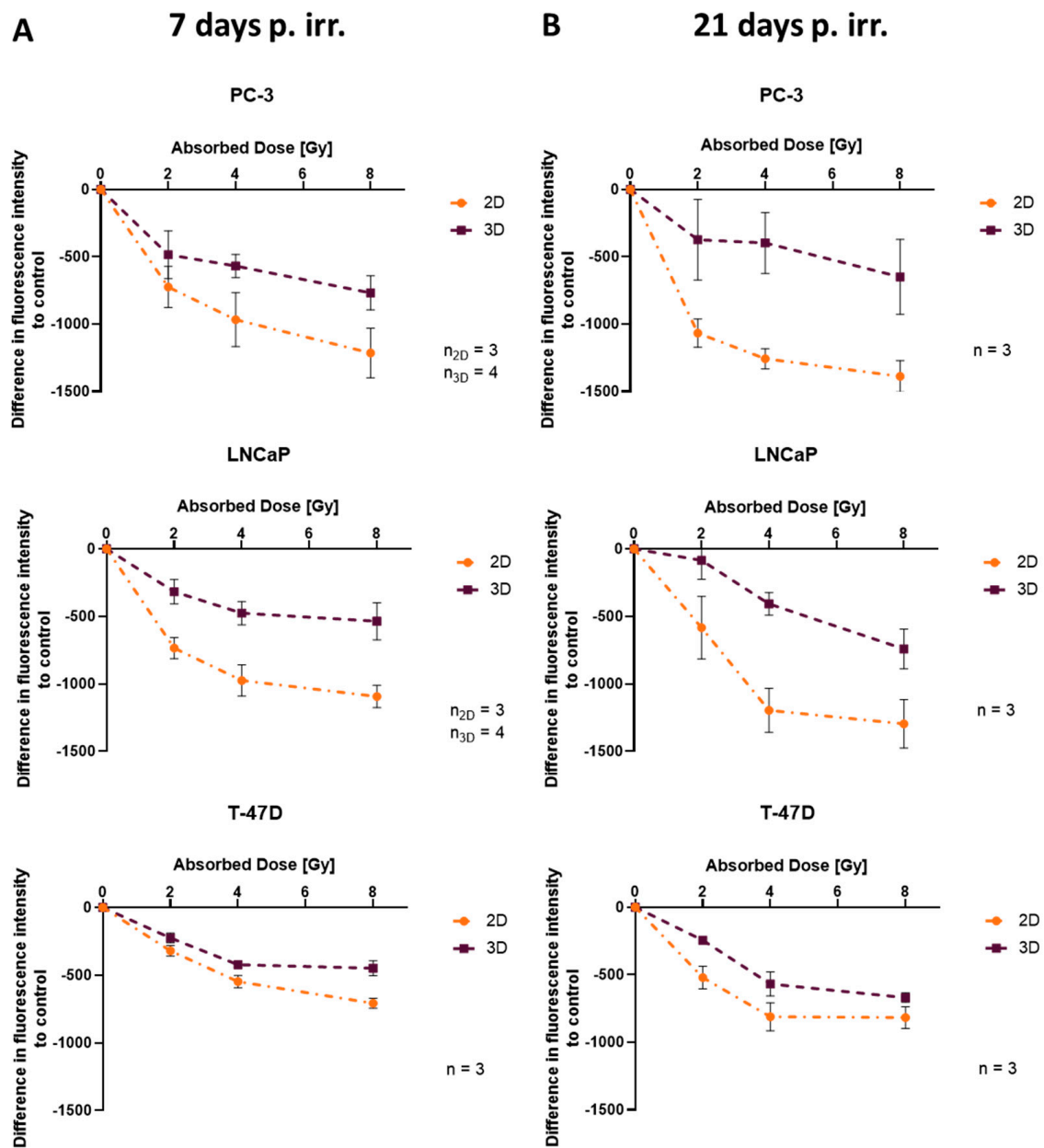


Figure 5. Assessment of difference in fluorescence intensity (metabolic activity) to control between 2D and 3D (A) 7 and (B) 21 days after irradiation; plotted as mean $\pm$ standard deviation from three to four independent experiments ($n = 3$ –4), each performed in triplicate; p. irr. = post irradiation.

3.7. DNA Damage Assessment: γ H2AX Directly after Irradiation

By measuring the amount of double-strand breaks, insight into molecular mechanisms is given. As shown in Figure 6, all cell lines show an increase in γ H2AX with increasing radiation doses. Furthermore, more γ H2AX was detected in 2D compared to 3D (except for 8 Gy in T-47D), with highest differences in PC-3. Corresponding western blots are depicted in the (Supplementary Figure S5).

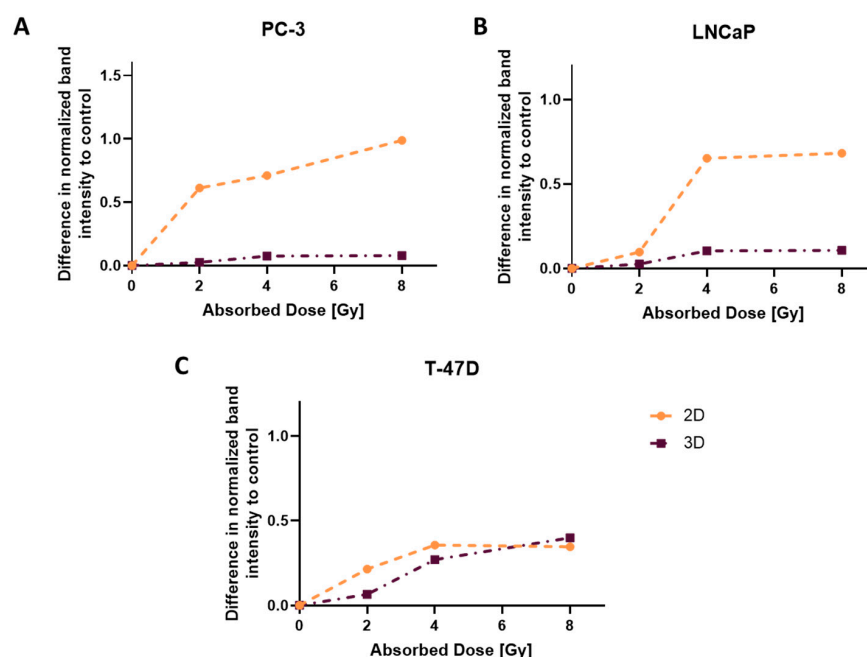


Figure 6. Differences in normalized γ H2AX band intensity to control, compared between 2D and 3D directly after irradiation for (A) PC-3, (B) LNCaP and (C) T-47D.

4. Discussion

In recent years, there has been increasing interest in implementing three-dimensional cell cultures as tumor model systems, being superior to 2D cell cultures when compared to *in vivo* models. However, there is still a lack of systematic, long term radiobiological studies and, until now, no regard to differences between 2D and 3D cell cultures in the widely used prostate cancer cell lines PC-3 and LNCaP, as well as the breast cancer cell line T-47D. In this study, a radiation response of up to 20 Gy was compared between monolayers and spheroids of these three different human cancer cell lines. 2D and 3D cell culture models were cultivated, treated with X-rays (1, 2, 4, 6, 8 and dose escalation to 20 Gy) and examined on growth behavior, amount of double-strand breaks and viability, using a range of assays. Distinct responses to radiation were shown not only by microscopic growth analysis, but also on various molecular levels.

The Trypan Blue Exclusion assay in monolayers showed no cell death upon irradiation up to 72 h, but, rather, impeded cell replicability in a dose-dependent manner. Single-cell replicability was further investigated by the commonly used clonogenic assay. Resulting data clearly supported a dose-dependent effect: a decrease in cell proliferation with increased radiation dose was observed in all cell lines in 2D. This effect was also shown for LNCaP and T-47D in 3D by means of growth analysis and was also supported by Cell Titer Glo[®] 3D assay. Similar observations have been made for 3D cultures of HCT116 and CAL27 cells [18]. Regarding the PC-3 cell line, this effect was only observable when comparing day 7 and day 15 after irradiation. 21 days after irradiation, PC-3 spheroids irradiated with up to 8 Gy showed no significant difference in size or viability, compared to the control, suggesting radioresistance up to 8 Gy.

The amount of DNA double-strand breaks correlates with viability data and size measurement of spheroids. Under the same experimental conditions, spheroids showed less double-strand breaks than monolayers. This is in agreement with the findings of Fazeli et al. in the prostate cancer cell line DU145 using Comet Assay [19]. The exception being T-47D spheroids and monolayers, which seem to have similar amounts of double-strand breaks after 8 Gy irradiation.

Comparing dimensionality, the resazurin assay showed big discrepancies between 2D and 3D in all cell lines, which increase with time. The monolayers showed higher difference

in metabolic activity to the control, compared to spheroids for all radiation doses, supporting earlier described lower radiosensitivity in spheroids compared to monolayers [13–19]. Furthermore, the results from the resazurin assay support the data obtained from growth analysis and viability assays: dose-dependent decline was observed in all cell lines 7 days after irradiation, but no significant difference appeared between 2, 4 and 8 Gy in PC-3 spheroids 21 days after irradiation (day 29).

Moreover, clonogenic survival and growth data provided valid indication of cell line specific treatment sensitivity. Our results evinced the highest radiosensitivity for LNCaP (steepest survival curve), followed by T-47D and PC-3 in 2D, and an order of T-47D (significant growth inhibition already with 2 Gy, most-significant difference to control at low doses), LNCaP and PC-3 in 3D. These results raise the knowledge on the differences in radiosensitivity not only between cell lines, but also regarding dimensionality.

Our study supports previous studies, adding data of a bigger repertoire of assays for therapeutic efficacy testing of radiation treatment as well as long term (up to 29 days) radiation response data for the widely used prostate cancer cell lines LNCaP and PC-3 and the breast cancer cell line T-47D. With this, we want to inspire further investigations to gain deeper knowledge on the biological effects of radiation.

However, a limitation of this study is the use of a single cell type in the 3D model. We envisioned utilizing a co-culture model comprising stromal, endothelial and immune cells. Co-culturing aims for better recapitulation of in vivo conditions, such as intercellular interactions between different cell types not represented in the current model. It also remains to be shown how fractionation affects treatment outcome, since this is the current clinical practice in cancer patients treated with external beam therapy, with the general trend of increasing doses per fraction (hypofractionation) enabled by precision radiotherapy techniques. Besides radio-oncological applications, a future step should also be the translation of the current findings into a setup of radionuclide therapy dosimetry.

5. Conclusions

This study demonstrates that the radiobiological response to X-rays measured in 2D is not reflected in 3D. The spheroid model shows higher radioresistance in all cell lines, compared to the respective monolayers. This effect is immense when using PC-3 cells, which showed resistance to X-ray radiation up to 8 Gy in spheroids, whereas a dose-dependent decrease in cell proliferation was shown for monolayers. Furthermore, the order of radiosensitivity was determined, observing highest radiosensitivity in LNCaP, followed by T-47D and PC-3 in 2D, but reversed order between LNCaP and T-47D (highest radiosensitivity in T-47D) in 3D. To further foster the understanding of radiobiology in radiation oncology and radionuclide therapy, more in vitro studies need to be performed with special regard to co-culturing methods.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells12030360/s1>, Figure S1: Growth analysis of spheroids LNCaP and T-47D + Geltrex after single irradiation with 0, 2, 4, 8 or 20 Gy on day 8 (Irr 0); Figure S2: Representative clonogenic assay evaluation; Figure S3: Time lapse growth analysis; Figure S4: Live/dead stainings of spheroids; Figure S5: Western Blots.

Author Contributions: Conceptualization, J.R. and M.M.; methodology, J.R.; validation, J.R. and B.B.; formal analysis, B.B. and J.R.; investigation, J.R. and B.B.; resources, M.M., M.H. and D.G.; data curation, B.B. and J.R.; writing—original draft preparation, J.R.; writing—review and editing, B.B., T.B., M.M. and D.G.; visualization, B.B. and J.R.; supervision, M.M. and T.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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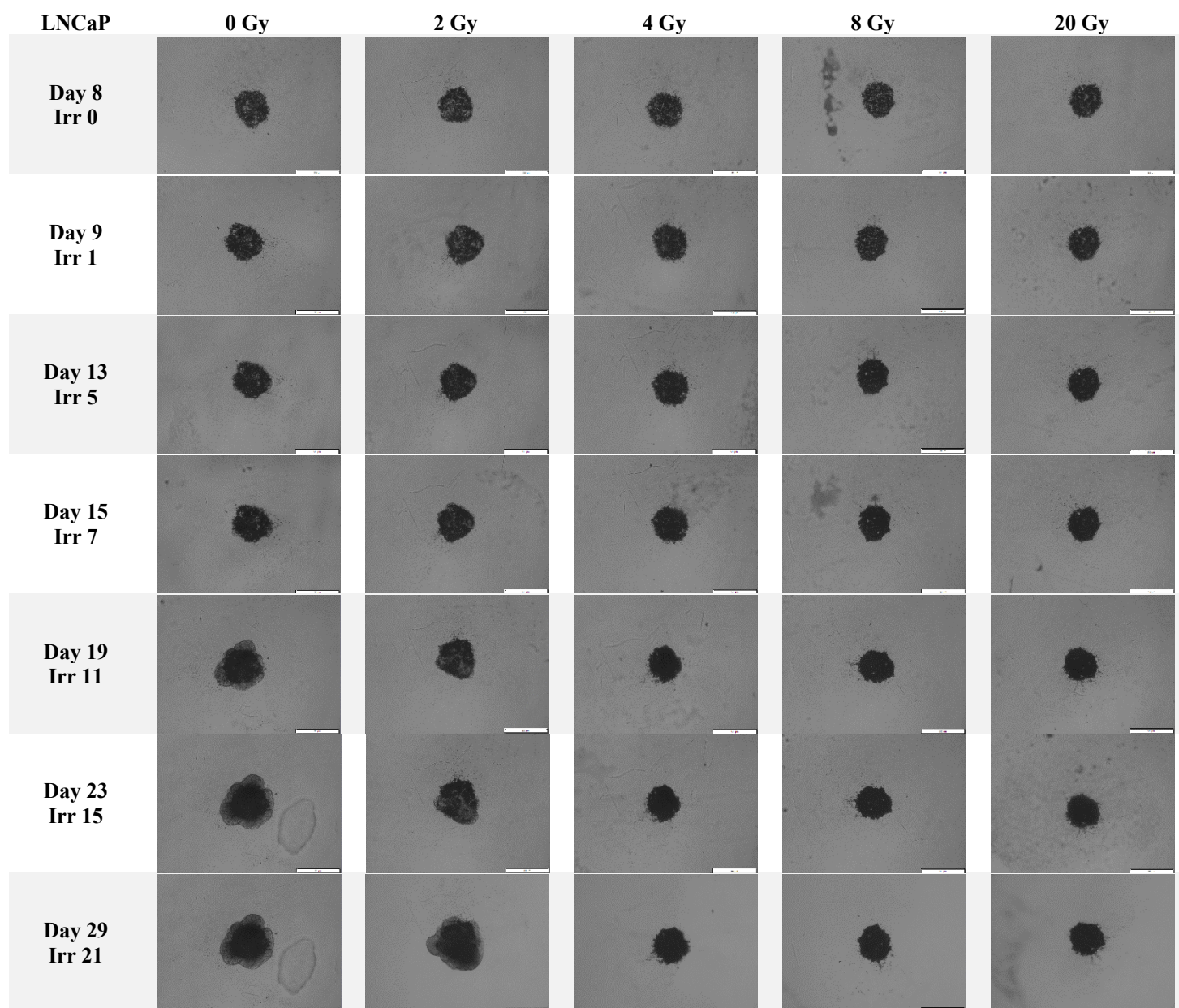
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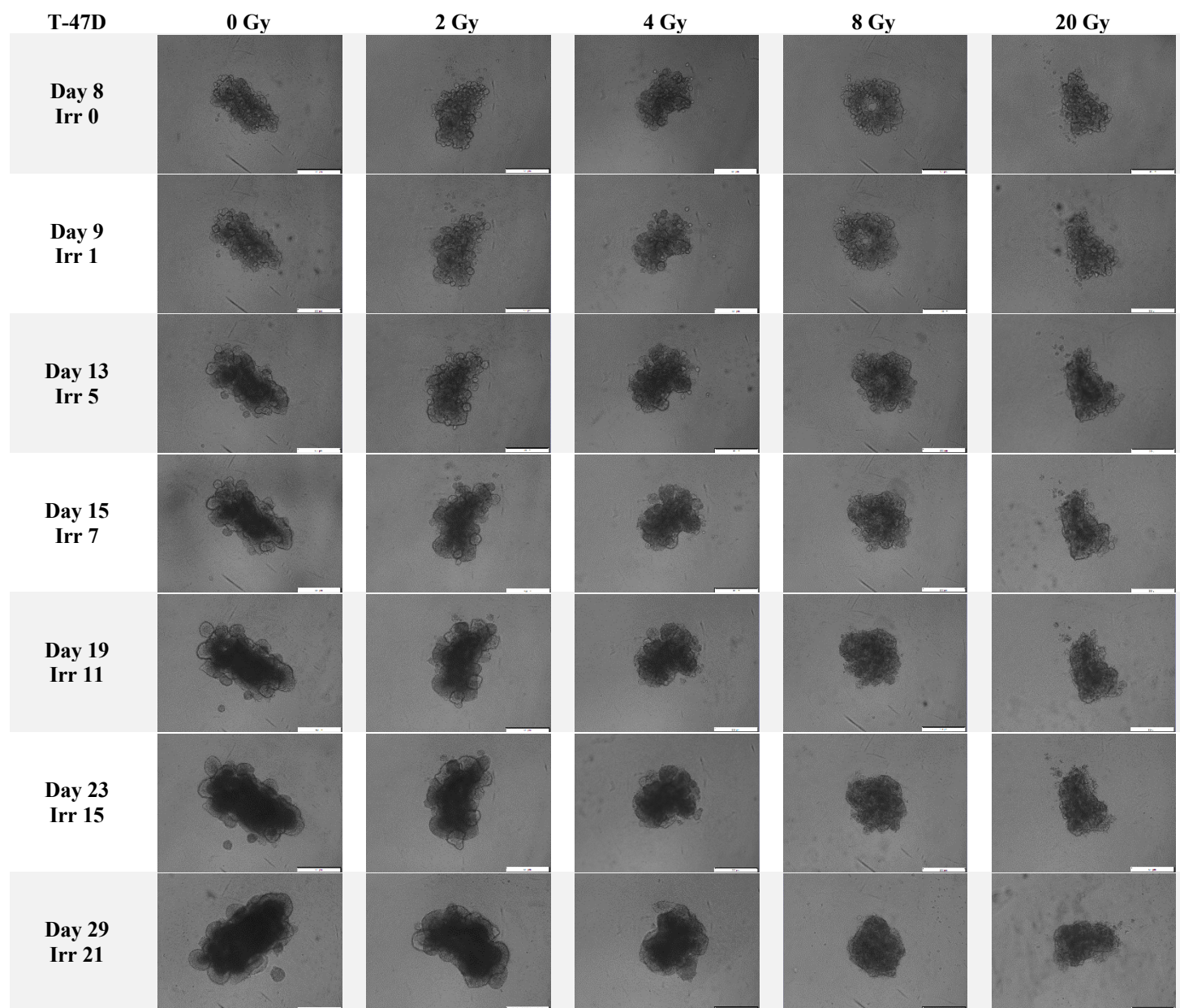
Supplementary information

Figure S1. Growth analysis of spheroids LNCaP and T-47D + Geltrex after single irradiation with 0, 2, 4, 8 or 20 Gy on day 8 (Irr 0). (A) Time lapse images of LNCaP + Geltrex (B) Time lapse images of T-47D + Geltrex (C) Diameter of spheroids; average over three diameter measurements per spheroid and time point plotted as mean $\pm$ standard deviation from three independent experiments (n = 3); Irr 0, 7, 15 and 21 indicate the time after irradiation in days

(A)



(B)



(C)

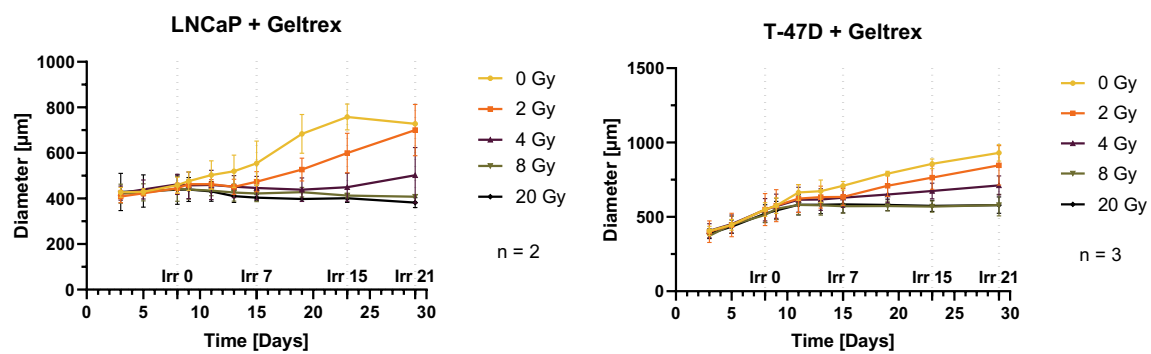
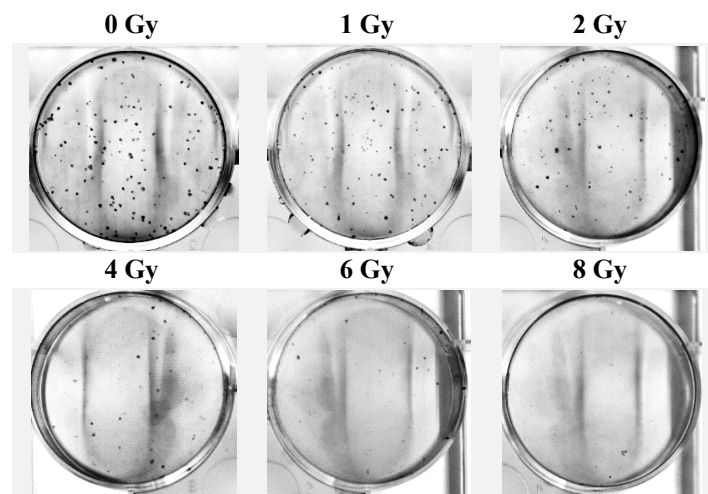
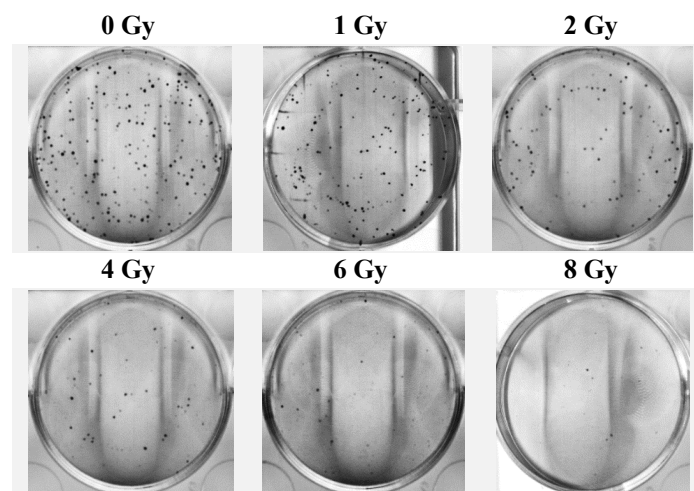


Figure S2. Representative clonogenic assay evaluation of (A) PC-3, (B) LNCaP and (C) T-47D wells irradiated with 0, 1, 2, 4, 6 or 8 Gy

(A)



(B)



(C)

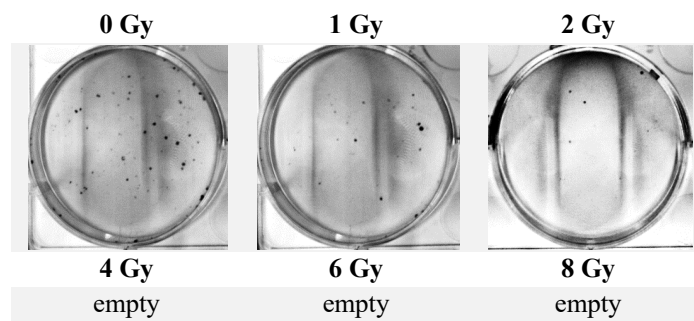
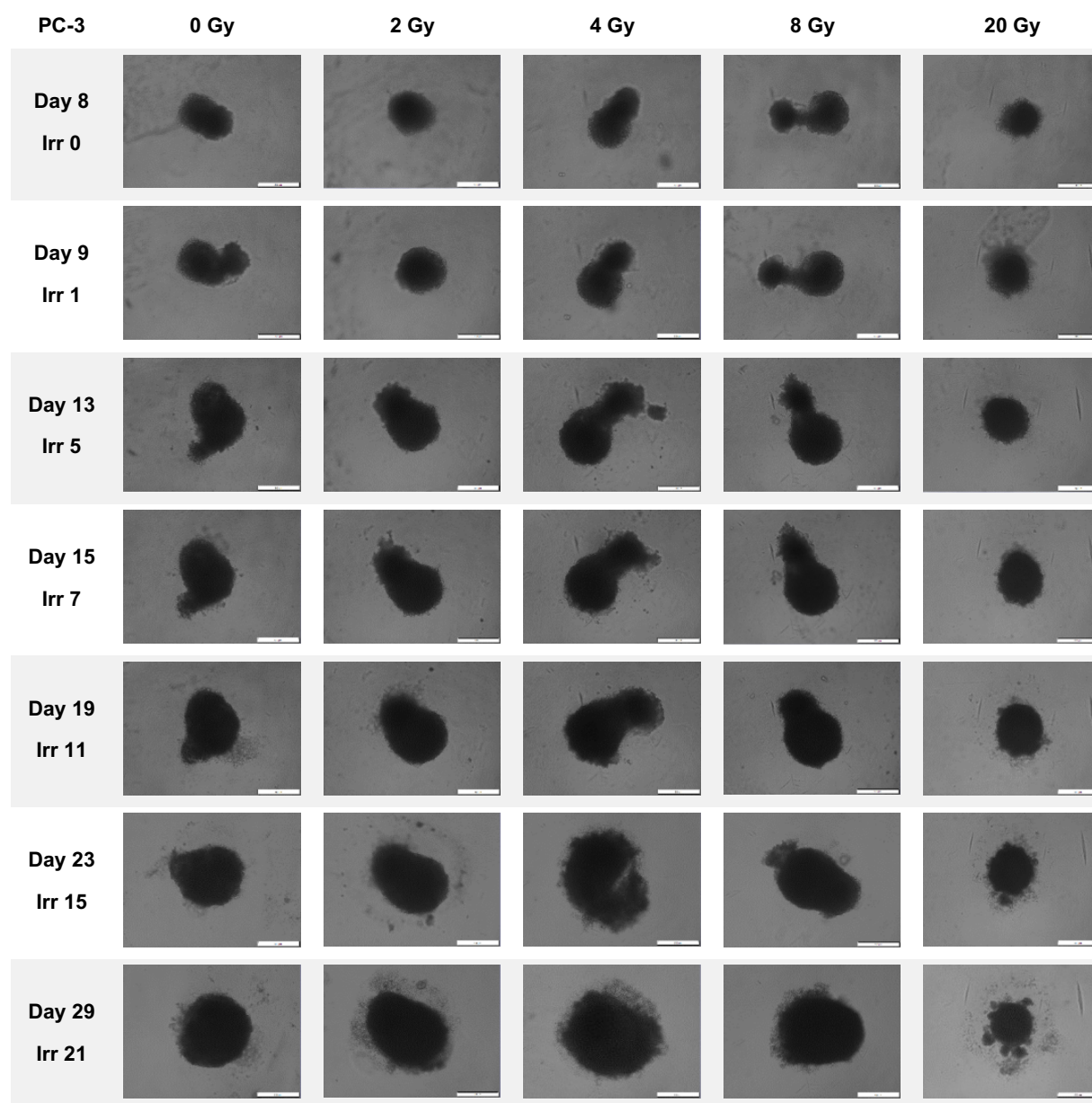
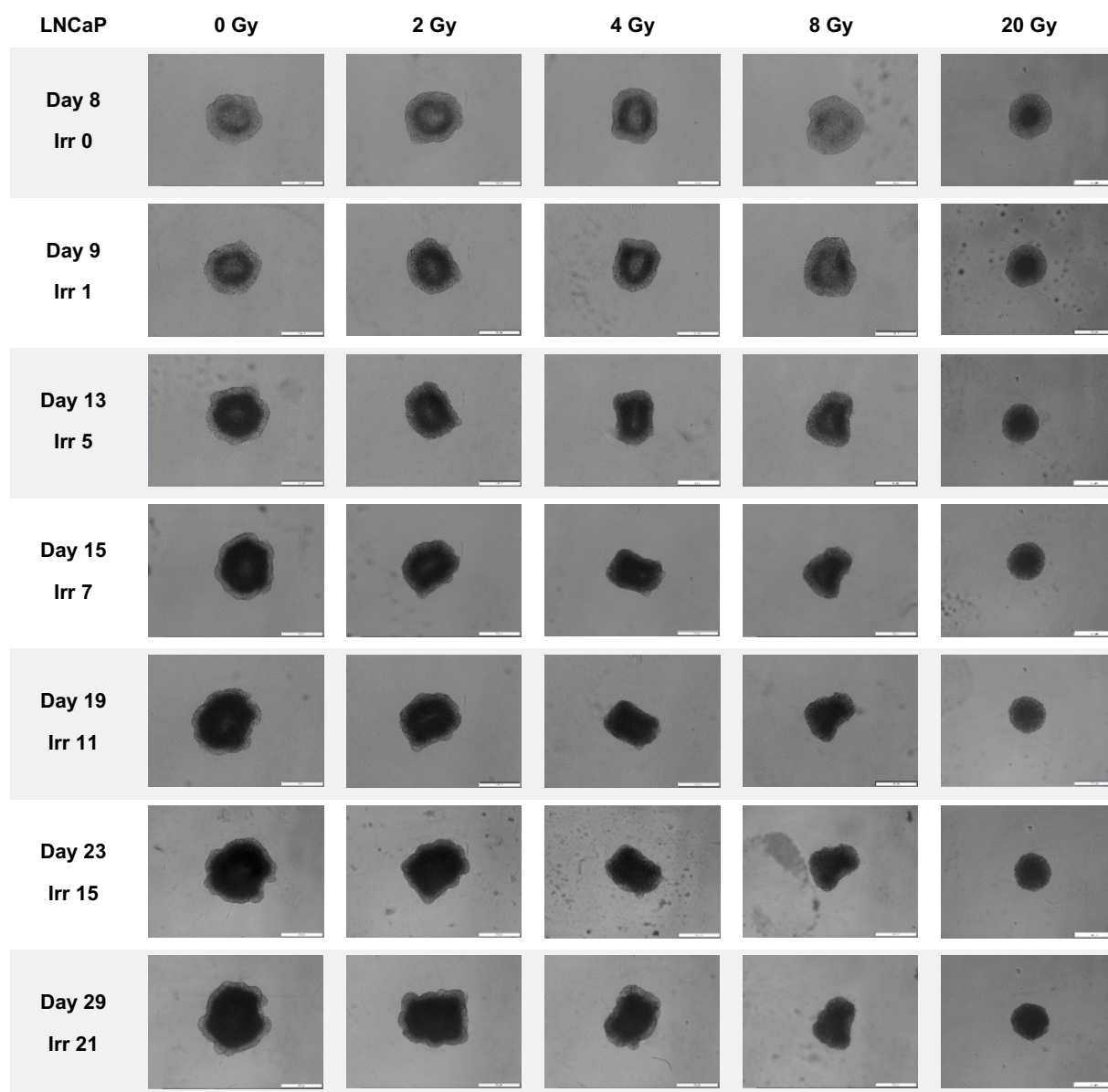


Figure S3. Time lapse growth observation of (A) PC-3 (with Geltrex), (B) LNCaP and (C) T-47D irradiated with 0, 2, 4, 8 or 20 Gy on day 8 (Irr 0); white scale indicates 500 μm

(A)



(B)



(C)

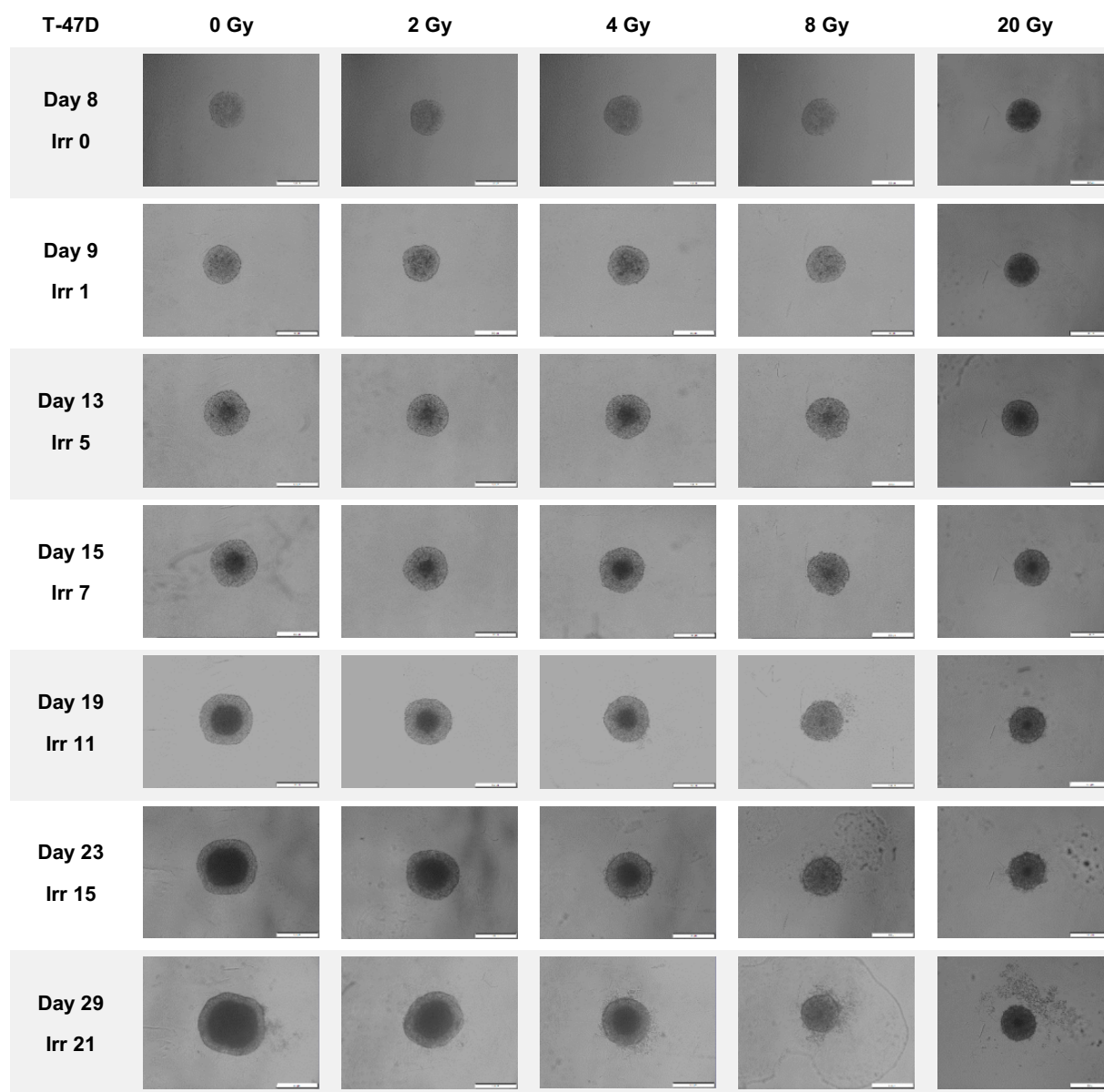
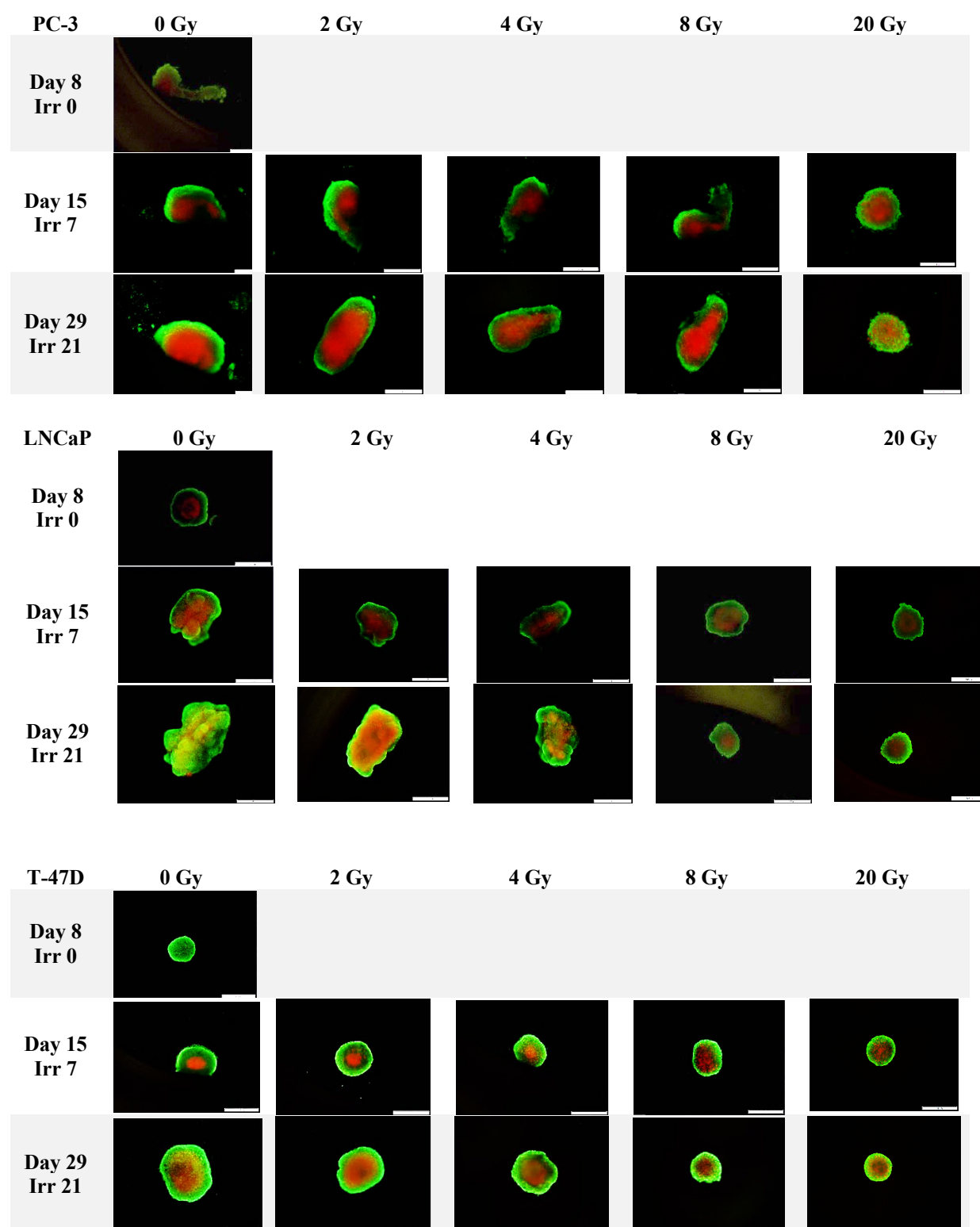


Figure S4. Live/dead staining of spheroids. (A) Propidium iodide (red) and calcein-AM (green) staining of spheroids on Day 8 (Irr 0), Day 15 (Irr 7) and Day 29 (Irr 21); white scale indicates 500 μ m (B) Ratio necrotic core to total spheroid size for PC-3, LNCaP and T-47D; plotted as mean $\pm$ standard deviation from one or two independent experiments (n=1-2)

(A)



(B)

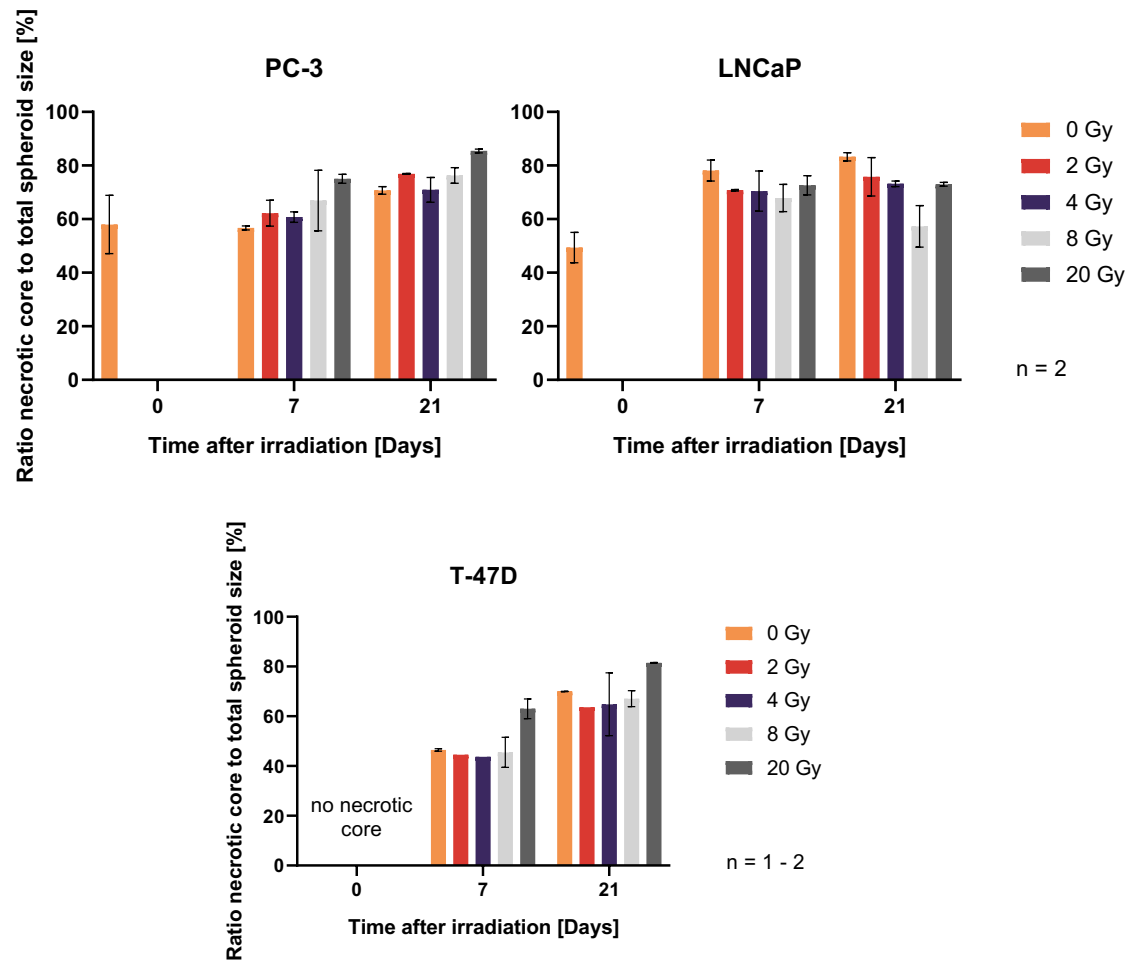
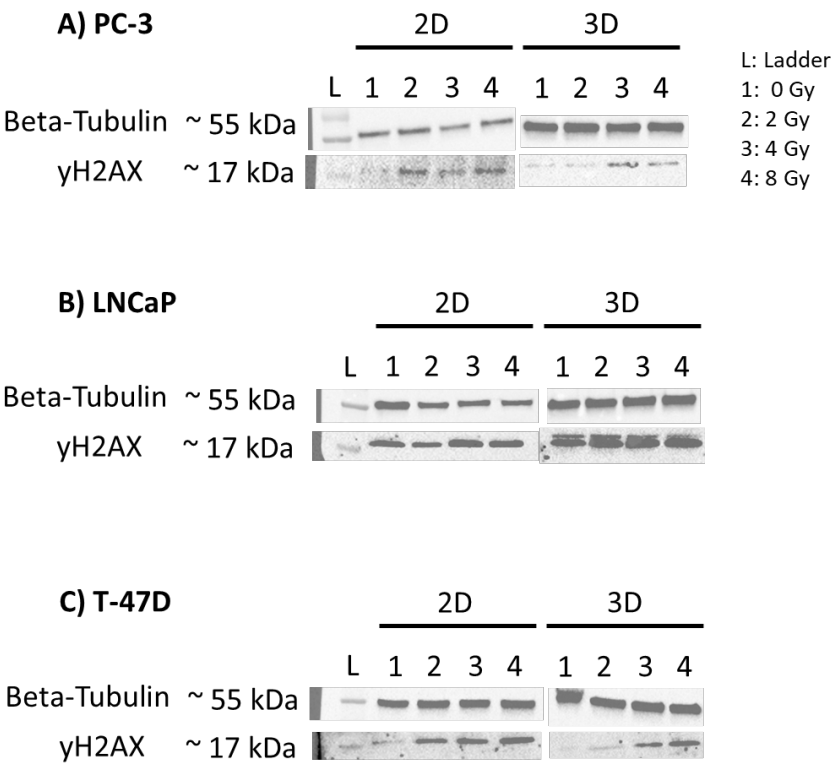


Figure S5. Western Blots for further analysis with ImageJ



3.1.2 Manuscript 2

Schmitl S, Raitanen J, Witoszynskyj S, Patronas E-M, Nics L, Ozenil M, Weissenböck V, Mindt TL, Hacker M, Wadsak W, Brandt MR, Mitterhauser M. Quality Assurance Investigations and Impurity Characterization during Upscaling of [^{177}Lu]Lu-PSMA^{I&T}. *Molecules*. 2023; 28(23):7696. <https://doi.org/10.3390/molecules28237696>

Article

Quality Assurance Investigations and Impurity Characterization during Upscaling of [^{177}Lu]Lu-PSMA^{I&T}

Stefan Schmitl¹, Julia Raitanen^{1,2,3,4} , Stephan Witoszynskyj¹, Eva-Maria Patronas¹, Lukas Nics¹, Marius Ozenil¹ , Victoria Weissenböck¹, Thomas L. Mindt^{2,3,5} , Marcus Hacker¹ , Wolfgang Wadsak¹, Marie R. Brandt^{2,3,5,*}  and Markus Mitterhauser^{1,2,3,5} 

¹ Department of Biomedical Imaging and Image-Guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, 1090 Vienna, Austria

² Ludwig Boltzmann Institute Applied Diagnostics, AKH Wien c/o Sekretariat Nuklearmedizin, 1090 Vienna, Austria

³ Department of Inorganic Chemistry, Faculty of Chemistry, University of Vienna, 1090 Vienna, Austria

⁴ Vienna Doctoral School of Chemistry (DoSChem), University of Vienna, 1090 Vienna, Austria

⁵ Joint Applied Medicinal Radiochemistry Facility, University of Vienna & Medical University of Vienna, 1090 Vienna, Austria

* Correspondence: marie.brandt@univie.ac.at

Abstract: [^{177}Lu]Lu-PSMA^{I&T} is widely used for the radioligand therapy of metastatic castration-resistant prostate cancer (mCRPC). Since this kind of therapy has gained a large momentum in recent years, an upscaled production process yielding multiple patient doses in one batch has been developed. During upscaling, the established production method as well as the HPLC quality control were challenged. A major finding was a correlation between the specific activity and the formation of a pre-peak, presumably caused by radiolysis. Hence, nonradioactive reference standards were irradiated with an X-ray source and the formed pre-peak was subsequently identified as a deiodination product by UPLC-MS. To confirm the occurrence of the same deiodinated side product in the routine batch, a customized deiodinated precursor was radiolabeled and analyzed with the same HPLC setup, revealing an identical retention time to the pre-peak in the formerly synthesized routine batches. Additionally, further cyclization products of [^{177}Lu]Lu-PSMA^{I&T} were identified as major contributors to radiochemical impurities. The comparison of two HPLC methods showed the likelihood of the overestimation of the radiochemical purity during the synthesis of [^{177}Lu]Lu-PSMA^{I&T}. Finally, a prospective cost reduction through an optimization of the production process was shown.

Keywords: [^{177}Lu]Lu-PSMA^{I&T}; radiolysis; radioligand therapy; upscaling; quality assurance; HPLC; UPLC-MS



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1. Introduction

[^{177}Lu]Lu-PSMA^{I&T} (INN: Lutetium (^{177}Lu) zadavotide guraxetan) [1] is one of the main PSMA-directed radiopharmaceuticals that are successfully used for the treatment of metastatic castration-resistant prostate cancer in nuclear medicine departments around the world [2]. Lutetium-177 decays via β^-/γ emission to its daughter nuclide hafnium-176 (^{176}Hf). With a physical half-life of 6.647 days and a maximum tissue penetration range of <2 mm of its emitted β particles, it displays beneficial properties for the deposition of high radiation doses to tumor lesions and metastases while sparing the surrounding tissue [3].

The success of PSMA-directed radioligand therapy is not only reflected by the very recent approval of [^{177}Lu]Lu-PSMA-617 (Pluvicto[®]) for mCRPC in the US and EU, but also by the increasing clinical demand for comparable agents [2,4,5]. In the literature, administered activities ranging from 3.7 to 9.3 GBq were described for [^{177}Lu]Lu-PSMA therapy in metastatic castration-resistant prostate cancer (mCRPC) [6]. In our clinic, 7.4 GBq of

$[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$ every six weeks represents the current standard in mCRPC treatment, based on the marketing authorization of Pluvicto[®]. However, lower levels have been administered in earlier stages of prostate carcinoma in recent pilot studies [7,8].

In the Division of Nuclear Medicine at the General Hospital of Vienna, the number of mCRPC patients referred to $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$ therapy under named patient use has constantly increased throughout recent years. This development is in accordance with a recent study that showed an increasing prevalence of mCRPC in the United States, with stable incidence rates between 2010 and 2017, mostly attributable to the introduction of novel therapies (e.g., taxane-based chemotherapy, androgen deprivation therapy) [9]. To ensure the supplying of all our patients with this treatment using the available resources, it became necessary to upscale our production process from two patients per batch to at least four patients per batch (18 to 36 GBq starting activity/batch). Due to the increasing amount of radioactivity, radiolysis was suspected to constitute a major problem with regard to radiochemical purity results during upscaling.

The degradation of a radiopharmaceutical under the influence of its incorporated radionuclide (radiolysis) is a phenomenon known to occur during the production of $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$ and other radiopharmaceuticals labeled with α or β^- emitters. The process is mediated predominantly by solvated electrons, radicals and highly reactive molecules that result from the irradiation of water and thereby formed reactive oxygen species (ROS). To a lower extent, radiolysis is mediated by direct effects of radiation on the radiopharmaceutical. Different radiolysis quenchers have been investigated to maintain the radiochemical purity of therapeutic radiopharmaceuticals during storage [10]. The major radiolysis product of $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$ was recently suggested to be the result of deiodination of the iodotyrosine moiety (see Figure 1) [11].

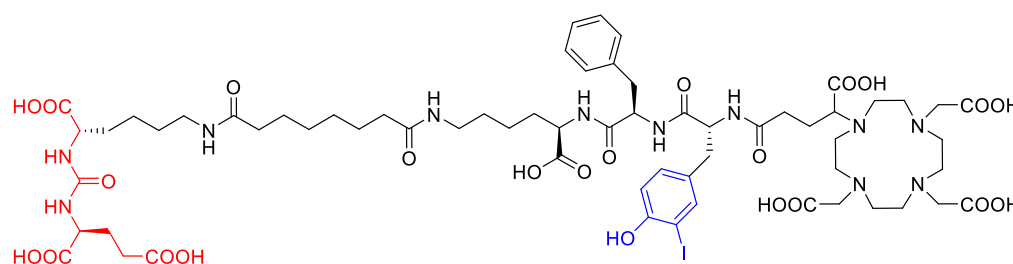


Figure 1. Molecular structure of $\text{PSMA}^{\text{I\&T}}$ with Glu-urea-Lys-binding motif (red, left) and iodotyrosine moiety (center, blue).

For $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$, the radiolabeling with up to 30 GBq in the presence of gentisic acid, ascorbic acid and sodium acetate followed by the dilution of the reaction mixture with an ascorbic acid solution showed excellent radiochemical purity results of >97% after storage for 30 h [12]. The effectiveness of ascorbic acid/gentisic acid combination buffers can be explained by the oxidation of gentisic acid via ROS and the subsequent reduction of the primary radicals via ascorbic acid, hence sparing the radiopetide [13].

Apart from radiolysis, the chemical rearrangement of the product was also considered to have a possible influence on the formation of radioactive side products, although not necessarily related to the increased radioactivity amounts. Recently, the heat-dependent formation of hydantoins via cyclization of the PSMA binding motif was shown to occur during the radiosynthesis of $[^{177}\text{Lu}]\text{Lu-PSMA-617}$. These hydantoins showed no binding affinity to PSMA and were rapidly excreted by the kidneys. It was shown that these byproducts constitute >2% of the sum of radiochemical impurities, even under optimized conditions [14]. To the best of our knowledge, the formation of hydantoins in the PSMA binding motif has not been described for $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$ yet. Given the likelihood of the formation of these by-products in $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$, we suspect that an overestimation of radiochemical purity could be prevalent throughout the existing literature, possibly due to the use of unsuitable HPLC methods for the detection of the cyclization products.

The aim of this study was therefore to investigate the formation and identity of radiolysis and cyclization products during the upscaling of [^{177}Lu]Lu-PSMA^{I&T} production.

2. Results and Discussion

2.1. Investigation of the Production Process of [^{177}Lu]Lu-PSMA^{I&T}

Due to the recent publications on radiolysis-generated side products [10,12,15] in Lutetium-177-labeled radiopharmaceuticals, the radiochemical purity (RCP) depending on the radioactivity concentration was defined as a key parameter to be investigated and prioritized in the upscaling process. Hence, several batches with different starting activity concentrations (AC, 0.5–2.5 GBq/mL) were analyzed with HPLC method 1 (see chapter “Methods”) for possible radiolysis products. The batches were produced according to the method referred to as “original method” (see Section 3.4). A pre-peak ($R_t = 3.88$ min) was detected in all cases and all activity concentrations, with a slightly shorter retention time than the main product ($R_t = 4.20$ min; see Figure 2). Its integral was below the in-house limit of 5% by that time for ACs up to 1 GBq/mL. No other peaks were detected in the radioactivity channel.

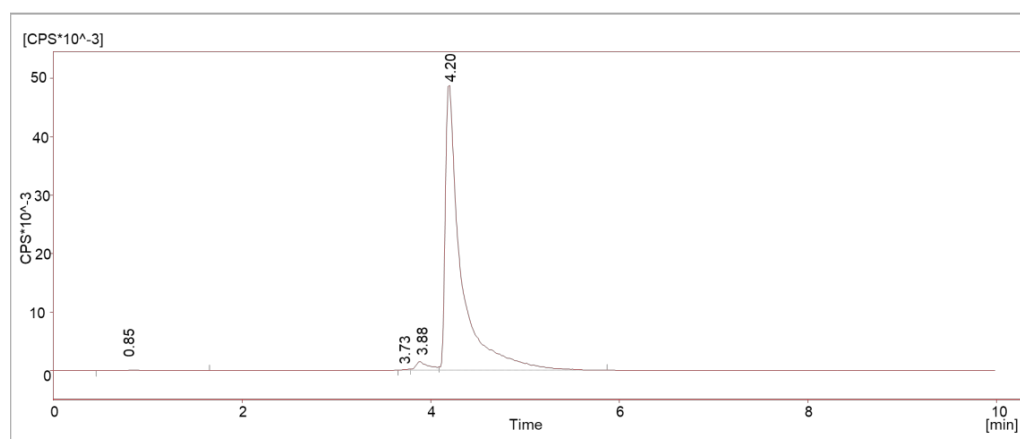


Figure 2. Typical γ -HPLC chromatogram (method 1) of [^{177}Lu]Lu-PSMA^{I&T} with main product ($R_t = 4.20$ min) and radiolysis-induced side product ($R_t = 3.88$ min).

Depending on the AC, the integral of the impurity was rising, revealing a correlation between the pre-peak and the AC (see Figure 3).

2.2. HPLC Optimization Studies and HPLC Validation

In the next step, our HPLC quality control methods were revised, as the relatively poor separation of the product from the radiolysis pre-peak was identified as problematic. Hence, the HPLC method described as “method C” by Martin et al. [14] was slightly modified and the resulting method will be called “method 2” throughout this text [14] (for details see the chapter “Methods”). The method was originally used to detect cyclization products of [^{177}Lu]Lu-PSMA-617 in patient urine samples. For comparison, simultaneous measurements of test batches in two identical HPLC setups were conducted to compare the performance of method 1 and method 2. The direct comparison showed significant underestimations of the presence of impurities by method 1 (see Figure 4).

The validation results of HPLC method 2 are displayed in Table 1.

To improve the radiochemical purity of the product, the need for a modified production process and storage formulation to suppress the suspected radiolysis became obvious. As described in the section “Methods: Radiosyntheses”, a manual process described by Di Orto et al. [12] was modified and automatized (called “adapted method” in this text, whereas “original method” describes our previously used procedure, for details see Section 3.4).

The implementation of the new production method combined with HPLC method 2 showed a maximum of $\leq 10\%$ total impurity for syntheses with a maximum AC of 1.8 GBq/mL in the product solution, as displayed in Figure 5.

It is also shown that, in addition to the baseline separated radiolysis product, hydrophobic substances eluting after the main product additionally appeared in HPLC method 2 (contribution of $2.87 \pm 0.85\%$ to total impurities) and furthermore formed independently from the activity concentration. A typical HPLC chromatogram acquired with method 2 is displayed in Figure 6.

Stability measurements were performed on three batches containing 8.6, 26.3 and 26.5 GBq. All batches showed $>95\%$ radiochemical purity (HPLC) after 4 h. Long term stability studies were not conducted since no shipment to other facilities or commercial use was intended.

In the next step, the radiochemical by-products were identified.

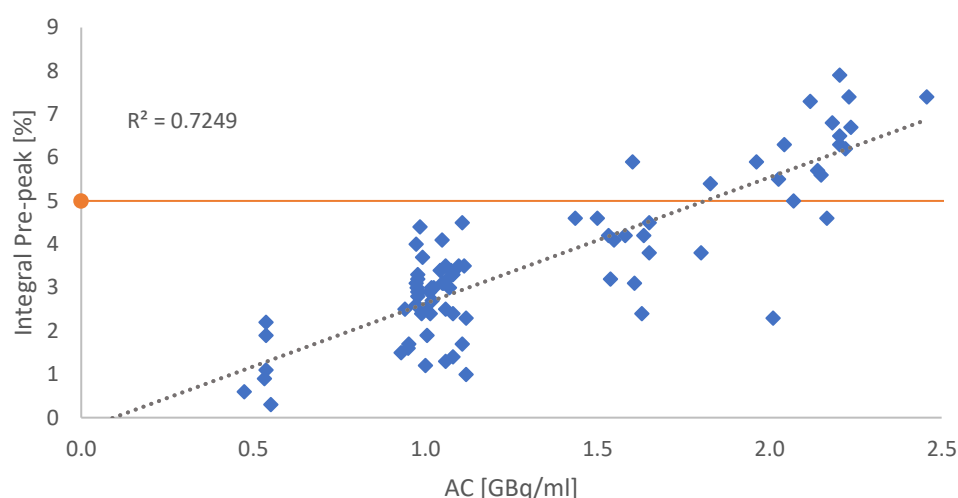


Figure 3. Graphical correlation between the integral of the suspected radiolysis-induced side product and the AC. Each blue dot refers to a single production batch. The orange line indicates the in-house specification benchmark of 5% impurities. All dots above the red line indicate batches with RCP $< 95\%$.

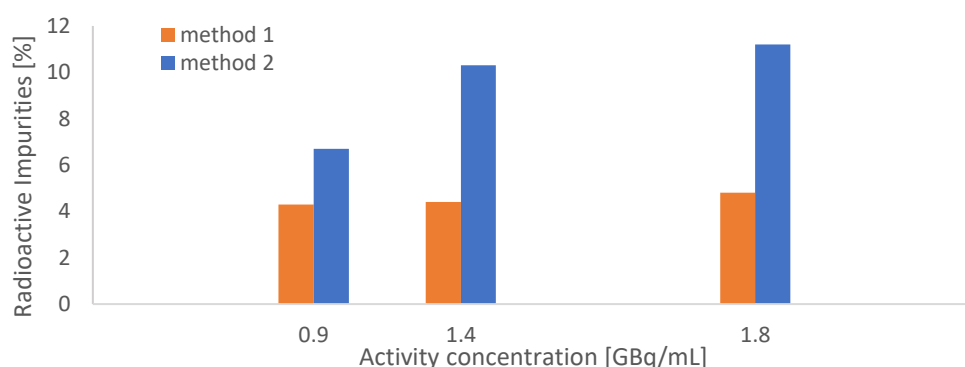
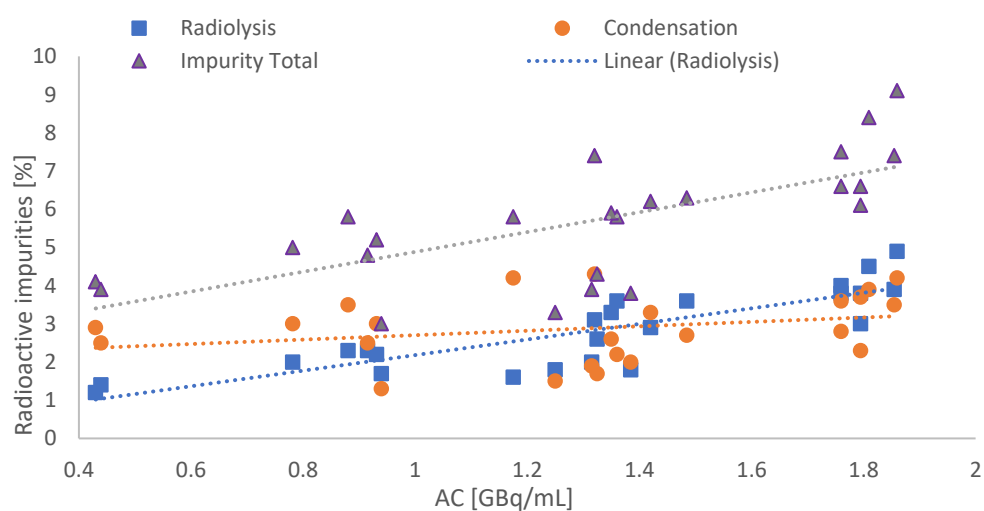
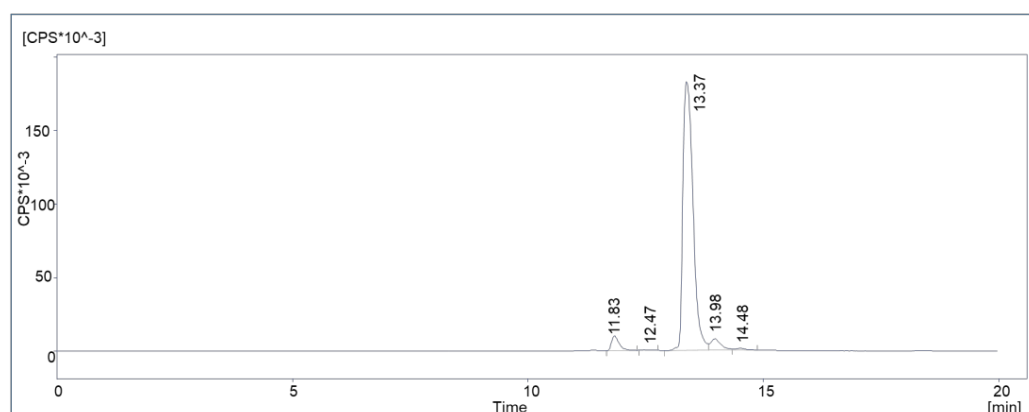


Figure 4. Comparison between HPLC methods 1 and 2 through simultaneous measurements of $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$ batches; three different batches with increasing activities (18–36 GBq, AC = 1–2 GBq/mL, N = 1) were synthesized according to the original synthetic procedure.

Table 1. Validation of HPLC method 2.

Radiodetector				
Parameter	Radiochemical Identity	Radiochemical Purity	Acceptance Criteria	Results
Precision (repeatability)		$CV\% \leq 5\%$		complies
Specificity (radiolysis product)		$R_s \geq 1.5$		complies
Linearity		$R^2 \geq 0.99$		complies
UV detector				
Parameter	Acceptance Criteria			Results
Precision (repeatability)	$CV\% \leq 5\%$			complies
LOD ($[^{nat}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$)	Based on calibration curve			0.0361 $\mu\text{g}/\mu\text{L}$
LOD ($\text{PSMA}^{\text{I\&T}}$)	Based on calibration curve			0.0149 $\mu\text{g}/\mu\text{L}$
LOQ ($[^{nat}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$)	Based on calibration curve			0.1269 $\mu\text{g}/\mu\text{L}$
LOQ ($\text{PSMA}^{\text{I\&T}}$)	Based on calibration curve			0.0531 $\mu\text{g}/\mu\text{L}$
Linearity $[^{nat}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$	$R^2 \geq 0.99$			complies
Linearity $\text{PSMA}^{\text{I\&T}}$	$R^2 \geq 0.99$			complies

**Figure 5.** Total amount of radioactive impurities by using the new production and HPLC method in dependence of AC (blue dots: radiolysis pre-peak; orange circle: condensation side products; purple triangles: total impurities).**Figure 6.** Representative chromatogram of a routine batch of $[^{177}\text{Lu}]\text{Lu-PSMA}^{\text{I\&T}}$ with HPLC method 2.

2.3. Identification of the Radiolysis By-Products: Irradiation Experiments and Coelution

At this stage of the project, the identity of the pre-peak in [^{177}Lu]Lu-PSMA^{I&T} was not yet clear (a study by Kraihammer et al. with a similar experimental setup has been published since then [11,13]). Due to the almost linear correlation between the intensity of the by-product and the radioactivity concentrations as described above, radiolysis was highly likely to be the cause of the formation. The dissociation of the iodine atom in the iodotyrosine moiety of the molecule (see Figure 7), which was shown to be caused by reactive oxygen species (ROS) induced by external radiation in aqueous media [16], was suspected to be the most probable cause.

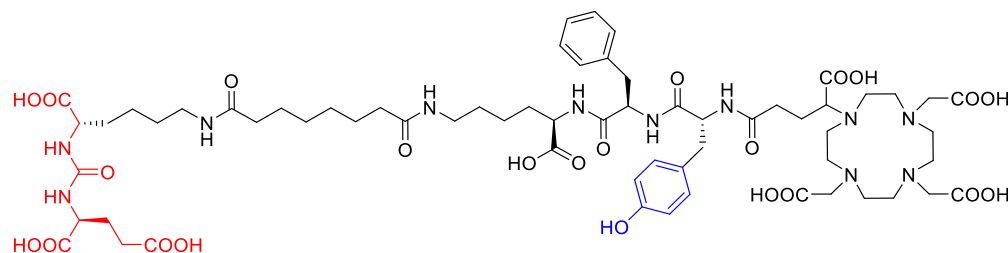


Figure 7. Molecular structure of the deiodinated PSMA^{I&T} by-product with the intact binding motif and missing iodine atom on the tyrosine side chain.

Hence, radiolysis was investigated by simulation. The intact Gd-PSMA^{I&T} was at first subjected to LC-MS and showed a retention time of 7.721 min and $[\text{M}+2\text{H}]^{2+}$ was found to be 828.30 (calculated: 828.31).

Then, the non-radioactive reference compound Gd-PSMA^{I&T} was irradiated in both the reaction buffer (ascorbate buffer from POLATOM) as well as in the reaction buffer plus ethanol with our in-house X-ray irradiation device (see “methods: Radiosyntheses”). Due to the similar chemical behavior and lower cost, a gadolinium salt was used as a surrogate for lutetium.

First, a simplified dose calculation was performed under the following four assumptions: 1. The β^- emitter Lutetium-177 was causing substance damage comparable to X-rays. 2. The radionuclide was evenly distributed throughout the reaction vessel in 19.6 mL total volume. 3. Only β^- particles contributed to the total dose. 4. The storage time was 45 min. The physical dose for the ACs of 0.4, 0.9, 1.8 and 2 GBq/mL, corresponding to batch activities of 7, 18, 36 and 40 GBq per batch (for the calculation, see the chapter “Dose Calculations” in the Section 3), was calculated. Then, the samples were irradiated with the calculated doses and analyzed via LC-MS to identify potentially formed side products. In the samples without ethanol, two side products could be detected, whose intensities increased with rising dose equivalents (Figure 8) and were barely present in the ethanol containing samples kept for the same time in the same buffer.

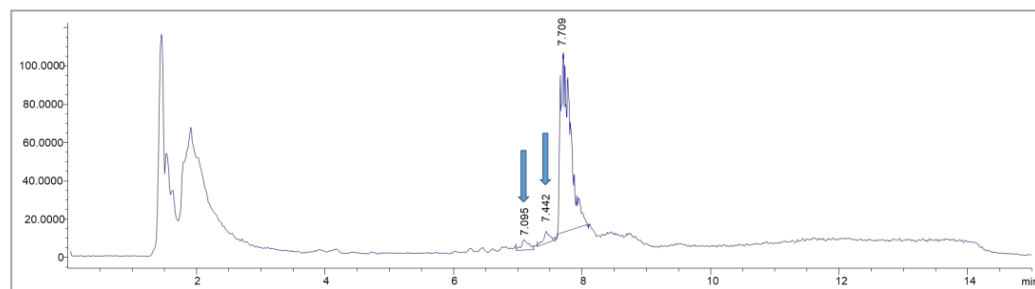


Figure 8. MS trace of an exemplary LC-MS measurement of a sample without buffer adjustments, irradiated with X-rays equivalent to 130 Gy. The blue arrows indicate the pre-peaks newly formed due to the irradiation.

In the corresponding MS spectra, one of the pre-peaks ($R_t = 7.095$ min) could in fact be identified as the deiodinated Gd-PSMA^{I&T} (see Figure 9) with a molecular mass of 1527 ($m/z = 763.8$ $[M-I+H]^+2$). The other side product, with a retention time of $R_t = 7.442$ min, showed multiple mass peaks ($m/z = 104.4$; 740.8; 521.2) and could not be identified unambiguously (Figure 9). The main peak, however, showed intact Gd-PSMA^{I&T} ($R_t = 7.709$ min; $m/z = 828.8$ $[M+2H]^+2$; 839.1 $[M+H+Na]^+2$; see Figures 8 and 9).

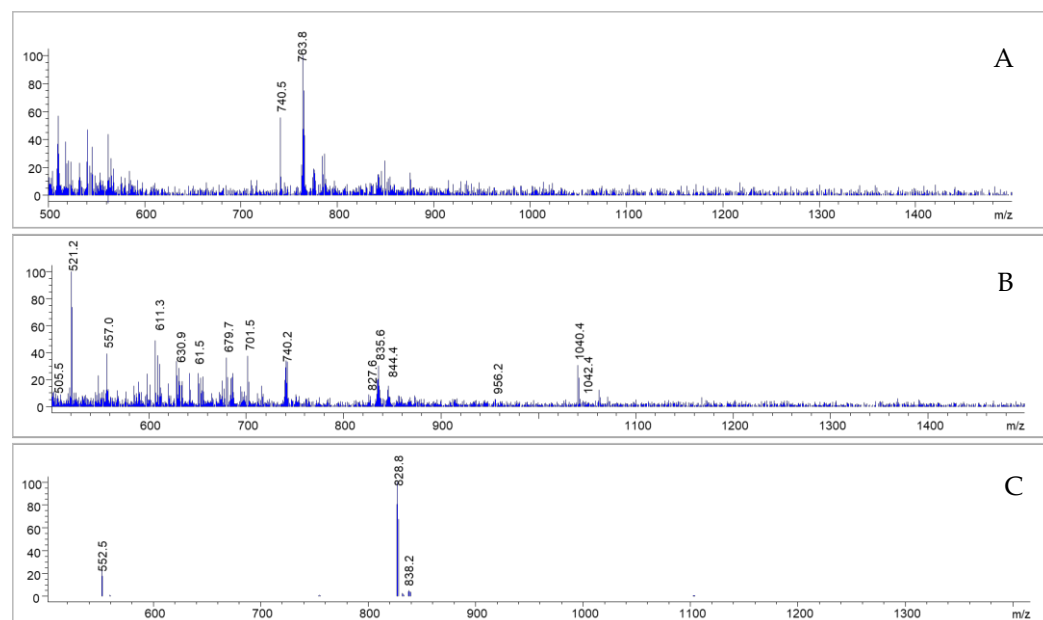


Figure 9. MS spectra corresponding to the impurities detected ($R_t = 7.095$ (A); 7.442 (B) and 7.709 (C)); after irradiation of Gd-PSMA^{I&T} without adjusted buffer system, as described further above.

To quantify the effect of the irradiation on Gd-PSMA^{I&T} in the two different reaction buffers, the peak of the identified deiodinated compound was integrated and correlated with the radiation dose (Figure 10). The effect of the radiolysis quencher EtOH was shown to be very effective in the dose ranges up to 117 Gy (equivalent to 36 GBq or 1.8 GBq/mL). In the batch with the highest irradiation dose, the deiodinated compound could be detected in both buffer formulations. Since the primary goal was to identify the major radiolysis product, the irradiation experiment was performed only once (in duplicates).

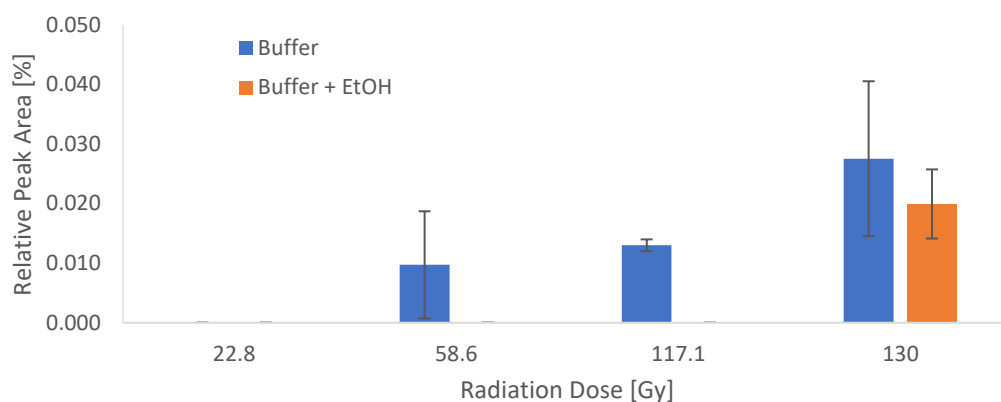


Figure 10. Integrals of the deiodinated compound determined via UPLC-MS in correlation with the applied dose in the two different reaction buffers. N = 1, performed in duplicate.

To further prove the suspicion of deiodination as a radiolysis mechanism, customized “deiodinated” PSMA^{I&T} was labeled with Lutetium-177. Using the optimized synthesis method, the overlay chromatogram showed highly comparable retention times, $R_t = 11.967$

vs. 11.950 min, for the radiolabeled custom peptide and the radiolysis peak of the [^{177}Lu]Lu-PSMA^{I&T} routine batch from the same day (Figure 11).

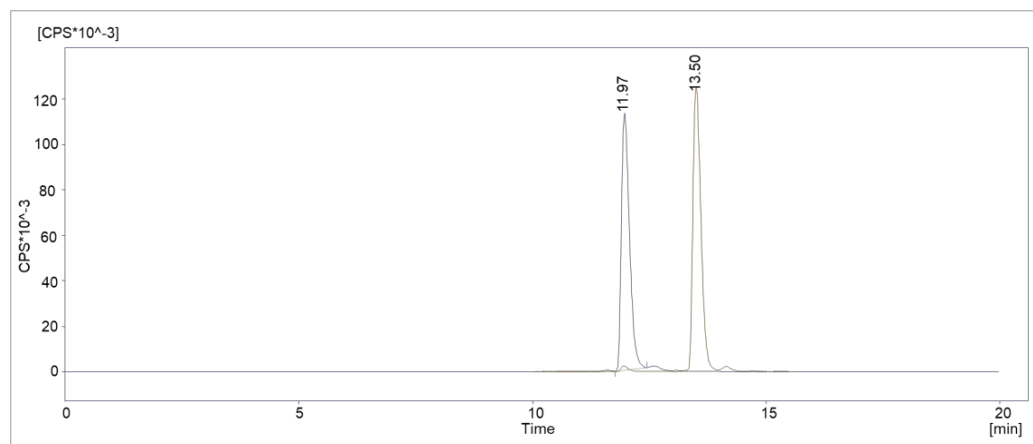


Figure 11. Overlay chromatogram of deiodinated [^{177}Lu]Lu-PSMA^{I&T} (blue) and a routine [^{177}Lu]Lu-PSMA^{I&T} batch (orange) from the same day.

Next to the radiolysis side products, a side product in the peak tail of the main product was also found by UPLC-MS. The area showed a mass difference of 18 in comparison to the parent compound, indicating a cyclization process under the dissociation of water (Figure 12).

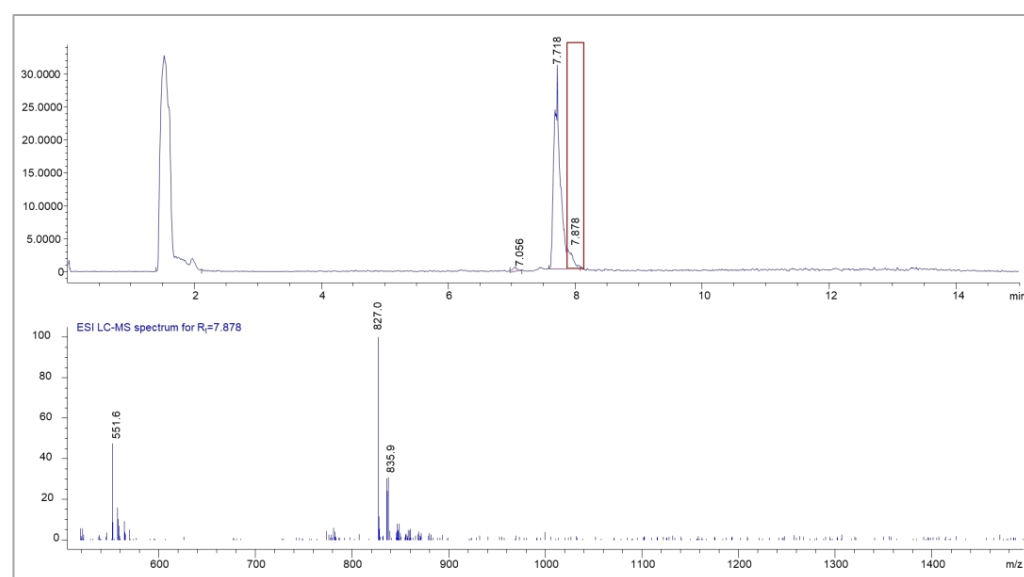


Figure 12. UPLC-MS chromatogram of [^{nat}Lu]Lu-PSMA^{I&T} with the MS spectrum of the peak tail ($R_t = 7.878$ min); clearly visible are condensation products of ^{nat}Lu -PSMA ($m/z = 835.0 + [\text{M}+2\text{H}]^{2+}$) with the mass of $m/z = 827 [\text{M}-\text{H}_2\text{O}+2\text{H}]^{2+}$.

This product was attributed to the heat-dependent condensations of the PSMA-binding motif, as published earlier by Martin et al. [14] for [^{177}Lu]Lu-PSMA-617, resulting in three possible side products (Figure 13). As already mentioned in Section 2.2, the condensation products did not correlate with the activity concentration.

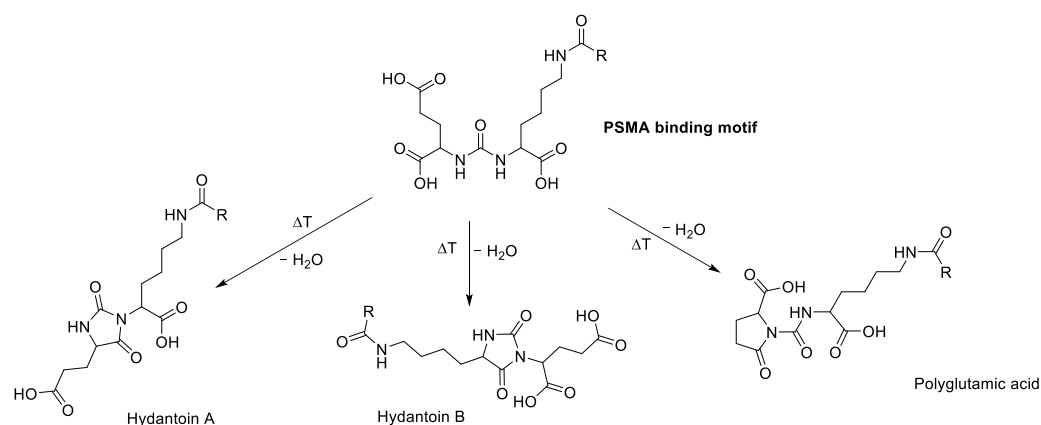


Figure 13. The PSMA binding motif and its possible heat-dependent condensation products, as proposed by Martin et al. [14].

2.4. Economical Optimization

Lastly, a radiochemical yield analysis of the new production method yielded a possible reduction in starting activity per patient from 9 to 8 GBq. Given the daily limit of 42 GBq Lutetium-177 in our department, serving up to 5 patients per batch became possible due to the successful upscaling process, reducing relative radiation exposure for the involved staff, overall costs and the relative formation of the deiodination product at the same time (Table 2).

Table 2. Retrospective analysis of 26 routine batches of [^{177}Lu]Lu-PSMA^{I&T} with average yields shows excellent yields with the possibility of reduction in starting activity; SA = starting activity; * 1 patient receives a therapeutic dose of 7.4 GBq.

Patients */Batch	SA Original Method [GBq]	SA Adapted Method [GBq]	av. RCY [%]	av. Absolute Yield [GBq]	Minimum Final Activity [GBq]
5	40	-	97.7 ± 0.5	41.5 ± 0.2	37.0
4	36	32	96.3 ± 1.2	36.8 ± 0.8	29.6
3	27	24	96.5 ± 2.6	27.4 ± 2.0	22.2
2	18	16	93.6 ± 5.3	17.6 ± 1.3	14.8

3. Methods

3.1. Chemicals

All chemicals and substances were used as received without further purification. GMP-grade PSMA^{I&T} was purchased from SCINTOMICS (Fürstentfeldbruck, Germany). Custom-synthesized Glu-CO-Lys(Sub)DLys-DPhe-DTyr-DOTAGA trifluoroacetate was obtained from piCHEM Forschungs- und Entwicklungs GmbH (Raaba-Grumbach, Austria). Sodium ascorbate buffer (Polatom; kit ASC-01 containing 50 mg ascorbic acid and 7.9 mg NaOH) was received from Polatom (Warsaw, Poland) and diluted as indicated with Trace SelectTM water (Honeywell Austria, Vienna, Austria). N.c.a. [^{177}Lu]LuCl₃ was purchased either from Isotope Technologies Munich (Munich, Germany) or Isotopia (Petah Tikva, Israel) in GMP quality. For dilutions and HPLC, deionized water generated from a MilliQ device (Merck Millipore) or Aqua ad injectabilia (B. Braun, Maria Enzersdorf, Austria) was used.

3.2. Chromatography

3.2.1. UPLC-MS Measurements

Analytical UPLC-MS runs were performed on an Agilent 1260 Infinity II system equipped with a flexible pump, an Agilent 1260 UV detector with variable wavelength ($\lambda = 220$ nm) and an LC/MSD mass detector, in combination with either a Chromolith

performance RP-18 (100 × 4.6 mm, flow = 1 mL/min) or an Acquity BEH C18 column, 1.7 µm, 3.0 × 50 mm (flow = 0.6 mL/min). A binary mobile phase of H₂O + 0.1% TFA (A) and acetonitrile + 0.1% TFA (B) was used.

3.2.2. HPLC Measurements

All HPLC measurements of the radioactive products were performed on a VWR Hitachi Chromaster System which included a column oven and UV detection unit, equipped with a Ramona Star Beta radiation detector (Elysia Raytest, Straubenhardt, Germany). HPLC measurements were performed at RT with a UV detection wavelength of 250 nm with a binary mobile phase of H₂O + 0.1% TFA (A) and acetonitrile + 0.1% TFA (B).

For method 1, a chromolith performance RP-18 (100 × 4.6 mm, flow = 2 mL/min) column (Merck, Darmstadt, Germany) was applied. The respective gradient was 5% to 50% B for 5.5 min.

For method 2, a Jupiter Proteo 4 µm RP (250 × 4.6 mm, flow = 1 mL/min, Phenomenex, Phenomenex Inc., Torrance, CA, USA) was used with the gradient displayed in Table 3. The validation of HPLC method 2 was analyzed via Validat[®] software (v1) (GUS LAB GmbH, Gera, Germany) and based on the current ICH Q2(R1) and EANM guidelines [17,18].

Table 3. Solvent gradient for HPLC method 2 (flow = 1 mL/min).

Time [min]	A (%)	B (%)
1	90	10
2	88	12
3	84	16
5	80	20
7	76	24
8	74	26
9	72	28
10	71	29
11	70.5	29.5
12	70	30
14	69.5	30.5
17	5	95
18	95	5
20	95	5

3.3. Validation of HPLC Method 2

Calculations were performed automatically via Validat[®] software. Intermediate blank injections were performed between measurements and checked for residuals of the respective test compounds. Injection volume was 20 µL for all measurements. Acceptance criteria are displayed in Table 1. Specificity regarding [¹⁷⁷Lu]Lu-PSMA^{I&T} and the deiodinated product was calculated by Validat[®] according to the retention times of the substances and the resulting resolution.

For the determination of linearity and LOD/LOQ of the precursor, seven different concentrations between 1 µg/µL and 0.005 µg/µL were prepared by dilution in water for injection. Each concentration was measured as triplicate.

Precision measurement of the reference standard was performed as follows: 10 µL (1 µg/µL) reference standard were mixed with 10 µL water for injection and the measurement was repeated 6 times.

For linearity and LOD/LOQ of the reference standard, seven different concentrations between 1 µg/µL and 0.005 µg/µL were prepared by dilution in water for injection. Each

concentration was measured as triplicate. For the determination of the linearity of the radiodetector, 5 different activities of Lutetium-177 between 3.780 and 0.034 MBq were injected. To determine the repeatability of the radiodetector, a sample of 3.8 MBq was injected 5 times and total peak areas were analyzed.

3.4. Radiosyntheses

Radiosyntheses were performed on a Modular-Lab PharmTracer (Eckert & Ziegler GmbH, Berlin, Germany) using the corresponding single-use disposable cassettes.

Before each synthesis, an automated cassette pressure test was performed to ensure the leak-proofness of the synthesis cassette. The production was performed automatically and step-wise by the Modular Lab (version 6.2). The steps are displayed in Table 4.

Table 4. Automated radiosynthesis procedure; the reaction buffer, radiosynthesis conditions in step 3 and the final formulation buffer and volume in step 6 vary between the original and adapted method and are described in Table 5.

#	Step
1	Conditioning of the Sep-Pak [®] C18 Plus cartridge with water/ethanol (50/50 mixture) followed by the formulation buffer
2	Transfer of radioactivity to reactor and rinsing of the activity vial with 1.4 mL of reaction buffer
3	Radiosynthesis
4	Transfer of the reaction mixture to the Sep-Pak [®] C18 Plus Cartridge
5	Elution of the product with EtOH/H ₂ O (2.5 mL, 50/50 mixture)
6	Formulation of the product to a final volume of 20 mL with formulation buffer

Table 5. Differences between the original and the adapted synthetic procedure.

Parameter	Original Method	Adapted Method
Product vial preparation	Sterile filtration of 0.15 mL (=30 mg DTPA) Ditripentat-Heyl [®] (200 mg/mL) solution into product vial	Sterile filtration of 0.15 mL (=30 mg DTPA) Ditripentat-Heyl [®] (200 mg/mL) solution and 1.4 mL reaction buffer into product vial
Reaction buffer	35.7 mg/mL L (+)-ascorbic acid, 11.1 mg/mL NaOH (commercial buffer kit, Polatom [®]) in 1.4 mL Trace Select [®] water	92.1 mg/mL L (+)-ascorbic acid, 111.4 mg/mL sodium acetate trihydrate, 34 mg/mL gentisic acid in 1.4 mL water for injection, adjusted to pH 5.2 with 2 N NaOH
Precursor amount	125 µg/~9 GBq n.c.a. Lutetium-177	
EtOH present during synthesis	200 µL+ 0.5 µL per µg precursor	0.5 µL per µg precursor
T [°C]	90	95
t [min] labelling reaction	10	30
Formulation buffer	16 mL phys NaCl 0.9%	24 mg/mL sodium ascorbate + 2.4 mg/mL L (+)-ascorbic acid in 16 mL phys NaCl 0.9%
Total volume EOS [mL]	18.6	20.0

After each synthesis, a fully automated filter test of the product sterile filter, as programmed in the software, was performed. Differences between the old and new synthesis method are displayed in Table 5.

3.5. Syntheses of $^{nat}\text{Lu-PSMA}^{\text{I\&T}}$ and $^{nat}\text{Gd-PSMA}^{\text{I\&T}}$

Briefly, 250 μg (167 nmol) $\text{PSMA}^{\text{I\&T}}$ was dissolved in 200 μL of sodium ascorbate buffer (1 M, pH 4.5). Then, 10 equivalents (1.67 μmol) of either Gadolinium(III) nitrate or Lutetium(III) chloride in 1 mM aqueous solution were added. The vial containing the mixture was heated at 95 $^{\circ}\text{C}$ in an aluminum heating block for 10 min. After cooling to RT, qualitative LC-MS was performed without further workup. LC-MS conditions were RP-LCMS, Chromolith performance; 5–50% MeCN + 0.1% TFA over 10 min.

3.6. Dose Calculations

To estimate the radiation dose absorbed in a sample, the contributions of all particles resulting from any decay have to be considered. ^{177}Lu decays to ^{177}Hf by four possible β^- transitions. The relative intensities of these transitions are 11.6%, 0.016%, 8.89% and 79.44% [19]. The average energies $\langle E_{\beta} \rangle$ of the corresponding β particles are 47.23 keV, 78.12 keV, 111.20 keV and 148.84 keV, respectively. During the subsequent transition of the resulting excited state to the ground state additional conversion electrons, Auger electrons, γ photons and X-rays are emitted. The total energy of β particles and electrons is 146 keV (2.34×10^{-14} J) per decay and the total energy of photons is 33 keV (5.29×10^{-15} J) per decay. If a sample is sufficiently large, the energy of all β^- particles and electrons is deposited within the sample. For the dose estimation, the dose of the photons will be neglected, since only a small fraction of the energy will be absorbed within the sample. Thus, the absorbed dose D is given by

$$D = \frac{NE_{e^-}}{m} \quad (1)$$

where N is the number of decays, E_{e^-} is the total energy per decay of β particles and electrons and m is the mass of the sample.

The number of disintegrations within a time interval t is given by

$$N = \int_0^T A e^{-\frac{t \ln 2}{t_{1/2}}} dt = A \frac{\ln 2}{T_{1/2}} \left(1 - e^{-\frac{t \ln 2}{t_{1/2}}} \right) \quad (2)$$

with A being the activity and $t_{1/2}$ the half-life.

If t is large with respect to the half-life $t_{1/2}$, N approaches $N = A \frac{\ln 2}{t_{1/2}}$. For t much shorter than $t_{1/2}$, N can be approximated as $N \approx A T$ using the Taylor series of the exponential function. Assuming an approximate density of 1 g/cm^3 and t much shorter than $t_{1/2}$, the dose can be calculated as

$$D = t \times A_c \times 2.34 \times 10^{-2} \text{Gy} \cdot \text{mL} \quad (3)$$

where t is the time in seconds, D is the dose in Gy and A_c is the activity concentration in GBq/mL .

3.7. Irradiations

After the preparation of one batch of $\text{Gd-PSMA}^{\text{I\&T}}$ and another batch of $\text{Gd-PSMA}^{\text{I\&T}}$ mixed with 280 μL of ethanol, each batch was divided into 8 portions. The samples were irradiated in duplicates with 4 different doses of X-rays, according to Table 6.

For that, an YXLON reference irradiator (Maxishot, YXLON International GmbH, Hamburg, Germany) was used, as previously described [20]. In brief, irradiation was performed at 200 kV, 20 mA with a focus size of 5.5 mm, using a 0.5 mm copper filter and a 3 mm aluminum filter. The average dose rate was about 1.1 Gy/min. After irradiation, the samples were subjected to LC-MS measurements.

Table 6. Irradiation parameters (actual dose and calculated activity equivalents) of the irradiated samples.

Sample Number Gd-PSMA ^{I&T}	Sample Number Gd-PSMA ^{I&T} + EtOH	Dose [Gy]	Activity Equivalent for 45 min Storage Time [GBq]
1.1	2.1	22.8	7
1.2	2.2	58.6	18
1.3	2.3	117.1	36
1.4	2.4	130	40

3.8. Conclusions

In this study, a thorough analysis of the quality control parameters of [¹⁷⁷Lu]Lu-PSMA^{I&T} was performed. Major optimizations in HPLC analyses and irradiation experiments followed by UPLC-MS studies revealed a significant contribution of cyclization and radiolysis products to the amount of radioactive impurities in the production of [¹⁷⁷Lu]Lu-PSMA^{I&T}.

The pre-peak, now known to consist of deiodinated [¹⁷⁷Lu]Lu-PSMA^{I&T} and induced by radiolysis, is expected to show altered pharmacokinetics and might reduce the overall tumor dose, but still demonstrated cell uptake in a previous study [15]. Our findings regarding the identity of the pre-peak are in accordance with a recently published study [11] and corroborate them by the approach of radiolabeling of the suspected radiolysis product and comparing the HPLC retention time with that of a [¹⁷⁷Lu]Lu-PSMA^{I&T} routine batch.

The cyclization products were already shown to lack tumor cell binding, due to the altered PSMA binding motif. Further, NMR studies were performed to elucidate the definite structure of the cyclization products [14]. In our study, the conclusion was drawn based on the combination of UPLC-MS, HPLC elution profile and independency from activity concentration.

A risk-based approach resulted in the extension of our radiochemical purity thresholds for the sum of hydantoins and deiodinated product for 90%, given the fact that the cyclization products constitute the most critical impurity with respect to therapeutic efficacy and only represented $2.87 \pm 0.85\%$ of the total radioactivity in the samples. As expected, the formation of the main radiolysis product was shown to depend on the activity concentration and represented $2.8 \pm 0.1\%$ of the total radioactivity in the samples.

To our knowledge, this is the first time that the discussed cyclization peaks were shown to contribute to the overall amount of radiochemical impurities in [¹⁷⁷Lu]Lu-PSMA^{I&T} and the question of how adequate HPLC methods should be used to detect them was addressed.

Further, an overall improvement of processes for the routine supply of [¹⁷⁷Lu]Lu-PSMA^{I&T} was achieved and it was possible to keep up with the increasing demand for this radioligand therapy. Due to the reduction in starting activity, a possible cost reduction through quality assurance procedures was also shown in this work.

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3.1.2 Manuscript 3

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Article

Radiobiological Assessment of Targeted Radionuclide Therapy with [^{177}Lu]Lu-PSMA-I&T in 2D vs. 3D Cell Culture Models

Julia Raitanen ^{1,2,3,4} , Bernadette Barta ¹, Hermann Fuchs ⁵ , Marcus Hacker ² , Theresa Balber ^{1,2,6} , Dietmar Georg ⁵ and Markus Mitterhauser ^{1,2,4,6,*}

¹ Ludwig Boltzmann Institute Applied Diagnostics, 1090 Vienna, Austria; julia.raitanen@meduniwien.ac.at (J.R.)

² Department of Biomedical Imaging and Image-Guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, 1090 Vienna, Austria

³ Vienna Doctoral School of Chemistry (DoSChem), University of Vienna, 1090 Vienna, Austria

⁴ Institute of Inorganic Chemistry, Faculty of Chemistry, University of Vienna, 1090 Vienna, Austria

⁵ Department of Radiation Oncology, Division of Medical Radiation Physics, Medical University of Vienna, 1090 Vienna, Austria

⁶ Joint Applied Medicinal Radiochemistry Facility, Medical University of Vienna, University of Vienna, 1090 Vienna, Austria

* Correspondence: markus.mitterhauser@univie.ac.at

Abstract: In vitro therapeutic efficacy studies are commonly conducted in cell monolayers. However, three-dimensional (3D) tumor spheroids are known to better represent in vivo tumors. This study used [^{177}Lu]Lu-PSMA-I&T, an already clinically applied radiopharmaceutical for targeted radionuclide therapy against metastatic castrate-resistant prostate cancer, to demonstrate the differences in the radiobiological response between 2D and 3D cell culture models of the prostate cancer cell lines PC-3 (PSMA negative) and LNCaP (PSMA positive). After assessing the target expression in both models via Western Blot, cell viability, reproductive ability, and growth inhibition were assessed. To investigate the geometric effects on dosimetry for the 2D vs. 3D models, Monte Carlo simulations were performed. Our results showed that PSMA expression in LNCaP spheroids was highly preserved, and target specificity was shown in both models. In monolayers of LNCaP, no short-term (48 h after treatment), but only long-term (14 days after treatment) radiobiological effects were evident, showing decreased viability and reproductive ability with the increasing activity. Further, LNCaP spheroid growth was inhibited with the increasing activity. Overall, treatment efficacy was higher in LNCaP spheroids compared to monolayers, which can be explained by the difference in the resulting dose, among others.

Keywords: radiation oncology; radiobiology; multicellular tumor spheroids (3D); targeted radionuclide therapy; [^{177}Lu]Lu-PSMA; prostate cancer (PC-3; LNCaP)



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1. Introduction

Cancer is the second leading cause of death, after cardiovascular diseases [1]. According to the statistics of the World Health Organization (WHO), the number of new cancer cases in 2020 accumulated to over 19 million and is estimated to rise up to 30.2 million by 2040 [2,3].

Radiotherapy plays a fundamental role in cancer therapy [4]. Currently, around 50% of all cancer patients require radiotherapy, either during primary management or for symptom control in a palliative setting [5]. While external beam radiotherapy (EBRT) is highly effective in primary cancers and solitary metastasis, it is not suited for treating micro metastases. In this context, molecular radionuclide therapy (MRT) is the treatment of choice [6]. The National Institutes of Health (NIH) have predicted that by 2035, radiopharmaceuticals will be used in approximately 60% of all radiation therapy procedures [7].

A current MRT treatment option of metastatic castrate-resistant prostate cancer (mCRPC) is systemic therapy with [^{177}Lu]Lu-PSMA-I&T. Due to the high expression of the prostate-specific membrane antigen (PSMA) in this type of tumor and Lu-177 being an ideal radionuclide for the treatment of micro-metastases (mean range of 670 μm and 0.1–2.2 MeV energies of beta particles), [^{177}Lu]Lu-PSMA-targeted radionuclide therapy is a highly effective therapy for most mCRPC patients. However, clinical studies show that around 30% of prostate cancer patients do not respond to this form of treatment [8,9].

To further improve MRT, biological research for new or improved vectors and a better understanding of radiation effects is needed [10]. Findings from radiobiology can not only support optimization, but also improve predictions on efficacy and adverse effects [11]. Moreover, radiobiological data are essential for better dose–effect modeling to overcome the common problem of suboptimal therapy delivery [12].

To date, *in vitro* therapeutic efficacy studies are mostly performed using a two-dimensional (2D) cell culture. Although simple, fast, and well-established, this model fails to properly mimic *in vivo* tumoral behavior. To overcome this limitation, the first three-dimensional (3D) cell culture model was introduced in 1943 [13]. Multicellular tumor spheroids were found to better recapitulate *in vivo* tumors in terms of their structure and growth behavior. Their structure is characterized by an inner necrotic core, an adjacent quiescent zone, and a proliferating outer layer, establishing molecular gradients [14–17]. The phenomenon of hypoxia and, thereof, resulting necrosis was previously shown to be apparent in many *in vivo* tumors [18].

In previous studies, the cell killing efficacy of Ra-223 [19], Ac-225 [20], Pb-212 [21], and Cu-64 [22] with different carriers was investigated in spheroids, showing significant detrimental effects.

In this study, we showcased an evaluation of the short- and long-term radiobiological effects of the clinically established [^{177}Lu]Lu-PSMA-I&T therapy using 2D and 3D cell culture models. Herein, we present several methods and describe the observed differences between the models.

2. Results

2.1. Verification of PSMA Expression via Western Blot

To verify conserved target expression upon growth in spheroids, cell lysates from monolayers and spheroids were prepared and blotted. As depicted in Figure 1, the PSMA-negative cell line, PC-3, showed no band in 2D and 3D, while high PSMA expression was demonstrated in both models of the LNCaP cell line.

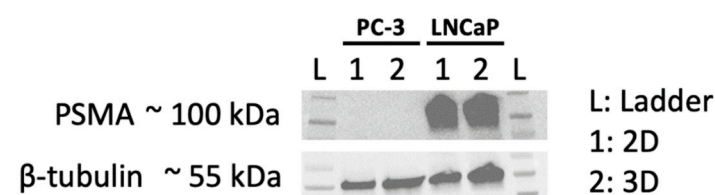


Figure 1. Western Blot of 2D and 3D samples of PC-3 and LNCaP cell lines; target = PSMA, loading control = β -tubulin.

2.2. Trypan Blue Exclusion Assay: Short-Term Cell Viability and Replicability in Monolayers

To investigate short-term cell viability and proliferation in monolayers, a dye exclusion assay was performed directly after treatment and 48 h after treatment over the range from 0 to 2 MBq for 3 h on LNCaP and PC-3 cells. No significant change in cell viability or cell number with increasing activity was observed: neither directly (0 h after treatment), nor 48 h after treatment with up to 2 MBq [^{177}Lu]Lu-PSMA-I&T for 3 h (Supplementary Figure S1).

2.3. Evaluation of Reproductive Potential in Monolayers by Standard Clonogenic Assay

To further evaluate the reproductive potential of cells cultivated in monolayers, clonogenic assays were performed. Figure 2 depicts a significant decrease in the surviving fraction with increasing activity in PSMA+ (PSMA positive) LNCaP cells. In contrast, PSMA- (PSMA-negative) PC-3 cells showed no significant change in the surviving fraction over the range from 0 to 2 MBq [^{177}Lu]Lu-PSMA-I&T treatment for 3 h.

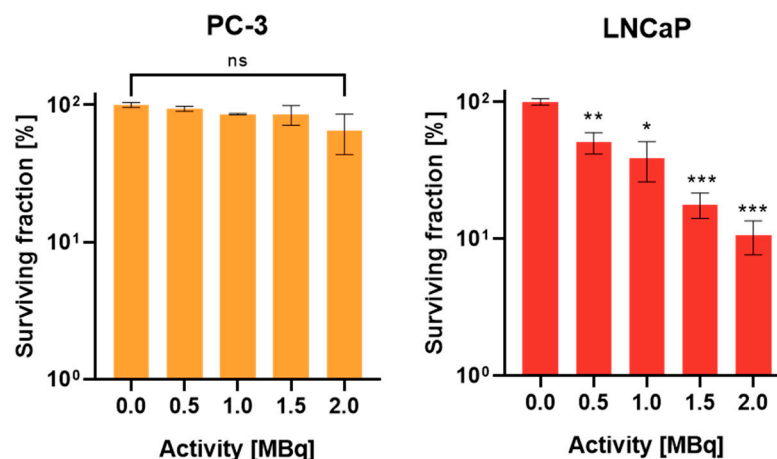


Figure 2. Survival of PC-3 (left) and LNCaP (right) cells; plotted as mean $\pm$ standard deviation from three to four independent experiments ($n = 3\text{--}4$) performed in triplicates. Statistical difference compared to control (ns = not significant, * = $p < 0.005$, ** = $p < 0.001$, *** = $p < 0.0001$).

2.4. Growth Analysis of Spheroids

While PC-3 spheroids showed no significant difference in size compared to the control up to 3.2 MBq, significant growth inhibition was evident in LNCaP spheroids already at the lowest applied dose of 0.01 MBq. Treatment with 0.05 MBq [^{177}Lu]Lu-PSMA-I&T showed a high treatment effect and was not significantly different to 0.2 or 0.4 MBq. Spheroid diameters 0, 7, 14, and 20 days after treatment are depicted in Figure 3. The spheroid shape and growth rate were cell line dependent. Time lapse images are given in the supplement (Supplementary Figure S2).

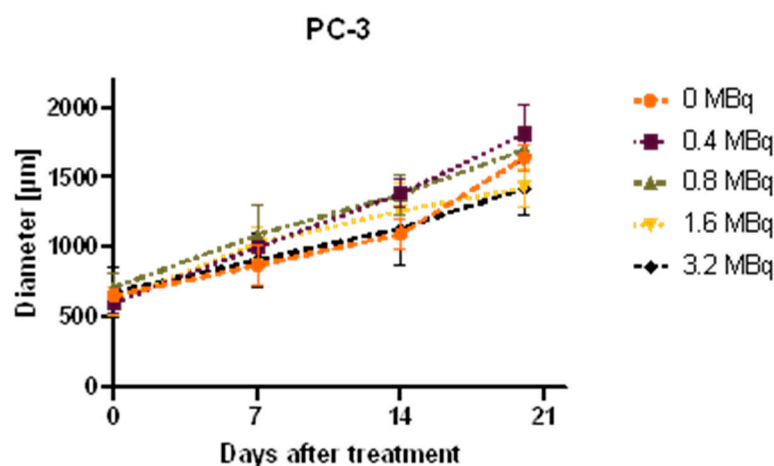


Figure 3. Cont.

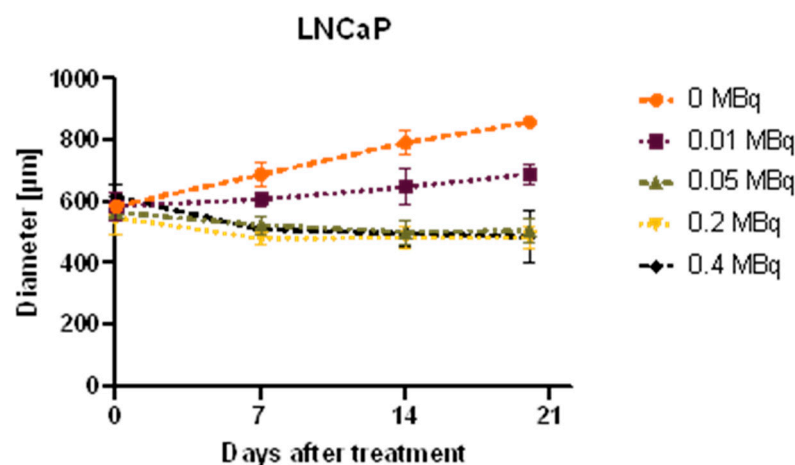


Figure 3. Growth observation of PC-3 (top) and LNCaP (bottom) spheroids after treatment. Diameter of spheroids; average over three diameter measurements per spheroid and time point are plotted as mean $\pm$ standard deviation from three independent experiments ($n = 3$).

2.5. Long-Term Cell Viability Determination Using Cell Titer Glo[®]: 2D vs. 3D

By means of the Cell Titer Glo[®] assay, ATP was measured as a surrogate for viability, detected by a luminescent readout. Figure 4 displays the cell viability comparison of 2D and 3D models of PC-3 and LNCaP cells. Neither monolayers nor spheroids of PC-3 showed a significant decrease in viability after treatment compared to the control, evident on day 7, 14, and 21 after treatment. For LNCaP, a significant decrease with increasing activity was detected. However, the viability in the LNCaP spheroids decreased already at much lower doses, compared to the monolayers. Further, the difference to the control increases over time. Please note the differences in the applied activity.

On day 7 after treatment with 0.2 MBq [¹⁷⁷Lu]Lu-PSMA-I&T for 3 h, a 2.4-fold difference between 2D and 3D was observed. This difference further increased over time, reaching a factor of 4.5 on day 21 after treatment, as shown in Table 1.

Table 1. Differences in cell viability in LNCaP between 2D and 3D after treatment with 0.2 MBq [¹⁷⁷Lu]Lu-PSMA-I&T for 3 h and observation on Day 7, 14, and 21.

Days after Treatment	X-Fold Difference (2D/3D)
7	2.4
14	2.9
21	4.5

2.6. Dosimetry Assessment Using Monte Carlo Simulations

To assess the influence of a geometrical effect on the resulting absorbed dose (in Gy) at the different models, Monte Carlo (MC) simulations were performed. Simulations were only used to assess the deposited dose on a macroscopic level. As shown in Figure 5, the cell monolayer was modeled using a cylinder of a 12 mm diameter and 0.01 mm height, and for the 3D spheroids, a sphere with a diameter of 0.6 mm was used. The surrounding medium was modeled by water and assuming evenly distributed activity for the treatment of 3 h.

Depending on the geometry of wells and models, considerable gradients were observed in the absorbed dose. Using an activity of 1 MBq and 3 h of treatment duration, the scoring region (depicted in green) in the 2D setting received 0.25 Gy, while the scoring region in the 3D setting received 4.54 Gy in the same conditions. A conversion table from the applied activity to the resulting absorbed dose is given in the supplement (Supplementary Table S1).

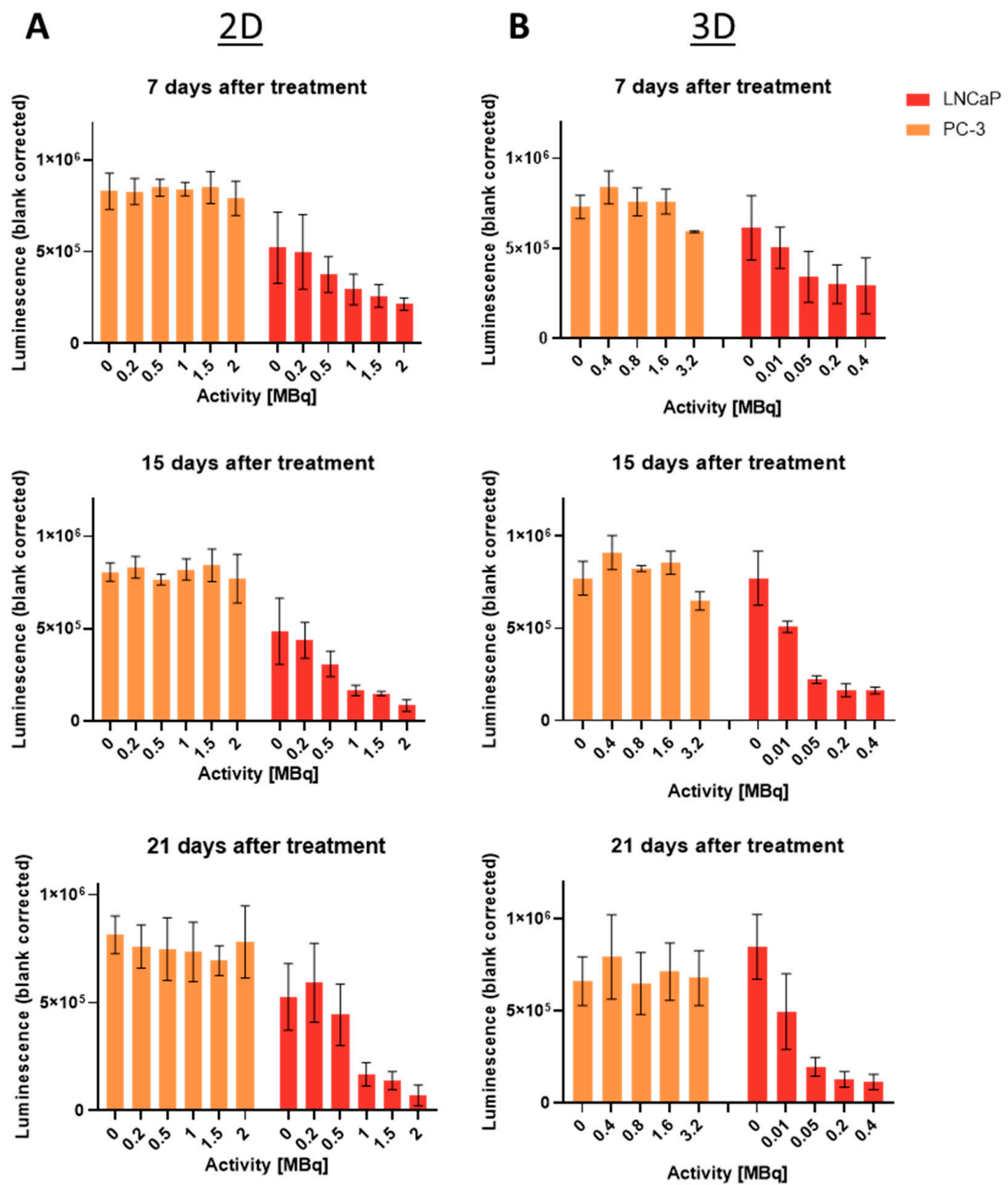


Figure 4. Cell viability assessment in 2D (A) and 3D (B) models of PC-3 and LNCaP cells on days 7, 15, and 21 after treatment, using Cell Titer Glo[®] 3D reagent. Plotted as mean $\pm$ standard deviation from three to four independent experiments ($n = 3-4$) in duplicates. Luminescence signal corresponds with viability. Note the differences in applied activity between 2D and 3D.

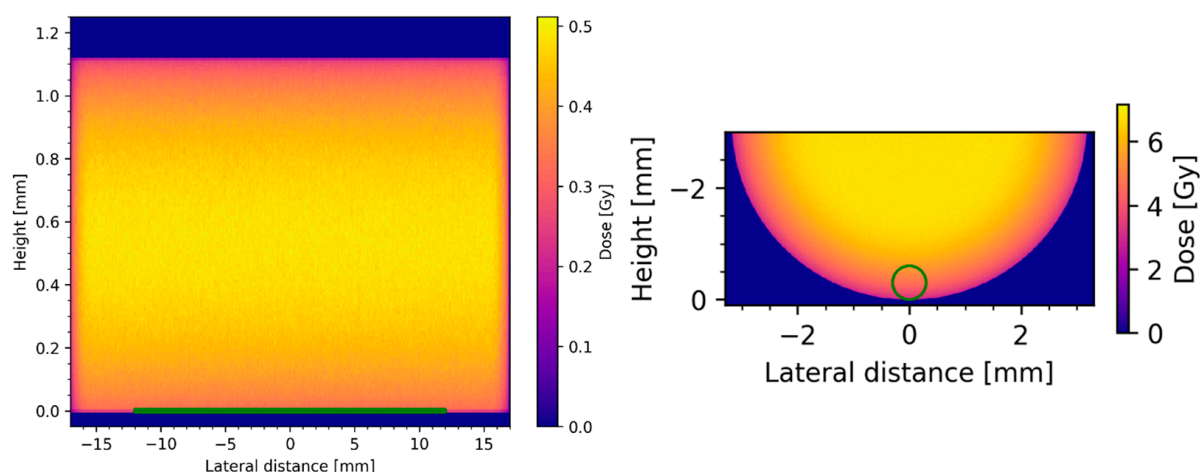


Figure 5. Visualization of dose distribution in 2D (left) and 3D (right) models. Green line/circle depicts monolayer/spheroid scoring region.

3. Discussion

In vitro radiobiological assessments of new therapeutics are currently mostly performed in 2D since it is affordable and already well-established. However, the lack of resemblance to in vivo tumors can lead to wrong estimations of efficacy. Multicellular tumor spheroids can enhance the predictive power and minimize the error by being a better mimic, ultimately reducing time and costs during drug development by the earlier detection of ineffective agents [23]. Thus, 3D models are credited to be fundamental tools in pharmaceutical development and biomedical research in the future [23,24].

This in vitro study investigated the differences in cell viability and proliferation between the 2D and 3D models of the two prostate cancer cell lines, PC-3 and LNCaP, after treatment with the already clinically used radioligand therapy agent, [^{177}Lu]Lu-PSMA-I&T. Thus, this study elucidates the differences between monolayers and spheroids in respect to radioligand therapy and provides the methodology for radiobiological studies of targeted radioligand therapy in spheroids.

After confirming preserved high PSMA expression in LNCaP spheroids and monolayers, which is the basis for the application of this targeted radionuclide therapy, we investigated cell viability and replicability in monolayers using a Trypan Blue Exclusion assay. No decrease in viability and cell number up to 48 h was observed, suggesting no effects of senescence or apoptosis in the first two days after treatment. However, as expected, a decrease in the surviving fraction with increasing activity was evident for the target expressing LNCaP cells after 14 days (clonogenic assay), but not in PSMA negative PC-3 cells, demonstrating the specificity of the therapeutic agent.

In 3D models, growth monitoring showed no treatment effect in the PSMA-lacking PC-3 cells, but significant growth inhibition over 20 days in PSMA+ LNCaP, confirming target specificity also in the 3D model. Interestingly, a significant treatment effect was evident already at the lowest activity of 0.01 MBq. Increasing the treatment from 0.05 MBq to 0.4 MBq showed no increase in growth inhibition. We assume that already with 0.05 MBq, most of the cells in the spheroid are either killed or senescent. In addition, we hypothesize that the higher dose of 0.4 MBq leads to different repair mechanisms, which let the cells survive in a similar extent as after treatment with 0.05 MBq.

The comparison of 2D and 3D in one assay was performed by means of a Cell Titer Glo[®] viability assay. When analyzing long-term cell viability after treatment with [^{177}Lu]Lu-PSMA-I&T, LNCaP spheroids were more effectively treated than the respective monolayers, apparent by the strong decrease in viability already at lower applied activities than in 2D. After treatment with 0.2 MBq [^{177}Lu]Lu-PSMA-I&T for 3 h and observation for 7 days afterwards, cell viability was 2.4-fold higher in 2D, compared to 3D. Over time, this effect increased, reaching a 4.5-fold difference on day 21 after treatment.

In order to evaluate the extent of the geometric effects, resulting from the large beta particle range of Lu-177, Monte Carlo simulations were performed. Our results showed that the cell monolayer received only about 0.25 Gy while the spheroids received 4.54 Gy (corresponding to 1 MBq for 3 h), explaining the lower viability in 3D versus 2D. As shown before [25], Monte Carlo simulations could in principle be used to go beyond the macroscopic dose distribution employed in this manuscript and provide insights into the micro- and nanodosimetric scale. However, such models are still under investigation and no clear recommendations have been published so far. When comparing these findings to our previously published results [26], where we irradiated 2D and 3D models of the same cell lines with X-ray, the calculated absorbed doses for the applied activities fit well into the respective clonogenic survival curves previously published, validating the simulation.

A possible explanation for the increase in the sensitivity of spheroids to radionuclide therapy over time could be a retention of the compound in the 3D construct, leading to an extended treatment duration with a concomitant elevated bystander effect. However, it remains to be investigated whether the extent of this effect is similar to in vivo tumors.

4. Materials and Methods

4.1. Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

4.2. Cell Culture

The cell lines of PC-3 (PSMA-negative: PSMA-) and LNCaP (PSMA-positive: PSMA+) were obtained from the American Type Culture Collection (ATCC) and cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium. Medium was supplemented with 10% fetal bovine serum, 2 mM Glutamine, and 1% penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL). All cell culture reagents were obtained from Gibco Life Technologies Ltd. (Paisley, UK). Culturing took place at 37 °C with 5% CO₂ and cells were routinely tested for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland), according to the manufacturer's instructions [27].

4.3. Generation of Spheroids

Spheroids were generated as previously described [26], using the liquid overlay technique. In short, 2000 cells/well (in 100 µL medium) were seeded into U-bottom 96-well plates, which were precoated with a thin layer of 1.5% low gelling agarose. LNCaP were cultured scaffold-free. For PC-3 spheroids, 3% Geltrex™ (LDEV-Free Reduced Growth Factor Basement Membrane Matrix, Gibco) was added to the cell suspension. Plates were centrifuged at 161 × g for 10 min at 4 °C before further incubation at 37 °C and 5% CO₂.

4.4. Western Blot

Whole cell lysates were prepared to assess the amount of prostate-specific membrane antigen (PSMA) in 2D and 3D cultures. Then, 2D and 3D lysate preparation as well as Western Blot were performed as described previously [26].

Membranes were incubated with the following primary antibodies: anti-PSMA, (D4S1F; Cell Signaling Technology Europe B.V., Leiden, The Netherlands; 1:1000 in 5% DMP in TBS-T) or anti-β-Tubulin (Cell Signaling Technology; 1:1000 in 5% BSA in TBS-T) overnight at 4 °C. After incubation with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature, detection of chemiluminescence (Pierce™ ECL Western Blotting Substrate Kit; Thermo Fisher Scientific, Waltham, MA, USA) was performed using a ChemiDoc Imaging System (Bio-Rad Laboratories Inc., Berkeley, CA, USA), according to the manufacturer [28].

4.5. Treatment Procedure

[¹⁷⁷Lu]Lu-PSMA-I&T (10 MBq/nmol) was provided by the routine production at the General Hospital of Vienna. Monolayers and spheroids were treated for 3 h in either 1 mL (2D) or 100 µL (3D) medium with activities up to 3.2 MBq (total activity per well). Medium was discarded and renewed after treatment. Spheroids were additionally washed with 100 µL DPBS before adding 100 µL of fresh medium. Medium was renewed once per week until the last experiment, 3 weeks after treatment.

4.6. Microscopic Growth Analysis

An Olympus IMT-2 Inverted Microscope with an XC50 Color Camera (Olympus, Tokyo, Japan) and cellSens Entry (Version 2.3, Olympus) software were used to monitor spheroid growth and obtain diameter measurements on days 0, 7, 14, and 20 after treatment. One representative spheroid per cell line and activity was chosen for each experiment and a mean value of three diameter measurements per spheroid was calculated. Data were then plotted in Prism Version 8 (GraphPad, San Diego, CA, USA).

4.7. Clonogenic Assay

By use of standard clonogenic assays, the reproductive ability after radiation treatment was determined [29]. For this, 250 PC-3 or 1000 LNCaP cells were seeded in 6-well plates in 2 mL medium per well and incubated overnight. On the following day, plates were treated with 0.5, 1, 1.5, or 2 MBq [¹⁷⁷Lu]Lu-PSMA-I&T per well. PC-3 cells were further cultured for 7 days and LNCaP for 14 days, with medium being changed once per week. After washing with PBS, cells were fixed with methanol for 30 min at 4 °C. This was followed by another washing step and final staining with crystal violet (Sigma-Aldrich; 1% in water) for 3 min. Excess stain was removed by submerging the plates in tap water and stained colonies were counted by eye.

Plating efficiency was determined by dividing the average number of colonies in the control plate by the number of total seeded cells. To obtain the surviving fraction (SF), the plating efficiency was multiplied by the number of colonies after treatment.

4.8. Trypan Blue Exclusion Assay

For evaluation after 0 h, 500,000 cells of both cell lines, and for evaluation after 48 h, 100,000 PC-3 or 250,000 LNCaP were seeded in 6-well plates. After overnight incubation, cells were treated with 0.5, 1, 1.5, and 2 MBq per well (in 1 mL medium) for 3 h. 0 and 48 h after treatment; supernatants, wash fractions, and harvested cells were combined and centrifuged for 4 min at 161 × g at room temperature. Supernatants were discarded, and cell pellets were resuspended in 0.5 mL medium. For determination of cell viability and cell number, single cell suspensions were mixed with Trypan blue (1:1; Gibco) and assessed with a LUNATM automated cell counter (Logos Biosystems, Anyang, Republic of Korea).

4.9. Viability Assessment Using Cell Titer Glo[®] 3D

The assay was performed according to the manufacturer's protocol [30]. For 2D assessment, 4000 PC-3 or 40,000 LNCaP cells were seeded in 6-well plates, incubated overnight and treated with up to 3.2 MBq of [¹⁷⁷Lu]Lu-PSMA-I&T (day 0). On day 6, supernatant and detached cells of control and each treatment condition were collected and counted, hereby supernatant was not discarded, but combined with the detached cells to also retrieve dead/detached cells. Further, the liquid volume to seed a total of 5000 cells was calculated from the control. This calculated volume was seeded for all treatment conditions (in total 100 µL/well) in white-walled 96-well plates and incubated overnight. To assess viability on day 15, 4000 PC-3 or 40,000 LNCaP cells were again seeded in 6-well plates and incubated until day 14. The same procedure was applied for viability assessment on day 21. This procedure needed to be done in order to prevent overgrowth in the control plates, but still providing enough cells for viability analysis in the condition with the highest activity treatment. On days 7, 15, and 21, plates and reagent

were first equilibrated to room temperature for 30 min before addition of 100 μ L reagent per well. This was followed by vigorous shaking of the plate for 5 min using the Synergy HTX multimode reader (Biotek, Winooski, VT, USA). After another incubation at room temperature for 25 min, luminescence was measured in the same reader. For 3D viability assessment, spheroids were transferred to white-walled 96-well plates on days 7, 15, and 21. Reagent addition and measurement was performed as described for monolayers.

4.10. Dosimetry

Monte Carlo (MC) simulations were performed using GATE v9.3 alongside GEANT4 v11.1. GATE is an open-source general-purpose MC tool-kit, developed by the OpenGATE collaboration since 2001 [31,32]. Both 2D as well as 3D wells were modeled in GATE based on the respective manufacturer's cell vessel specifications, using 1 mm-thick polystyrene walls. In the simulation, medium was modeled by water and the treatment was applied as specified above. Activity was evenly distributed within the water volumes. To improve simulation efficiency, the Lu-177 decay was modeled solely using electron sources with the corresponding energies (78.6% 497 keV, 9.1% 384 keV, and 12.2% 176 keV). Scoring regions approximating the corresponding biological samples were designed as follows: for the 2D cell layer, a cylinder of 12 mm diameter and 0.01 mm height; for the 3D spheroid, a sphere with a diameter of 0.6 mm. Voxel size was set to 0.01 mm in all directions for the 3D well, while for the 2D well, a voxel size of $0.1 \times 0.1 \times 0.01$ mm was used to improve the simulation performance while remaining at maximum resolution. Uptake and retention into the 2D or 3D cell structures could not be considered during simulations. Absorbed dose (in Gy) in the scoring regions was averaged over the whole scoring volume. Simulations were performed to achieve a statistical variance below 1.5%. MC parameters are listed in Table 2.

Table 2. MC parameters used for dosimetric simulations.

Parameter	Value
Physics list	QGSP_BIC_HP_EMZ
Ionization potential of water	78 eV
Production and stopping cuts in scoring region	0.01 mm
Maximum step size in scoring region	0.01 mm

4.11. Statistical Analysis

Data analysis was performed in Prism software version 8 (GraphPad, San Diego, CA, USA). For data comparison, multiple unpaired *t*-tests were performed and *p*-values < 0.01 were considered statistically significant.

5. Conclusions

We investigated the differences in radiobiological response when testing radionuclide therapy in monolayers and spheroids. Our results showed that the PSMA-targeted radionuclide therapy is specific for LNCaP in both models and more effective in 3D compared to 2D. MC simulations showed that geometric effects lead to higher doses for the 3D model compared to the monolayers.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms242317015/s1>.

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Supplementary information

Figure S1. Trypan Blue Exclusion Assay: evaluation of cell viability and proliferation in monolayers. Cell viability immediately (0 h; A) and 48 h (B) after treatment. Cell count 48 h (C) after treatment. Graphs are plotted as mean $\pm$ standard deviation from two independent experiments (n=2) performed in duplicates.

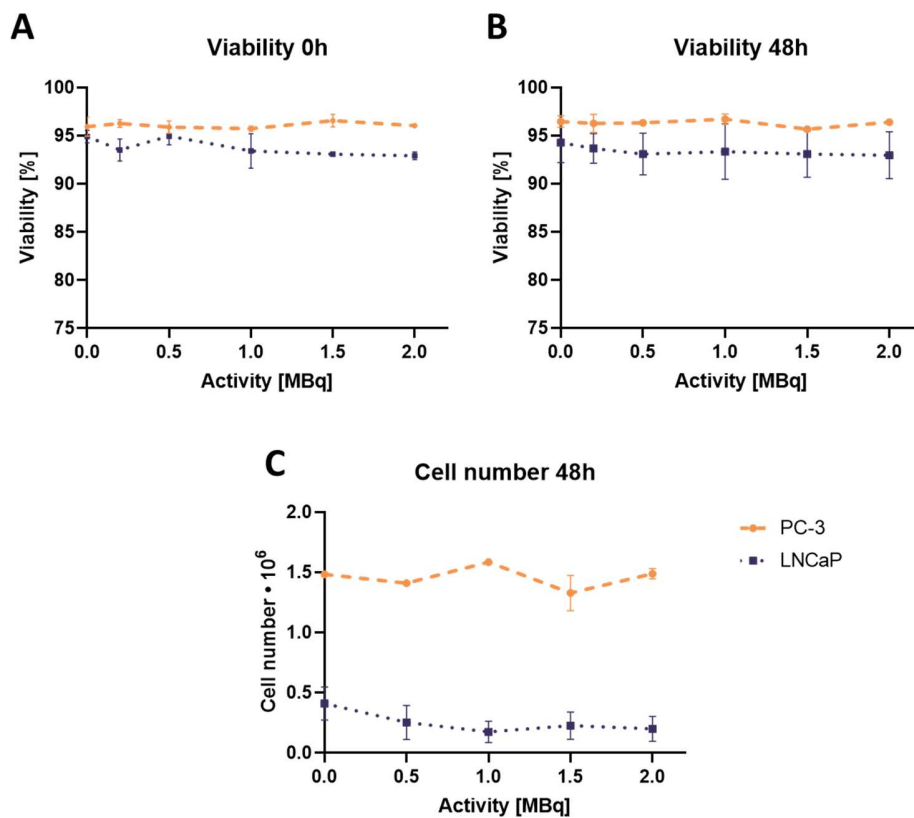


Figure S2. Time lapse growth observation of LNCaP and PC-3 spheroids treated with up to 3.2 MBq on day 0; white scale indicates 500 μm

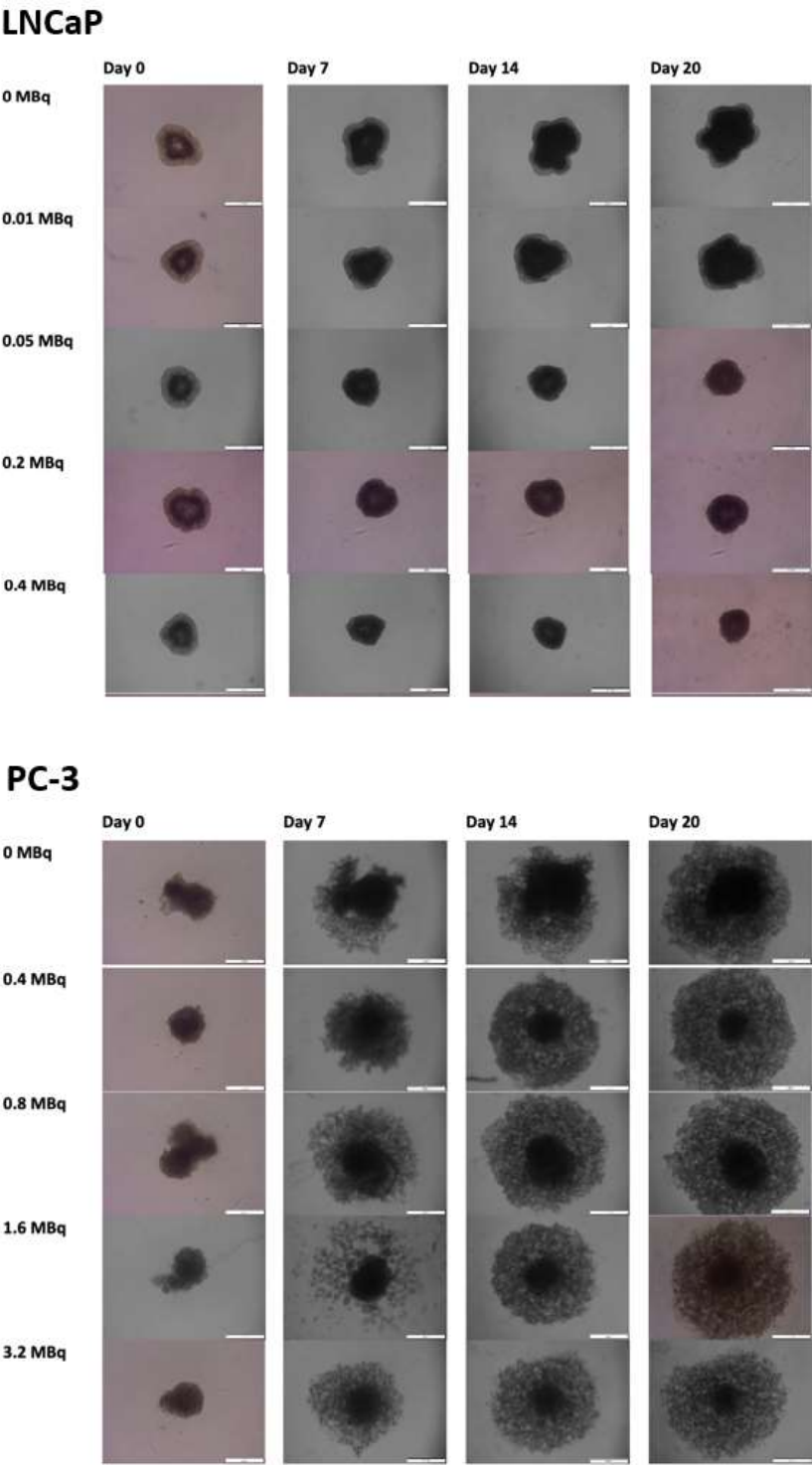


Table S3. Conversion table from applied activity to resulting dose

Applied activity [MBq]	Resulting dose 2D [Gy]	Resulting dose 3D [Gy]
0,01	0,002	0,05
0,05	0,01	0,23
0,2	0,05	0,91
0,4	0,10	1,82
0,5	0,13	2,27
0,8	0,20	3,63
1,0	0,25	4,54
1,5	0,38	6,81
1,6	0,40	7,26
2,0	0,50	9,08
3,2	0,80	14,53

3.2 Unpublished results

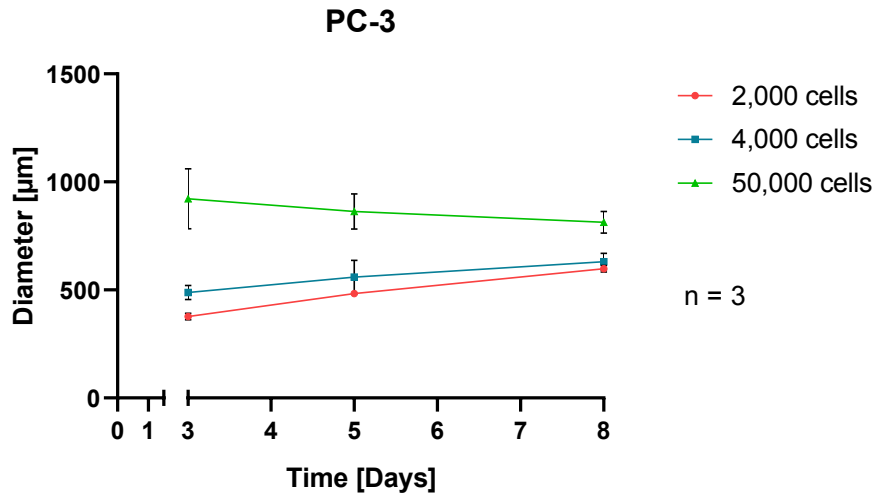
The *in ovo* model is an alternative *in vivo* model, representing an intermediate step between *in vitro* experiments and testing on rodents. The CAM-xenograft model was recently established at our facility. However, similar to *in vitro* testing, the CAM-xenograft model in the field of cancer research has mostly been used together with 2D cell culture. By combining our expertise in the fields of *in ovo* and 3D cell culture, we investigated the usability of prostate cancer tumor spheroids inoculated on the chick embryonal CAM.

The design of the study, the experimental work regarding egg incubation and CAM manipulation, as well as the subsequent histopathological evaluation of harvested xenografts were performed together with Katarína Benčurová, MSc. Further, Lena Palm, BSc. and Dionis Shoshaj, BSc. partially performed analyses.

Spheroid growth analysis

Before inoculation of the spheroids onto the chick embryonal CAM, spheroids of different sizes were generated. Therefore 2,000, 4,000 or 50,000 cells were seeded in agarose-coated round-bottom 96-well plates and spheroid growth was monitored over time. The respective methods are described in manuscript 1 & 3, corresponding data is presented in Figure 5 and Figure 6. Both, PC-3 and LNCaP spheroids of 50,000 seeded cells, did not increase in diameter over time but rather lead to higher cell density within 5 days of *in vitro* cultivation, visible in the corresponding photographs. A slight increase in size was observed for spheroids of the other groups.

(A)



(B)

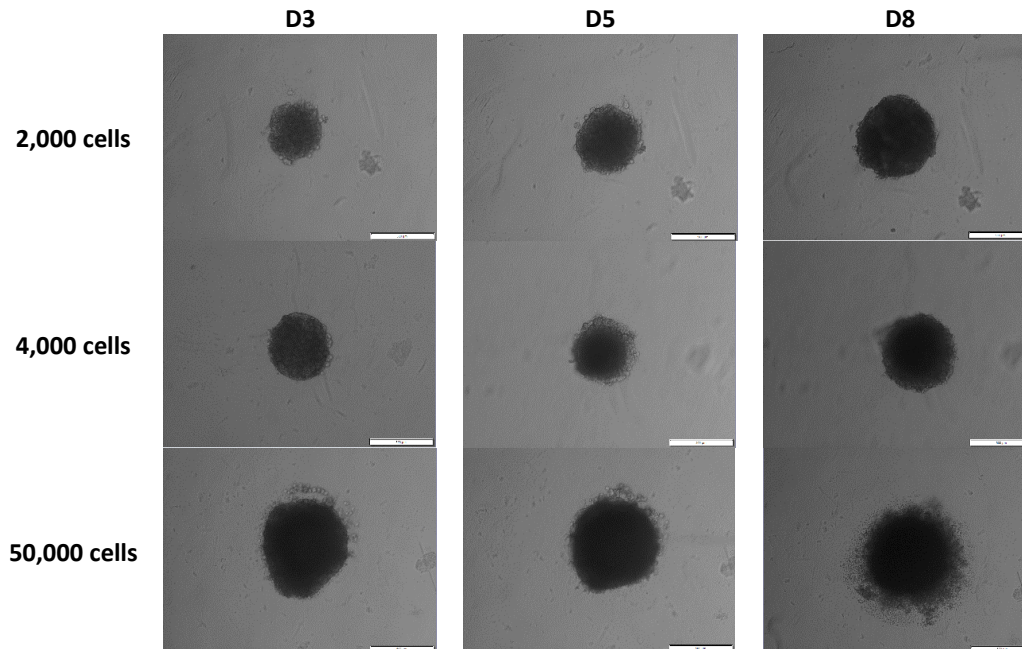
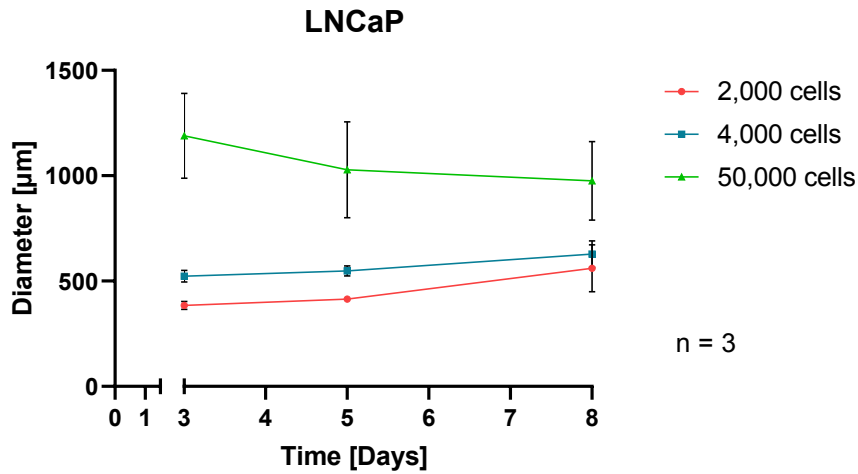


Figure 5: Growth observation of PC-3 spheroids, resulting from different seeding numbers (2,000, 4,000 or 50,000 cells on day 0). (A) Diameter measurements. Average over 3 diameter measurements per spheroid and time point, plotted as mean $\pm$ standard deviation from three independent experiments ($n=3$) in triplicates. (B) Photographs of representative spheroids on Day 3, 5 and 8 after seeding (D0). White scale indicates 500 μm .

(A)



(B)

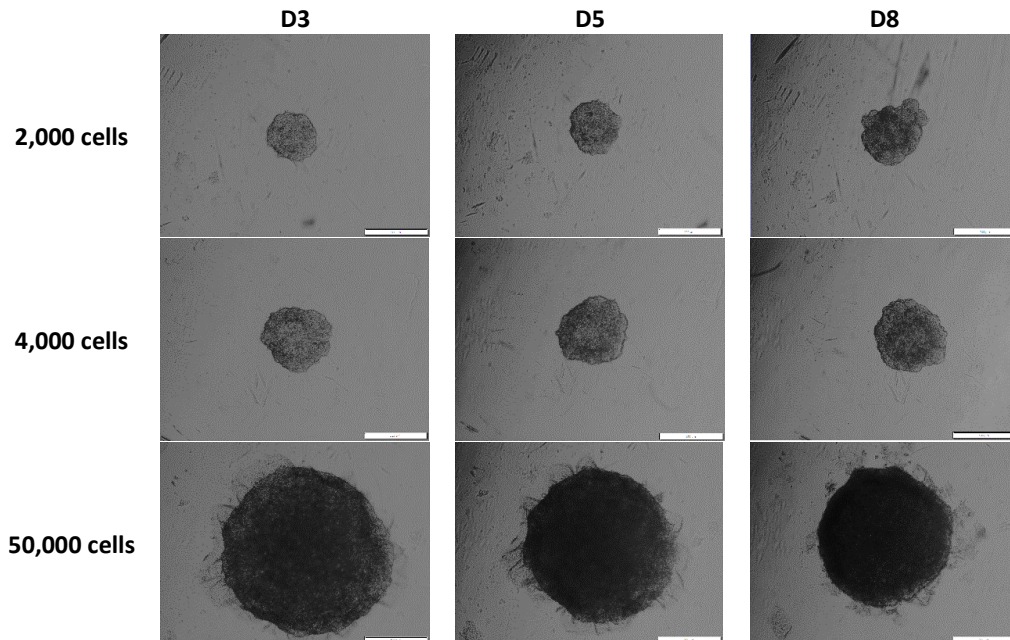
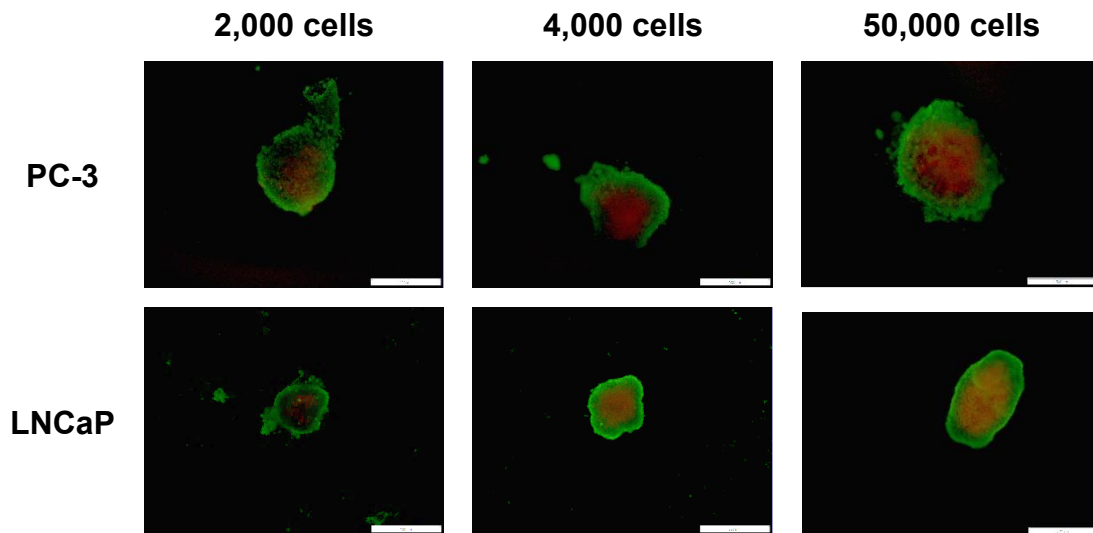


Figure 6: Growth observation of LNCaP spheroids, resulting from different seeding numbers (2,000, 4,000 or 50,000 cells on day 0). (A) Diameter measurements. Average over 3 diameter measurements per spheroid and time point, plotted as mean $\pm$ standard deviation from three independent experiments ($n=3$) in triplicates. (B) Photographs of representative spheroids on Day 3, 5 and 8 after seeding (D0). White scale indicates 500 μm .

Live/Dead Staining

To further characterize the spheroids, live and dead staining, using propidium iodide and calcein-AM were performed, as previously described in manuscript 1. The results are presented in Figure 7 and Table 3. The results showed a trend of increasing core-to-total-spheroid-diameter ratio with increasing cells seeded.

(A)



(B)

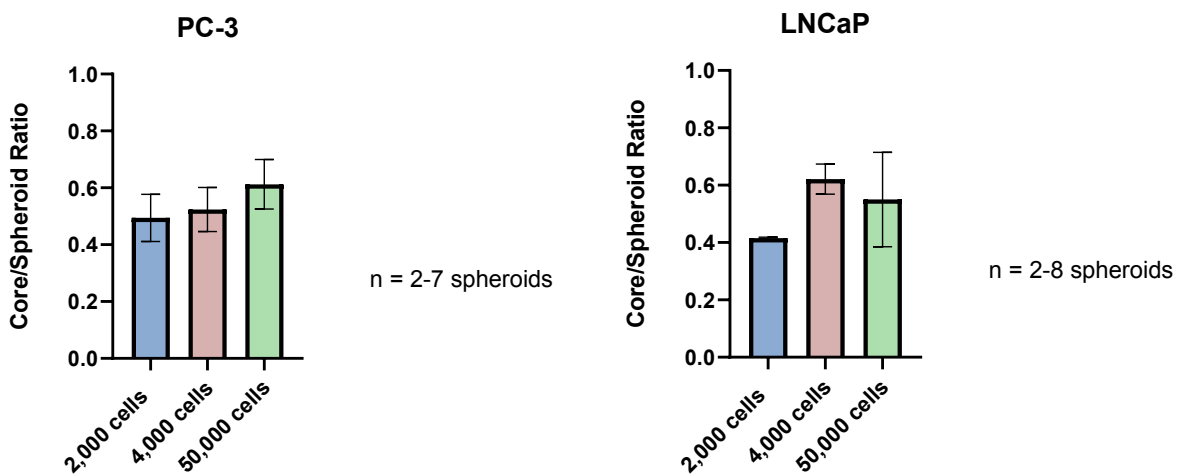


Figure 7: Live/dead staining of spheroids. (A) Representative images of propidium iodide (red) and calcein-AM (green) staining of spheroids on Day 8 after seeding; white scale indicates 500 μm

(B) Ratio necrotic core to total spheroid size for PC-3 and LNCaP; plotted as mean $\pm$ standard deviation from two to eight independent experiments (n=2-8)

Table 3: Diameter measurements (in μm) of total spheroid, necrotic core and calculated ratio for **(A)** PC-3 and **(B)** LNCaP. Average $\pm$ standard deviation.

(A) PC-3

	Spheroid diameter [μm]	Core diameter [μm]	Ratio (Core/spheroid)
2,000 cells (n = 2)	552.7 $\pm$ 29.1	274.9 $\pm$ 46.8	0.49 $\pm$ 0.06
4,000 cells (n = 5)	494.9 $\pm$ 49.5	261.8 $\pm$ 58.2	0.52 $\pm$ 0.07
50,000 cells (n = 7)	831.2 $\pm$ 116.7	512.8 $\pm$ 118.6	0.61 $\pm$ 0.08

(B) LNCaP

	Spheroid diameter [μm]	Core diameter [μm]	Ratio (Core/spheroid)
2,000 cells (n = 2)	380.6 $\pm$ 4.6	158.0 $\pm$ 0.9	0.41 $\pm$ 0.00
4,000 cells (n = 3)	457.0 $\pm$ 38.2	285.7 $\pm$ 44.2	0.62 $\pm$ 0.04
50,000 cells (n = 8)	586.9 $\pm$ 87.6	328.5 $\pm$ 127.9	0.55 $\pm$ 0.15

In ovo assay & histopathological evaluation of in ovo xenografts

Spheroids were inoculated on the chick embryonal CAM and further underwent histopathological evaluation, as described before [86].

Details on the take rate and average xenograft size, among others, are given in Table 4. The corresponding histopathological staining is depicted in Figure 8 and Figure 9. In our study half of the xenografts from PC-3 and 71% of the xenografts from LNCaP developed. However, the resulting xenografts were rather small in size (below 2 mm in diameter).

The performed histopathological evaluation showed viable tumor cells (Ki67), vascularization (Desmin) and little apoptosis (cleaved caspase 3) for xenografts grown from 2,000 and 4,000 seeded cells. In the representative images (see Figure 8) for PC-3, a dense population of tumor cells, with many viable cells (Ki67), blood vessel formation especially on the outer rim (Desmin) and low apoptosis (CC3) is shown. Regarding LNCaP, viable tumor cells were found foremost on the outer rim of the xenograft, as well as the blood vessels. Apoptosis was only found on a piece of the xenograft's center. For 50,000 seeded cells (see Figure 9), only H&E staining was performed, also showing a dense population of tumor cells surrounded by CAM.

However, a further evaluation was found to be not expedient, since no improvement to the current methodology, using cell suspensions, was observed. Nevertheless, progress in the field may provide bigger spheroids with smaller necrotic cores in the future and therefore may lead to better results.

Table 4: In ovo xenografts – facts and figures

		PC-3	LNCaP
Take rate of alive eggs		50% (5/10)	71% (5/7)
Average xenograft size (diameter in mm)		1.8 ± 0.1	1.9 ± 0.2
Histological staining	2,000 cells seeded	n=1	n=1
	4,000 cells seeded	n=1	n=1
	50,000 cells seeded	n=3	n=3

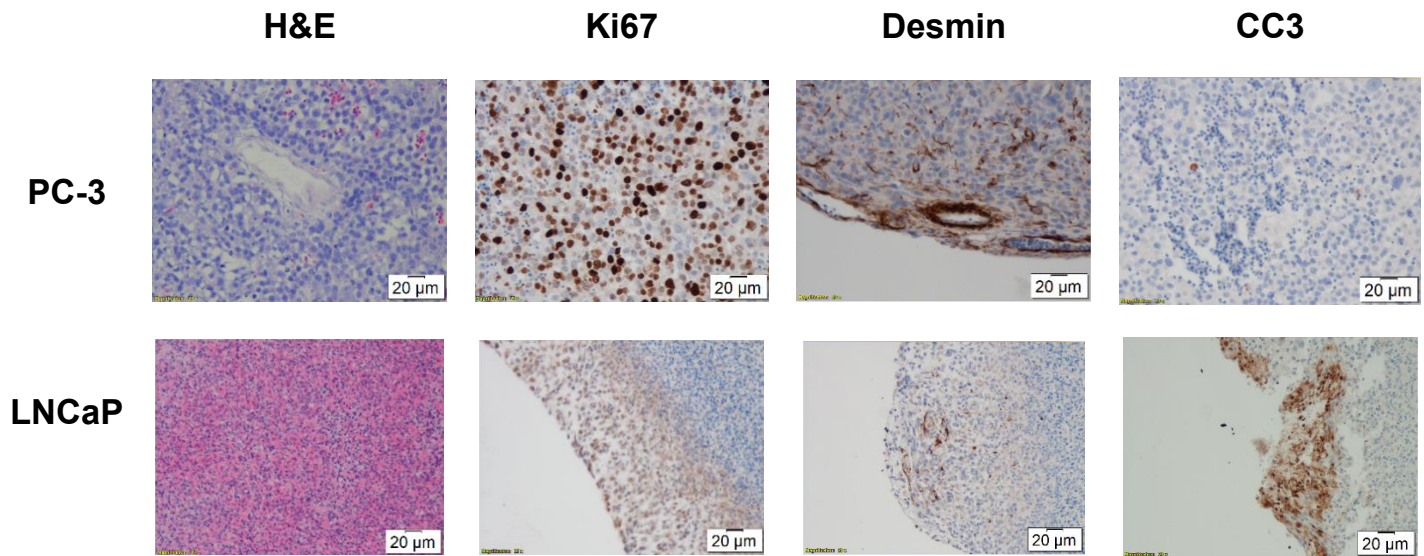


Figure 8: Representative images of the histopathological evaluation of *in ovo* xenografts. PC-3: spheroid with 2,000 cells seeded, LNCaP: spheroid with 4,000 cells seeded. (H&E = Hematoxylin & Eosin, CC3= cleaved caspase 3). Magnification 20x.

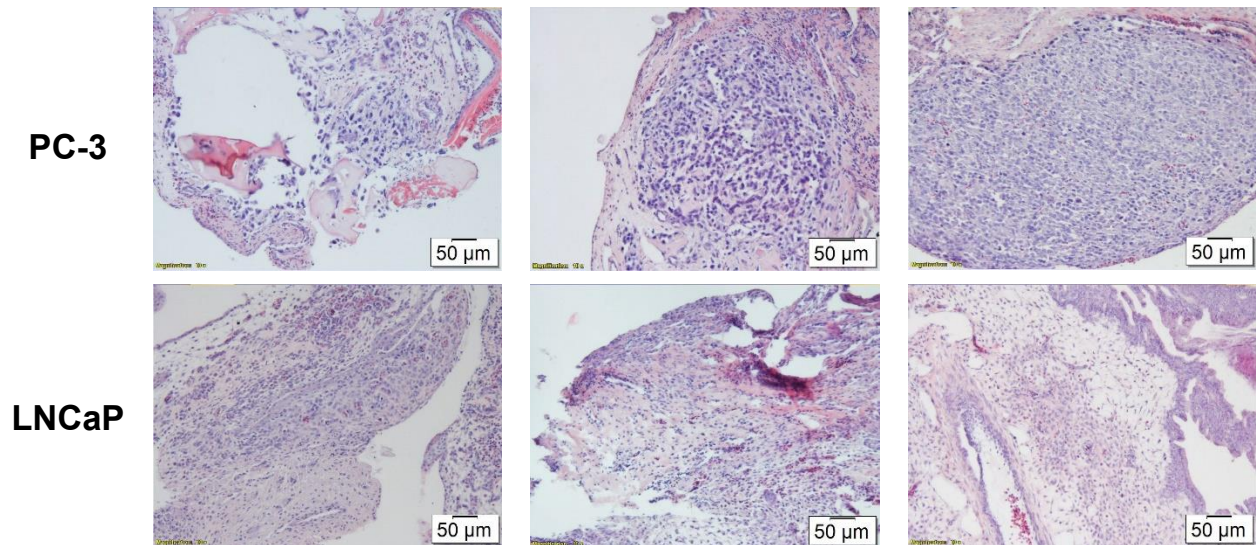


Figure 9: Representative images of the H&E staining from PC-3 and LNCaP with spheroids generated from 50,000 cells. Magnification 10x.

4. SUMMARY AND DISCUSSION

Since molecular radionuclide therapy is gaining increasing interest for the treatment of cancer and specifically in terms of metastasized cancer, efforts are being made to not only developing and synthesizing new ligands but also optimizing existing ones, which are already in (pre)clinical use. In this regard, deeper knowledge in the field of radiobiology, to understand which processes can be exploited by nuclear medicine for the treatment of cancers in the future, is essential.

So far, most of the radiobiological studies have been performed using EBRT. While the LQ model showed to be a reliable tool for the dose-response prediction in EBRT, the dose-response relationship in TRT is largely unknown. It depends on several factors. Not only the absorbed dose, but also the characteristics of the used radionuclides are important. Especially the heterogenous absorbed dose, due to heterogenous target expression, in TRT needs to be considered, also complicating dosimetry calculations. Since the LQ model is not a mechanistic but rather an empirical model to simulate DSB, it is of high importance to understand the underlying radiobiological mechanisms of TRT.

In general, each tumor cell has its intrinsic radiosensitivity, depending on the genetic background (DNA mutations, active repair mechanisms, etc.) and proliferation rate (cell cycle phase at time of irradiation, etc.). Moreover, the tumor microenvironment, as well as phenomena such as hypoxia largely influence the cell's radiosensitivity. Using spheroids as a basic 3D cell culture model, conditions like hypoxia, senescence and cell-cell interactions are modeled, leading to a more realistic *in vivo* tumor model.

In the manuscript “Comparison of Radiation Response between 2D and 3D Cell Culture Models of Different Human Cancer Cell Lines”, 2D and 3D models of the cancer cell lines PC-3, LNCaP and T-47D underwent external beam irradiation and were further assessed in terms of cell viability, replicability, growth inhibition and amount of double strand breaks. The results showed differences in radiosensitivity not only between the different cancer cell lines but also between monolayers and spheroids. Similarly to other cell lines from previously reported results [73–79], our investigations showed that spheroids were less radiosensitive compared to the respective monolayers. The most pronounced effect was observed in the PC-3 cell line, which showed no significant difference in spheroid size

compared to the control on day 21 after (up to) 8 Gy irradiation. In contrast, 8 Gy irradiation of PC-3 monolayers resulted in only 10% surviving fraction.

In order to investigate targeted therapy in comparison, several points needed to be considered. While EBRT leaves the sample non-radioactive after treatment and therefore simplifies the further handling, TRT results in radioactive specimens, contamination risks and body dose for the experimenter. Therefore, methods needed to be adjusted and dosimetry calculations for both models needed to be performed. For an initial assessment, the already clinically applied TRT using [^{177}Lu]Lu-PSMA-I&T was investigated in 2D and 3D models of PC-3 and LNCaP. The results are presented in the manuscript “Radiobiological Assessment of Targeted Radionuclide Therapy with [^{177}Lu]Lu-PSMA-I&T in 2D vs. 3D Cell Culture Models”. In short, target expression in LNCaP spheroids was preserved and target specificity of the radiopharmaceutical was shown in both models. Comparing monolayers with spheroids, spheroids showed to be treated more effectively compared to monolayers. This can be explained, among others, by dosimetry calculations using Monte Carlo methods. According to the simulation, the absorbed dose of the spheroids is approximately 19-times higher than in the monolayers. Moreover, a retention effect, leading to prolonged irradiation and by-stander effects, in the spheroids is assumed.

Moving towards *in vivo* applications of spheroids, we combined the expertise, we have in our group to investigate the usability of spheroids in the *in ovo* model. While successful xenograft growth from cell suspensions of the prostate cancer cell lines PC-3 and LNCaP has been shown before [89], only very limited data exists using spheroids. In our study, only half of the PC-3 xenografts and 71% of the LNCaP xenografts developed, and the resulting spheroids were too small of size to be properly used in further evaluations.

Although spheroid models provide a more realistic *in vivo* tumor model compared to monolayers, they also have limitations. Apart from the lack of vascularization, generation of spheroids is not trivial and sometimes matrices are needed for their generation. Those matrices can be very costly and therefore reduce the accessibility. In terms of analyzability, most assays are end-point assays and some methods, which are commonly used in monolayers, such as fluorescence microscopy, are not as easily usable for spheroids. Nevertheless, research into 3D models has made a lot of progress during the last decade,

and industry is trying to generate the models in high quantity to provide these to researchers, sparing them the time of establishment and characterization. Especially when investigating long-term effects, we found that spheroids are in fact more economic and less time consuming than monolayers. In contrast to monolayers, where many splitting and seeding steps are needed to prevent overgrowth in the flask, spheroid growth can easily be monitored over a long period of time without transfers. Also, there are already commercially available assays available, especially designed for 3D culture, aiding simple, fast and reproducible analyses. Although the use of our spheroids in the *in ovo* model was not successful, advancements in the field of spheroid generation might lead to better results in the future.

Lastly, external beam was also used for the impurity characterization during upscaling of [^{177}Lu]Lu-PSMA-I&T. Since Lu-PSMA radioligand therapy is a recognized treatment option against mCRPR in nuclear medicine departments around the globe, the demand is increasing and ultimately leading to the need of production upscaling. During our in-house upscaling, the formation of a by-product was observed, which correlated with specific activity. To simulate radiolysis, a non-radioactive reference standard was irradiated by external beam, and by means of HPLC-MS, amount and identity of the impurity (deiodinated product) were assessed. By optimization of the HPLC method, cyclization products were observed, contributing to the total amount of radiochemical impurities. Overall, the process of [^{177}Lu]Lu-PSMA-I&T supply and quality control was improved.

5. CONCLUSIONS AND FUTURE CONSIDERATIONS

In the course of this thesis, more information about radiosensitivity of different human cancer cells to external beam and targeted radionuclide therapy has been gained. Further, the advantages and limitations of spheroid models in nuclear medicine have been elucidated, the use of spheroids in the *in ovo* model has been investigated and external beam was utilized to simulate radiolysis in the production of a radiopharmaceutical in TRT.

The established methodology is currently used for the evaluation of a novel TRT, with many more to come, and therefore aids not only the development of new therapeutic radiopharmaceuticals but also serves to give deeper insights into radiobiology.

Regarding spheroids, I believe that commercialization of spheroid models, generated with various techniques to customized shapes, as well as the current progress in the field, will make 3D models a standard in radiation research in the future.

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List of abbreviations

2D	two-dimensional
3D	three-dimensional
53BP1	p53-binding protein 1
ATP	adenosine triphosphate
BER	base excision repair
CAM	chorioallantoic membrane
CC3	cleaved caspase 3
DDR	DNA damage response
DNA	desoxyribonucleic acid
DOTATATE	tetraxetan-(Tyr3)-octreotate
DSB	double strand break
EBRT	external beam radiation therapy
EC	electron conversion
EMA	European Medicines Agency
FDA	Food and Drug Administration
H & E	hematoxylin & eosin
HET	hen's egg test
HR	homologous recombination
IC	internal conversion
IR	ionizing radiation
IRT	internal radiation treatment
LET	linear energy transfer

LNCaP	lymph node metastasis of human metastatic prostate cancer
LQ	linear quadratic
MC	Monte Carlo
mCRPC	metastatic castrate-resistant prostate cancer
MMR	mismatch repair
MN	micronucleus
MR	magnetic resonance
NER	nucleotide excision repair
NET	neuroendocrine tumor
NHEJ	non-homologous endjoining
PC-3	bone metastasis of human metastatic prostate cancer
PET	positron emission tomography
PRRT	peptide receptor radiation therapy
PSMA	prostate specific membrane antigen
PSMA-I&T	prostate specific membrane antigen- imaging and therapy
RBE	radiobiologic effectiveness
RNS	reactive nitrogen species
ROS	reactive oxygen species
SF	surviving fraction
SSB	single strand breaks
SSTR	somatostatin receptor
T-47D	pleural effusion of breast cancer
TRT	targeted radionuclide therapy

List of figures

Figure 1: Latest version of the hallmarks of cancer. Graphic reproduced with permission from Cancer Discovery [3].	4
Figure 2: Types of Cancer Treatment. Created with BioRender.com (adapted template).5	
Figure 3: Structure of a multicellular LNCaP tumor spheroid, stained with propidium iodide (red; necrotic cells) and calcein-AM (green; viable cells). From the outermost layer (proliferating zone, green) to the center (necrotic core, red): decreasing gradient of nutrients, oxygen (O ₂), and adenosine triphosphate (ATP); increasing gradient of metabolic waste and carbon dioxide (CO ₂).....	18
Figure 4: Exemplary experimental procedure of the CAM-xenograft model (adapted from Katarína Benčurová); EDD=embryo development day	20
Figure 5: Growth observation of PC-3 spheroids, resulting from different seeding numbers (2,000, 4,000 or 50,000 cells on day 0). (A) Diameter measurements. Average over 3 diameter measurements per spheroid and time point, plotted as mean ± standard deviation from three independent experiments (n=3) in triplicates. (B) Photographs of representative spheroids on Day 3, 5 and 8 after seeding (D0). White scale indicates 500 µm.	82
Figure 6: Growth observation of LNCaP spheroids, resulting from different seeding numbers (2,000, 4,000 or 50,000 cells on day 0). (A) Diameter measurements. Average over 3 diameter measurements per spheroid and time point, plotted as mean ± standard deviation from three independent experiments (n=3) in triplicates. (B) Photographs of representative spheroids on Day 3, 5 and 8 after seeding (D0). White scale indicates 500 µm.	83
Figure 7: Live/dead staining of spheroids. (A) Representative images of propidium iodide (red) and calcein-AM (green) staining of spheroids on Day 8 after seeding; white scale indicates 500 µm (B) Ratio necrotic core to total spheroid size for PC-3 and LNCaP; plotted as mean ± standard deviation from two to eight independent experiments (n=2-8)	84
Figure 8: Representative images of the histopathological evaluation of in ovo xenografts. PC-3: spheroid with 2,000 cells seeded, LNCaP: spheroid with 4,000 cells seeded. (H&E = Hematoxylin & Eosin, CC3= cleaved caspase 3). Magnification 20x.	87

Figure 9: Representative images of the H&E staining from PC-3 and LNCaP with spheroids generated from 50,000 cells. Magnification 10x.	87
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List of tables

Table 1: Characteristics of EBRT vs TRT.....	6
Table 2: Characteristics and examples of therapeutic radionuclides. Adapted from [8,9].	8
Table 3: Diameter measurements (in μm) of total spheroid, necrotic core and calculated ratio for (A) PC-3 and (B) LNCaP	85
Table 4: In ovo xenografts – facts and figures	86

List of equations

Equation 1: LQ model.....	16
Equation 2: Extension of the LQ model	16

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