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Abstract

Besides cardiovascular diseases, cancerous diseases are one of the leading causes of morbidity and mortality worldwide. Consequently, efforts in developing new methods for tumor imaging and effective treatment have been increased. A decisive limiting factor in cancer treatment, however, is the decreased responsiveness to anti-cancer drugs and radiation, which can be reduced to typical characteristics of tumor tissue. In contrast to 2D cell culture methods, the cultivation of multicellular tumor spheroids allows a physiological simulation of tumor tissue. Hence, the use of multicellular tumor spheroids rapidly gained popularity in the evaluation of chemotherapeutical strategies and the development of anti-cancer drugs. In addition, an increased application of spheroids in the preclinical evaluation of PET-tracers, used for tumor imaging, would enable a targeted selection of malfunctioning substances, which consequently offers savings potential for animal experiments. In order to guarantee a targeted application of multicellular tumor spheroids, prior examinations on their biochemical effects with the use of well-established PET-tracers are necessary.

Therefore, the aim of this work was, on one hand, the cultivation of multicellular tumor spheroids and consequently the characterization of the obtained cell aggregates, regarding growth behavior and the appearance of hypoxic as well as necrotic regions. On the other hand, the preclinical applicability of multicellular tumor spheroids in PET-tracer evaluation was established. Therefore, the accumulation of [^{18}F]FDG, [^{18}F]FMISO, [^{18}F]FE@SUPPY as well as [^{18}F]NaF in HT-29 and HCT-116 spheroids, in comparison to 2D cell monolayers, was determined. For determination of the temperature dependence of PET-tracer accumulation, the incubation was performed at 37°C and 4°C, respectively. To gain further information on the intracellular uptake of [^{18}F]FDG, [^{18}F]FE@SUPPY and [^{125}I]I-AB-MECA, internalization assays were performed using HT-29, CHO-hA3R, CHO-K1 and PC-3 cell monolayers. Moreover, real-time accumulation kinetics of [^{18}F]FDG in HT-29, HCT-116 and PC-3 cells were measured using the LigandTracer® device.

Referring to the obtained results, it can be concluded, that spheroid formation as well as their growth behavior depends on the selection of the cell line, cell concentration and production method. Further, with the characterization of the obtained spheroids the postulated relation between spheroid size and the appearance of necrotic regions was confirmed, whereas neither with the performance of immunohistochemically staining of Hif2 α , nor western blot analysis, the appearance of hypoxic regions was shown. Moreover,

in accumulation experiments temperature down-regulation resulted in a decrease in radiotracer accumulation, if the used radiotracer was linked to mechanisms of active transport. In addition, [^{18}F]FE@SUPPY, a tracer for A₃ receptor imaging, showed high non-specific binding. However, the percentage of non-specific bound radiotracer could be reduced by additional washing steps. Referring to the results, an internalization of the A₃ receptor after [^{125}I]I-AB-MECA binding was determined, which could be blocked by adding FE@SUPPY as well as I-AB-MECA.

In conclusion, the obtained data show an outstanding applicability of multicellular tumor spheroids for the development of radiotracers for tumor imaging.

Zusammenfassung

Tumorerkrankungen stellen nach Herz-Kreislauferkrankungen die häufigste Todesursache dar. Als Folge dessen gibt es ein sehr großes Bestreben neue Ansätze zu finden, um Tumorgewebe schnellst möglich zu erkennen und gezielt zu behandeln. Limitierende Faktoren für eine erfolgreiche Krebstherapie stellen jedoch die verminderte Ansprechbarkeit auf Chemotherapeutika und Bestrahlung dar, welche auf Charakteristika des Tumorgewebes zurückzuführen sind. Mit der Kultivierung von multizellulären Tumor Sphäroiden gelingt, im Gegensatz zu 2D Zellkulturmodellen, eine physiologische Simulation von Tumorgewebe, was deren Einsatz in der Evaluierung von chemotherapeutischen Strategien und Wirkstoffen zunehmend beliebter macht. Zusätzlich würde der vermehrte Einsatz von Sphäroiden in der präklinischen Evaluierung von PET-Tracern zur Tumorerkennung eine gezieltere Selektion von wirksamen Substanzen ermöglichen und somit zu einer Reduktion von Tierversuchen beitragen. Um eine gezielte Anwendung von Sphäroiden etablieren zu können, muss zuvor die biochemische Wirksamkeit anhand bereits etablierter PET-Tracer geprüft werden.

Das Ziel dieser Arbeit war daher einerseits die Kultivierung von Multizellulären Tumor Sphäroiden und deren anschließende Charakterisierung in Hinblick auf Wachstumsverhalten und Gradienten bedingter Ausbildung von hypoxischen sowie nekrotischen Regionen. Andererseits sollte die präklinische Anwendung multizellulärer Tumor Sphäroide in der PET-Tracer Entwicklung evaluiert werden. Hierfür wurde die Akkumulation von [^{18}F]FDG, [^{18}F]FMISO, [^{18}F]FE@SUPPY sowie [^{18}F]NaF in HT-29 und HCT-116 Sphäroiden im Vergleich zum 2D Zellkulturmodell bestimmt. Die Inkubation mit dem PET-Tracer erfolgte bei 37°C beziehungsweise 4°C, um eine eventuelle Temperaturabhängigkeit der Akkumulation feststellen zu können. Um Rückschlüsse auf die intrazelluläre Aufnahme von [^{18}F]FDG, [^{18}F]FE@SUPPY und [^{125}I]I-AB-MECA ziehen zu können, wurden Internalisierungsexperimente mit HT-29, CHO-hA3R, CHO-K1 und PC-3 Zellen durchgeführt. Darüber hinaus wurde unter Verwendung des LigandTracers® die [^{18}F]FDG Akkumulationskinetik in HT-29, HCT-116 und PC-3 Zellen bestimmt.

Die erhaltenen Resultate bezüglich Sphäroid Bildung und Wachstumsverhalten zeigten eine deutliche Abhängigkeit von der verwendeten Zelllinie, Zellkonzentration und Herstellungsmethode. Die Charakterisierung der erhaltenen Sphäroide bestätigte zudem den postulierten Zusammenhang zwischen Sphäroid-Größe und der Ausbildung nekrotischer Regionen, wohingegen die Präsenz hypoxischer Regionen weder mittels HIF2 α immunhistochemischen Färbungen noch Western Blot Analyse bestätigt werden

konnte. In Akkumulationsexperimenten konnte darüber hinaus durch Herunterregulation der Temperatur eine verminderte Akkumulation von Radiotracern, die an aktive Transportprozesse gebunden sind, festgestellt werden. Der auf den A₃ Rezeptor abzielende Tracer [¹⁸F]FE@SUPPY zeigte darüber hinaus eine hohe unspezifische Bindung. Jedoch konnte der Anteil des unspezifisch gebundenen Radiotracers durch zusätzliche Waschschrte reduziert werden. Des Weiteren, konnte anhand der Resultate eine Internalisierung des A₃ Rezeptors nach [¹²⁵I]I-AB-MECA Bindung festgestellt werden, welche sowohl durch Zugabe von FE@SUPPY als auch von I-AB-MECA blockierbar war. Zusammenfassend zeigen die Ergebnisse eine hervorragende Anwendbarkeit von multizellulären Tumor Sphäroiden in der Tumor-Tracer Entwicklung.

TABLE OF CONTENTS

1. Introduction.....	2
1.1. Multicellular tumor spheroids.....	2
1.2. Hypoxia.....	10
1.3. Adenosine A ₃ receptor.....	15
1.4. Tumor imaging via PET.....	18
2. Aim.....	20
3. Materials and Methods.....	21
3.1. Cell lines and culture conditions.....	21
3.2. Multicellular tumor spheroid formation (3D cell culture).....	22
3.3. Embedding of multicellular tumor spheroids.....	24
3.4. Characterization of multicellular tumor spheroids.....	25
3.5. Determination of intracellular calcium concentration.....	27
3.6. PET-tracer synthesis.....	28
3.7. Accumulation of PET-tracers ([¹⁸ F]FDG, [¹⁸ F]FMISO, [¹⁸ F]NaF, [¹⁸ F]FE@SUPPLY) in multicellular tumor spheroids.....	29
3.8. Internalization assays.....	31
3.9. Real-time [¹⁸ F]FDG accumulation experiments.....	32
3.10. Western Blot.....	34
3.11. Immunohistochemistry staining of HIF2α.....	36
4. Results.....	38
4.1. Spheroid formation.....	38
4.2. Spheroid growth behavior.....	41
4.3. Visualization of necrotic regions within spheroids.....	44
4.4. PET-tracer accumulation experiments.....	46
4.5. Internalization assays.....	49
4.6. Real-time [¹⁸ F]FDG accumulation experiments.....	56
4.7. Western blot.....	59
4.8. Detection of Hif2α via immunohistochemical staining.....	63
5. Discussion.....	66
6. Conclusion.....	75
7. Abbreviations.....	77
8. References.....	78

1.Introduction

1.1. Multicellular tumor spheroids

The term multicellular tumor spheroid describes self-assembled dense cell aggregates, which are characterized by close cell-cell interactions and serve as a 3D cell culture model.



Figure 1: **Schematic representation of the aggregation of endodermic cells. Illustration adapted from Holtfreter.** ⁽¹⁾

Historical background

In 1944 Holtfreter observed the aggregate building of embryonic cells (Figure 1) for the first time, which opened the way for a new dimension of cell culturing.⁽¹⁾ Years later Sutherland *et al.* recognized the potential benefit of multicellular tumor spheroids in the development of cancer treatments and established this *in vitro* cell culture model for tumor cell lines. Their studies provided fundamental knowledge about the characteristics of multicellular spheroids, which is important to understand their high complexity. They described the cellular heterogeneity within different regions of the spheroid, the growth dependence on the microenvironment as well as the appearance of oxygen and nutrient gradients, to name only a few. Furthermore, the higher resistance of multicellular tumor spheroids, compared to 2D cell monolayers, to anti-cancer drugs and ionized radiation was revealed.⁽²⁻⁸⁾

Characteristics of multicellular tumor spheroids

2D tumor cell monolayers are mainly used for *in vitro* experiments, despite the insufficient simulation of real tumor tissue, regarding to physiological and anatomical aspects. Due to the fact, that cells, cultured in a flask, are forced into a monolayer morphology, they are not fully able to reflect the functional behavior of cells, found *in vivo*. 2D monolayers lack

cell-cell interactions, cell-matrix interactions and the appearance of molecular gradients, which determine important properties in physiological tumor tissue.⁽⁹⁾

By contrast, the missing features of the 2D cell monolayers can be found in multicellular tumor spheroids, which enables a closer simulation of real tissue.

Cytoskeletal network and surface adhesion molecules, such as cadherins and connexins assist cells within spheroids in self-assembling and formation of cell-to-cell interactions. Connexins for example form gap junctions, which connect cells to each other and allow a communication of cells with their surrounding neighbours.⁽¹⁰⁾ This cell-to-cell communication is not fully given in 2D cell monolayers, since cell-to-plastic-interactions provide the majority of cell-interactions.

As multicellular tumor spheroids increase in size, the three-dimensional cell arrangement creates a penetration barrier for soluble nutrients, oxygen, metabolites, as well as drugs and encourages the appearance of molecular gradients (Figure 2), similar to real tumor tissue.^(2,4-6,11)

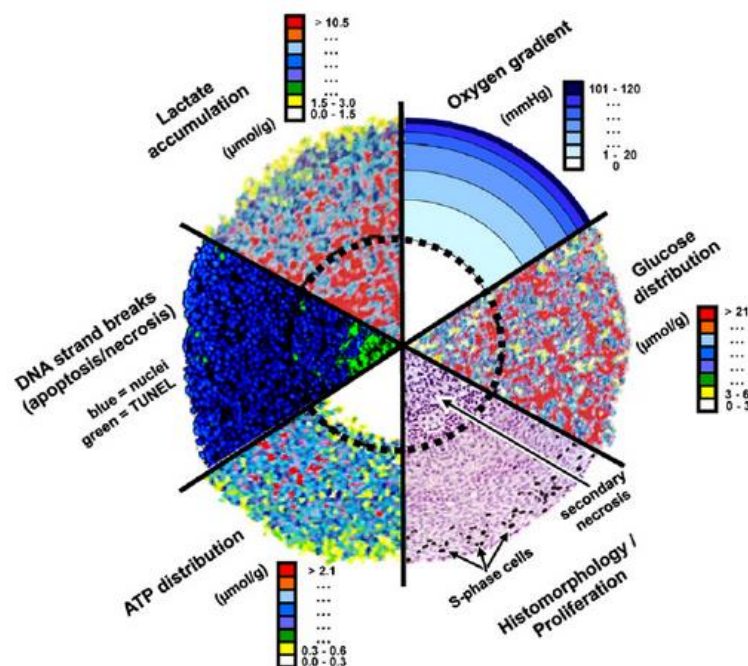


Figure 2: Visualization of different gradients, observed in multicellular tumor spheroids. Image adapted from (11).

The presence of the gradients, outlined above, results in a cellular heterogeneity within cells of the spheroid, which cannot be observed in 2D cell culture models, since bidimensional growth implies optimal exposure to nutrients of the growth medium and oxygen.

The heterogeneity of cells reflects the fact that different regions can be observed within multicellular tumor spheroids. Therefore, tumor spheroids are characterized by a viable rim, consisting of a layer of proliferating cells, called the surface layer, an intermediate layer and a layer of non-proliferating hypoxic cells, called the perinecrotic region, which surrounds a necrotic core (Figure 3). Development of cell damage and necrosis was observed in spheroids greater than 500 μm , as a result of low oxygen and nutrient exposure within the cells of the inner core. ^(12,13,14)

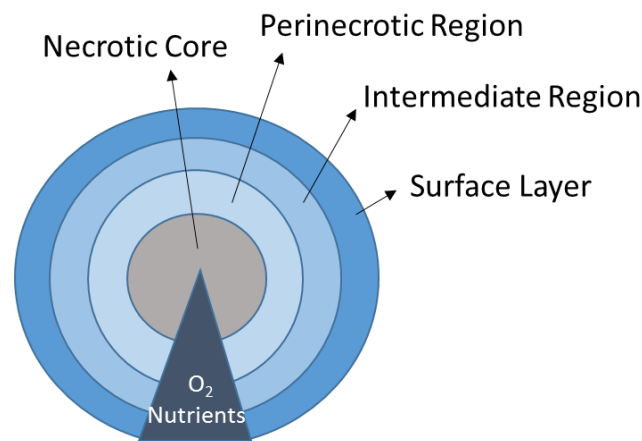


Figure 3: Illustration of different regions appearing in multicellular tumor spheroids.

In recent studies, McMahon *et al.* additionally revealed the changes of protein expression within different regions of multicellular tumor spheroids. More precisely, it was shown that cell death associated proteins, proteins, which participate in homeostasis mechanisms and enzymes, involved in carbohydrate and lipid metabolic pathways, like enoyl-CoA-hydratase, were up-regulated in necrotic regions. This founding confirms the theory that cells in the inner core are already dead or are about to die, as well as the existence of molecular gradients, since cells of the inner core try to prevent cell damage by increasing expression of proteins, necessary for energy production.

Further, an up-regulation of enzymes involved in steroid biosynthesis, glycolysis and tricarboxylic acid (TCA) cycle, like malate dehydrogenase, citrate synthase and pyruvate kinase was revealed within perinecrotic regions as well as in the inner core.

Through staining of the proliferation marker Ki67 the same group showed that cell proliferation decreases from regions of the viable rim to the necrotic core, resulting in a missing proliferation of cells within the perinecrotic region.⁽¹³⁾

A similar observation was made in real tumor tissue, where proliferating cells are located nearby blood vessels, whereas quiescent and necrotic cells are found in areas distant from vascularization.^(80,81)

However, not only the creation of an *in vivo*-like microenvironment, but also the growth kinetics, observed in multicellular tumor spheroids, mimic real tumors. After a phase of exponential growth, restrictions in nutrient and oxygen supply result in a retardation of growth. In addition to limitations in glucose and O₂ distribution, the increase of non-proliferating cell regions within the spheroid has an impact on the growth behavior, since perinecrotic as well as necrotic cells emit growth inhibiting factors, which debilitate proliferating cells of the intermediate and surface layer.^(12,15)

A further advantageous feature of multicellular tumor spheroids is the connection of cells with an extracellular matrix, which is also observed in tumors as well as in physiological tissue as a result of three-dimensional cell arrangement.

The extracellular matrix, also known as basement membrane, serves as a depot for growth factors and gives the cells polarity, which is important for the maintenance of normal tissue architecture and protective against drug-induced apoptosis. A key mechanism of the antiapoptotic effect is the binding of the cell-adhesion receptor $\alpha 6 \beta 4$ -integrin to the basement membrane protein laminin, which reminds cells, surrounded by the membrane, to stay alive. The importance of this connection can be pointed out in the spheroid model, since apoptotic mechanisms are induced in cells, located within the inner core and therefore lack attachment to the extracellular matrix.

The protective effect, resulting from the cell-membrane interaction, is relevant for the survival of normal cells during chemotherapy, however, troublesome for efficient killing of tumor cells.

As soon as tumor cells start spreading into other tissues, the basement membrane of healthy tissues suffers damage and releases the stored growth factors. Consequently, the antiapoptotic properties get lost in non-cancerous cells, whereas three-dimensional organization of tumor cells is encouraged by the released growth factors, which ultimately results in resistance to chemotherapeutical agents.

The opposite effect occurs, when tumor cells are cultivated in a bidimensional monolayer. The non-physiological cell organization in 2D cell culture model lacks cell contacts to an

extracellular matrix, which increases the response to anti-cancer drugs during *in vitro* drug evaluation. This leads to *in vivo* testing of chemotherapeutical agents, which afterwards show low efficiency, whereas other agents are dismissed too soon from further studies. These facts highlight the need of 3D cell culture models for efficient *in vitro* drug testing.^(16,17,18)

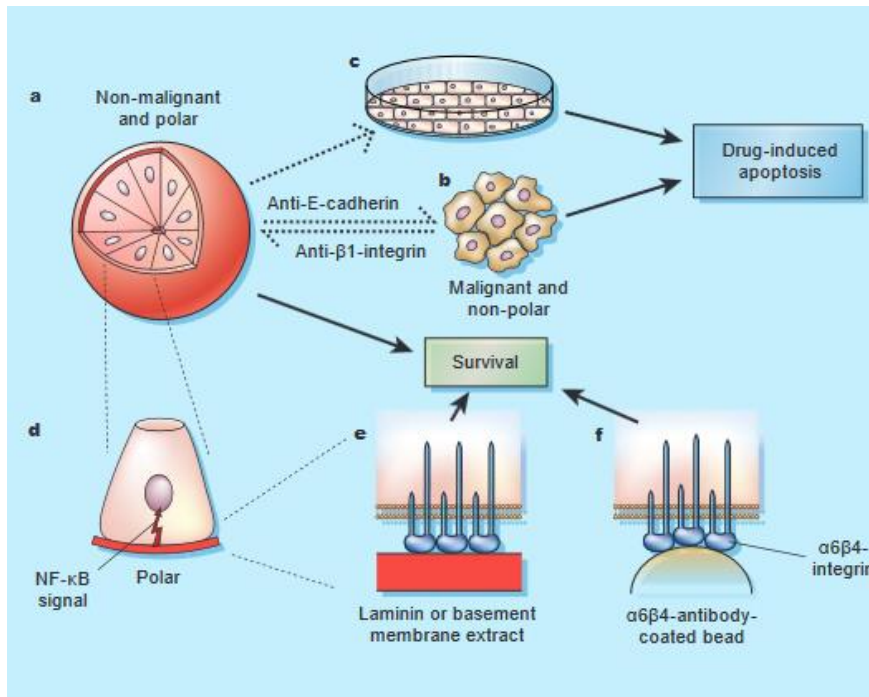


Figure 4: The differences between bidimensional and three-dimensional cell arrangement, regarding to the necessity of a basement membrane for resistance to anti-cancer drugs. Image adapted from (17).

Cultivation of multicellular tumor spheroids

The techniques, used for spheroid formation preferentially are based on cell aggregate building obtained through influencing effects of gravitational or rotational forces (Figure 5). In general, it can be distinguished between scaffold based methods and spheroid building without the addition of scaffold building agents.

To start with the scaffold-free methods, there are several 3D cell culture techniques available^(10,19-21), which greatly vary in spheroid reproducibility, time requirement, and in their costs.

At the very beginning of spheroid cultivation, the use of the spinner culture method was popular for aggregate formation. For this method, cell suspensions are constantly stirred

in bulbs, by the consideration that cell collisions lead to aggregate formation and the continuous movement prevents cells from settling. However, the constant presence of shear forces might cause damage to sensitive cells, which aggravate spheroid formation.

A gentler, but similar method is the application of rotating wall vessels. The slow rotation creates a microgravity environment, where cells are able to form aggregates in continuous free fall. In order to counteract the effects of gravity, which would lead to a sedimentation of the built spheroids within the vessel, rotation speed is increased as soon as aggregate formation is observed.

Despite the simplicity of both methods, the obtained spheroids are not uniform in size and shape, which might have adverse effects on experimental reproducibility. However, based on similar principals, the pellet culture method is widely performed to obtain homogenous and particularly large spheroids. Therefore, a cell suspension of an evaluated concentration is transferred to a conical centrifugation tube and centrifuged at approximately 500 g, which enables dense aggregate building.

Although all methods, using rotation forces for spheroid formation, are barely time-consuming, the disadvantage is the missing visualization of the aggregate formation process. Whereas, with the usage of methods, which are based on gravity-enforced cell self-assembling, the stages of spheroid formation can be observed.

The pioneering work in this field was done by Yuhas *et al.*, who established the liquid overlay method. Therefore, cell suspensions are seeded into Petri dishes, pre-coated with agarose to create a low-attachment surface, followed by slightly shaking, which encourages the aggregate formation. In a single processing step, a large number of spheroids can be obtained, however, size and shape uniformity is not given. The situation is different, when cell suspensions are seeded into 96-well plates, coated with gel building agents like agarose or gelatine, which mostly allows the formation of homogenous aggregates in each single well.

Similar results can be obtained with the use of the hanging-drop-method, where drops of cell suspensions are placed on the bottom of the inverted lid of a Petri dish. As soon as the lid is placed on the dish, cells start to settle to the bottom of the drop and assemble to aggregates. The built aggregates must be transferred to 96-well plates to enable further spheroid growth, which makes it the most time-consuming method.

Nowadays, spheroid formation can be simplified by using more expensive technologies, like Ultra-Low-Attachment (ULA) 96-well plates, which do not require additional coating to prevent cell settling, due to a covalently bound hydro layer.

Furthermore, spheroid formation could be obtained through the application of micro fluidics, where cells are partitioned in micro-chambers and exposed to micro-rotational, which allows the building of uniform and size controlled aggregates.

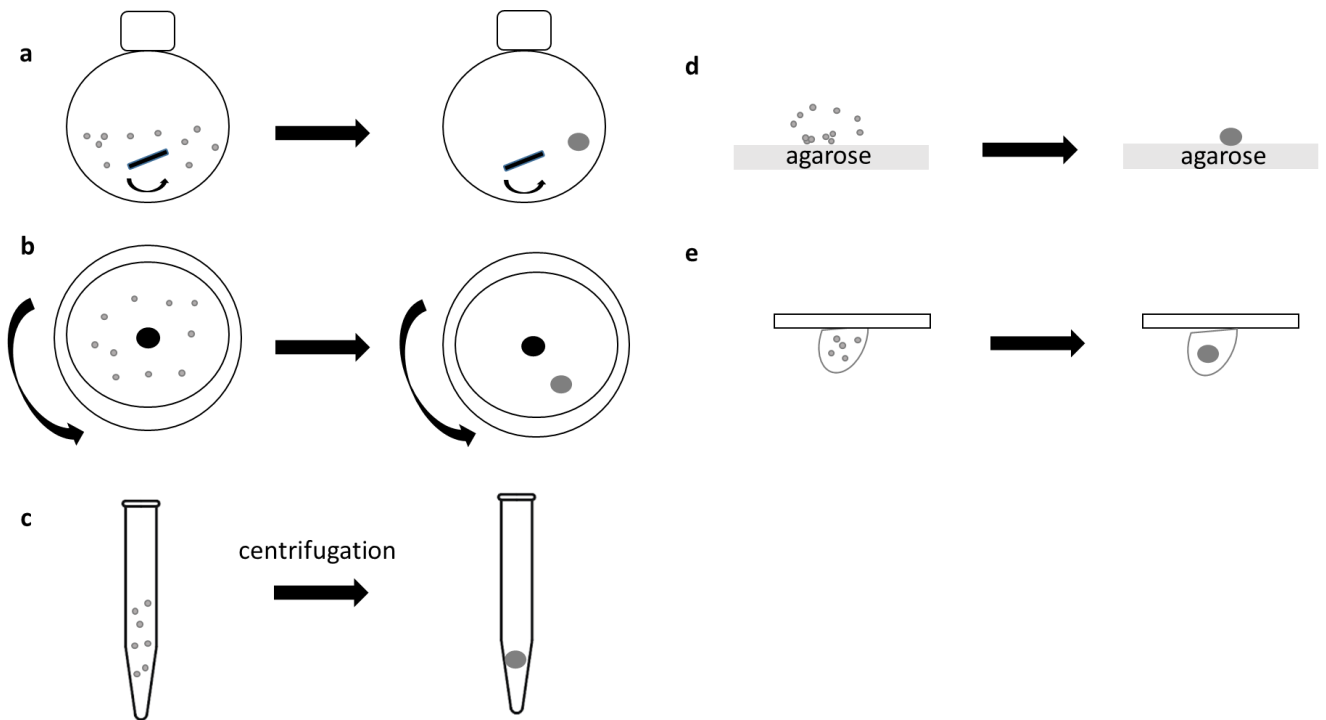


Figure 5: Schematic illustration of 3D cell culture techniques. (a) spinner culture method (b) rotating well vessel (c) pellet culture method (d) liquid overlay (e) hanging drop method.

While performing the 3D cell culture techniques as described above, cell settling to the surface or gel building agents is tried to be avoided, whereas with the use of scaffold based methods, the close contact between spheroids and hydrogels is explicit desired. With the use of scaffolds, like collagen type I, BME/Matrigel, or gelatine, an *in vivo*-like environment, such as roughness and wettability, is tried to be simulated. The used hydrogels are characterized by a high permeability for nutrients and oxygen, which makes them attractive for spheroid or cell embedding. Furthermore, for example the composition of BME/Matrigel is comparable to an embryonic basement membrane inclusive the stored growth factors, which facilitate three-dimensional cell organisation. Since tumor cells are able to cross the

extracellular matrix, they start spreading within the matrix, which mimics processes of metastasis. Therefore, scaffold based culture techniques are mainly used for angiogenesis and toxicity assays in preclinical anti-cancer drug development.^(9,22)

Imaging of multicellular tumor spheroids

Due to the three-dimensional cell arrangement imaging of spheroids is more challenging compared to 2D cell monolayers. However, conventional bright field microscopy can be used for observation of spheroid formation and growth behavior. Whereas scanning electron microscopy is required for surface imaging of the aggregates. When high resolution images are obtained, even further information about biological structures can be gained.

For certain issues, like the visualization of necrotic regions within the multicellular tumor spheroid, fluorescence based assays can be performed, which allows imaging of the whole aggregate.

To gain further information on their interior, cryo-sectioning and subsequent staining with antibodies or conventional dyes is currently inevitable.⁽¹⁰⁾

Application of multicellular tumor spheroids

In comparison to 2D cell monolayers, multicellular tumor spheroids closely resemble *in vivo* tumors, with regard to their growth behaviour and cellular heterogeneity, as a result of restrictions in supply of water-soluble nutrients, hypoxia and cell interactions.

Due to anti-apoptosis effects, which might be caused by hypoxic and necrotic regions as well as cell-matrix interactions, cells within real tumor tissue are often resistant to radiation and chemotherapeutical agents.⁽⁸⁾ Moreover, dense tissue architecture and the building of tight cell interactions create a penetration barrier, which results in an alteration of drug metabolism, additionally reinforcing drug resistance. Such properties can not be found in 2D tumor cell monolayers. Multicellular tumor spheroids, however, possess these important properties, suggesting their application in cancer research. Especially the use of spheroids in discovery of anti-cancer drugs and chemotherapeutical strategies became popular in the last few years. Convenient end points for determination of their response to radiation or chemotherapeutical agents are for example spheroid growth delay or cell survival. Therefore with the use of multicellular tumor spheroids, malfunctioning

substances can be early preselected in high-throughput drug screening studies, which offers a possibility to reduce animal testing.^(10,11,23,24)

Apart from drug discovery, the spheroid model can be also used in studies of drug transport and uptake mechanisms, which can be of high importance in PET-tracer evaluation. Monazzam *et al.* firstly introduced a method of [¹⁸F]FDG uptake studies in BT474 multicellular tumor spheroids, showing that PET-screenings could be used for selection and monitoring of breast cancer treatment.^(25,26) Their obtained results were promising, however, further data of PET-tracer accumulation in spheroids is still required.

Although multicellular tumor spheroids mimic physiological tissue better, compared to 2D tumor cell monolayers, they should not be completely equated with real tumors, since they are not surrounded by other human derived cells such as endothelial cells or fibroblasts. With embedding spheroids in hydrogels, like Matrigel or collagen, attempts are made to resemble physiological states. To obtain a better mimicry, spheroid co-cultures with endothelial cells or fibroblasts, become the focus of attention for evaluation of anti-angiogenic potentials of drugs.^(10,11,22,27)

Even co-culturing with lymphocytes, macrophages, T cells or natural killer cells is feasible in order to study the cellular toxicity and therapeutic effects of anti-tumor agents in the presence of physiological immune cells. Therewith, new immunotherapeutic strategies could be developed.^(11,28)

However, multicellular tumor spheroids as well as spheroid co-culture models are not only restricted to cancer research, but also have high potential for tissue engineering.⁽¹⁰⁾ Jun *et al.* for example revealed that hybrid spheroids, obtained from primary pancreatic islets and hepatocytes, are able to take over properties typical for the respective organs, like albumin secretion, glucose responsiveness and CYP450 activity. This observation highlights the possibility of cell-based therapies for liver disease and diabetes.⁽²⁹⁾

1.2. Hypoxia

As three-dimensional arranged tissues reach a particular size, a sufficient supply of nutrients and oxygen proves to be difficult. The chronically appearance of such restriction gradients lead to heterogeneity of cells, resulting in different regions within tumor tissue,

which can be also found in multicellular tumor spheroids. While small spheroids ($<200\ \mu\text{m}$) consist predominantly of normoxic and proliferating cells, with an increase in size hypoxic regions can be found. In addition to hypoxic regions, which are mainly located in the perinecrotic spheroid layers, necrotic areas occur within the inner core starting at a spheroid size of approximately $500\ \mu\text{m}$. As part of the appearance of hypoxic as well as necrotic cells, changes in gene expression and signal transduction can be observed, which are involved in tumor progression and drug or radiation resistance. Therefore, it can be concluded that hypoxia indirectly plays a key role in tumor growth and survival, which makes hypoxic cells attractive as a target for cancer imaging and chemotherapeutical strategies.^(4,5,11,31)

Alteration in gene expression within hypoxic regions

As mentioned above, the appearance of hypoxia is accompanied by alterations in cellular gene expression. While in healthy cells limitations in oxygen supply result in necrosis or programmed cell death, tumor cells respond to changes in O_2 pressure by the transcription of proteins, which help cells adapting to the changed conditions and protect them from apoptosis. The shift towards an up-regulated expression of hypoxia related genes is predominately regulated by a class of transcription factors, the so-called hypoxia-inducible factors (HIFs).

Hypoxia-inducible factors represent a family of heterodimers, consisting of a α - (HIF1 α , HIF2 α , HIF3 α) and a β -subunit (HIF1 β). HIF1 α and HIF2 α have similar protein structures and share 48% amino acid identity.^(31,32)

While the HIF β -subunit is constantly expressed within the nucleus, the activation of the α subunits is induced by restrictions in oxygen supply. Under normoxic conditions, their prolyl residues are hydroxylated, which facilitates the binding of the von Hippel-Lindau protein (pVHL). The pVHL serves as a marker for an E3 ubiquitin ligase complex, which ubiquitinate the bound substrate, resulting in a proteasomal destruction of the HIF α subunits (Figure 6).^(33,47) In the absence of oxygen, the activity of the prolyl hydroxylase-enzymes is inhibited, consequently, the pVHL binding does not take place, which results in a stabilization of the HIF α subunits. The stabilized HIF α subunit is able to enter the nucleus, where it dimerizes with HIF1 β and binds to a conserved DNA-sequence, the hypoxia-response element (HRE). This binding is important for gene activation and further

transcription of hypoxia related genes, involved in hypoxia response like the regulation of glucose uptake, glycolysis, and angiogenesis.^(34,36)

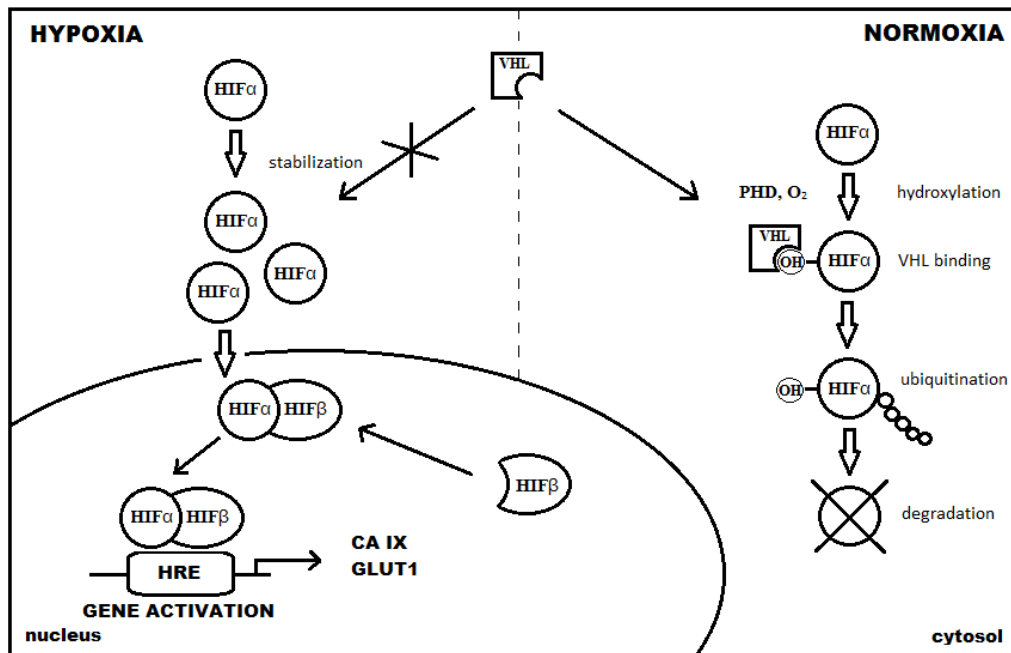


Figure 6: **Regulation of the HIFα pathway during normoxia and hypoxia.** Image adapted from (35).

Although activation mechanisms of HIF1α and HIF2α are similar, their expression and roles in hypoxic gene regulation varies within both subunits.

For HIF1α it was shown that it can be detected in nearly all cell types, even under normoxic conditions, however, undergoing hypoxic states, the expression is enforced.⁽³⁷⁾ In general, HIF1α activation is the key mechanism of response to acute hypoxia, since the expression of glycolytic genes and glucose transporters, necessary for ATP production due to rapid metabolic rates in tumors, is predominately regulated therewith. Further, it is important for cell proliferation, vessel sprouting, tumorigenesis and erythropoiesis, by inducing the transcription of VEGF and EPO. In relation to this, studies revealed, that stimulation of the adenosine A₃ receptor is correlated with HIF1α expression and therewith facilitates new capillary formation.⁽⁹⁵⁾ Moreover, increasing levels of the HIF1α subunit go hand in hand with overexpression of the multidrug resistance transporter P-glycoprotein (Pgp), which partly explains the ineffectiveness of some chemotherapeutical agents.^(38,39,40) Additionally, striking is the relation between HIF1α and the expression of the carbonic anhydrase IX (CA IX), which is important for pH regulation and used by tumor cells to adapt to environmental changes, resulting in tumor cell invasion. Since CA IX is rarely found in normal tissue, it can serve as a hypoxia marker and potential target for chemotherapy.⁽³⁵⁾

Studies have additionally shown that HIF1 α stabilization needs more severe hypoxia, compared to HIF2 α and that a down-regulation of HIF1 α expression can be observed after long-term exposure to hypoxia, which might be due to the fact that some prolyl hydroxylase-enzymes are also inducible by restrictions in oxygen, resulting in negative feedback regulation of HIF stabilization.^(41,42)

By contrast, HIF2 α expression can not be found in normoxic tissue and is restricted to endothelial cells and highly vascularized tissues, since high levels were found in lung, heart, liver, kidney, brain, and intestine.^(38,41) In comparison to HIF1 α , which is down-regulated during chronically hypoxia exposure, HIF2 α seems to have the key role in controlling responses to long-term hypoxia.⁽⁴²⁾ Koh *et al.* recently showed, that the E3 ubiquitin ligase, called hypoxia associated factor (HAF), which is important for the proteosomal destruction of HIF1 α during normoxic phases, is increased during chronic hypoxia. HAF is able to bind HIF2 α as well, however, this binding increases the activation of HIF2 α related gene activation. In short, during long-term hypoxia HAF switches the transcription of hypoxia related genes from HIF1 α -dependent mechanisms to HIF2 α -dependent regulation.⁽⁴³⁾ This switch in hypoxia response is accompanied by highly aggressive tumor growth, since HIF2 α -dependent signalling pathways are connected with cell proliferation, tumorigenesis, angiogenesis as well as cancer metastasis. The binding of HIF2 α to the HRE is connected with an increase in the transcription of c-Myc, TOR, and cyclin D1, resulting in promotion of cell cycle progression and consequently cell proliferation and the transcription of Oct-4, resulting in altered differentiation of hematopoietic stem cells.^(39,44,45) Participation of HIF2 α in JNK signalling pathways, explains tumor metastasis, whereas tumor angiogenesis can be explained by up-regulation of VEGF expression. Although HIF1 α is also associated with an increase of VEGF transcription, HIF2 α has a higher transactivation activity on the VEGF promotor.⁽³¹⁾ Similar to HIF1 α , HIF2 α activation leads to an increase in the transcription of the glucose transporter GLUT1. Nevertheless, Hu *et al.* revealed that HIF2 α is not associated with the up-regulation of glycolytic pathways.⁽³⁸⁾

Summarized, hypoxia-inducible factors are mainly involved in tumor progression, whereby both subunits are closely related and complementing each other in different phases of hypoxia. This close relation can be confirmed by the fact, that knockdown of one subunit

increases the expression of the other α subunit and shifts the transcription activity towards the signalling pathways, regulated by the respective HIF.⁽⁴⁶⁾

Imaging of hypoxia via PET

Changes in protein expression during hypoxia, result in survival of the tumor cells, which is, however, accompanied by poor outcome in patient's treatment. Imaging of hypoxic regions within tumor tissue is therefore important for the selection of chemotherapeutical strategies.

With the observation, that radiolabeled nitroimidazoles are trapped within hypoxic cells without losing their electron-transport activity, Chapman *et al.* pointed out their potential in hypoxia imaging.⁽⁴⁸⁾

Consequently, nitroimidazole derivatives, commonly labeled with PET radionuclides, like ^{18}F , are widely applied for hypoxia imaging via PET. The best investigated representative is [^{18}F]Fluoromisonidazole ([^{18}F]FMISO), which is already in use for imaging hypoxia in brain tissue, to identify ischemic stroke areas as well as chronic myocardial hypoxia. Further, it could serve as a potential radiotracer in image-guided cancer treatment. In addition to [^{18}F]FMISO, ^{18}F -fluoroazomycin-arabinofuranoside ([^{18}F]FAZA) or ^{18}F -fluoroetanidazole ([^{18}F]FETA) could serve as potential PET-tracers for hypoxia imaging.

The mechanism of all mentioned tracers is based on the fact, that the nitro functionality of nitroimidazoles has an affinity for electrons. The electron binding leads to formation of a radical anion, which reacts with oxygen and reduces the tracer to its original form (Figure 7). During hypoxia, the radical anion can not react with O_2 and accepts another electron, therewith the nitro functionality can be reduced to a highly efficient alkylating agent, which is covalently bound to macromolecules. This binding results in trapping the nitroimidazole within hypoxic cells.^(49,50) More precise, Masaki *et al.* revealed that especially the glutathione conjugate of the FMISO metabolite, amino-FMISO, is important for the binding to macromolecules and subsequently the accumulation in hypoxic cells, which allows imaging of such regions.⁽⁵¹⁾

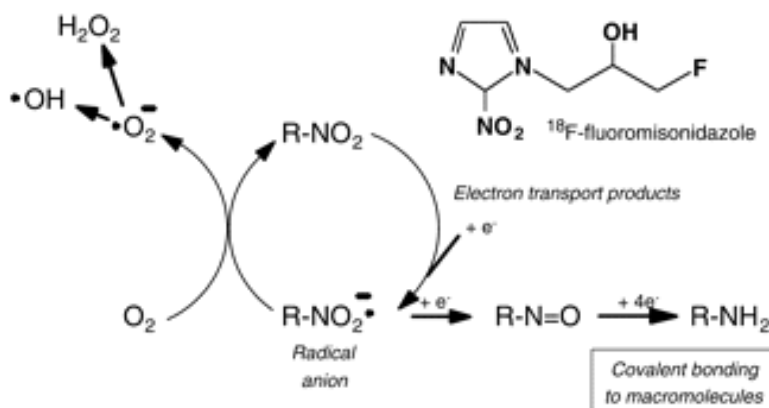


Figure 7: **Mechanism of nitroimidazole accumulation within hypoxic regions.** Image adapted from (50).

1.3. Adenosine A_3 receptor

In addition to the A_1 , A_{2A} , and A_{2B} receptor, the adenosine A_3 is a member of the superfamily of G-protein-coupled receptors. Adenosine receptors are major targets of caffeine or theophylline, however in comparison to the other receptor subtypes, receptor antagonists like xanthines show low binding affinities for the A_3 receptor.⁽⁵²⁾ It was found, that especially dihydropyridine derivatives, substituted with aromatic rings, like FE@SUPPY or MRS1334, serve as potent receptor antagonists. Whereas adenosine derivatives like IB-MECA represent prototypical receptor agonists.^(65,66,68,94)

The A_3 receptor couples to signalling pathways, involved in MAPK and other cell cycle activities, which explains their relation to cell differentiation, proliferation, survival or apoptosis induction, respectively. Especially breast, colon, lymphoma and prostate carcinoma tissue show high expression of the receptor subtype. Cell proliferation studies for example, using PC-3 prostate carcinoma cells and HCT-116 colon carcinoma cells, revealed that A_3 receptor activation inhibits tumor cell growth. In addition, A_3 receptor agonists increase the number as well as the activity of white blood cells, such as NK cells. Consequently, NK cell-mediated tumor cell destruction might be enhanced by A_3 receptor activation. These findings suggest the use of A_3 receptor agonists, such as IB-MECA, for cancer treatment.

Nevertheless, the receptor-mediated apoptosis induction is dose dependent. While high concentrations of receptor agonists initiate processes of programmed cell death, low concentrations block apoptosis. However, the dose-dependence of anti-tumoral effects

could not be confirmed for all cell lines, since colon carcinoma cell lines HT-29 and Caco2 showed an increased cell proliferation after A₃ receptor stimulation.

Further pro-tumoral effects are explained by the involvement of the A₃ receptors in hypoxic conditions. As it was mentioned above, A₃ receptor activation is correlated with HIF1 α expression and is therewith directly involved in angiogenesis by the stimulation of VEGF expression. In order to counteract the new capillary formation, the application of A₃ receptor antagonists might be indicated.

Summarized, the A₃ receptor could serve as a specific target for chemotherapeutical strategies in prostate, colon or breast carcinoma treatment. Since the receptor upregulation correlates well with the disease severity, it additionally might serve as a biological tumor marker.^(53,61,62,95)

Furthermore, receptor activation initiates second messenger pathways, like the stimulation of PLC, calcium mobilization and inhibition of adenylyl cyclase.⁽⁵³⁾ The therewith resulting vasodilatation is of high importance for protection of cardiac and cerebral ischemia.^(54,55) From another point of view, alterations in the A₃ receptor expression, which result in the reduction of coronary vasodilation, might play a role in the development of essential hypertension. This findings suggest the use of receptor agonists for hypertension treatment.⁽⁵⁶⁾

Additionally, receptor activation relates to the activation of K_{ATP} channels and pre-conditioning in cardiac cells, which points out their cardioprotective function. Since A₃-mediated heart protection occurs without side effects like hypotension, the therapeutical application of receptor agonists is conceivable. For heart protection, however, studies have shown that only low receptor expression is effective, whereas high expression results in the development of a dilated cardiomyopathy, which can be reduced to their vasodilator function.⁽⁵⁷⁻⁶⁰⁾

However, A₃-mediated vasodilatation might have a negative side effect too, since the kidney requires a particular pressure, which is obtained through vasoconstriction, to work properly. Prevailing vasodilatation due to alterations in the A₃ receptor expression might be detrimental for effective glomerular filtration, resulting in renal functional disorders. Therefore, studies have revealed that A₃ receptor antagonism shows renal protection.⁽⁶⁷⁾

Moreover, the A₃ receptor is involved in allergic responses, since receptor activation leads to degranulation of mast cells, resulting in a release of allergic mediators, like histamine.

With a selective application of A₃ antagonists the mast cell degranulation could be inhibited, which provides a possible application in asthma treatment.⁽⁶³⁾

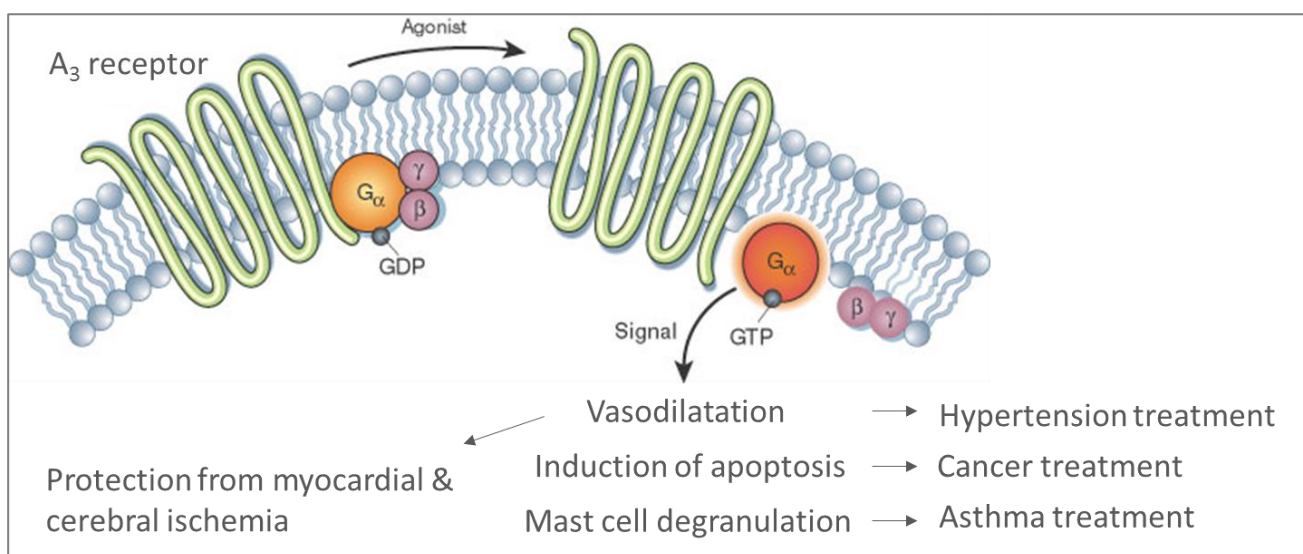


Figure 8: **Schematic illustration of the A₃ receptor as a potential target in different treatments.** Image partly adapted from (64).

Visualization of the A₃ receptor via PET

Since the A₃ receptor is involved in neurological, cardiovascular and tumor-related diseases, PET imaging is of high interest for diagnosis and therapy monitoring. The radio-iodinated 4-aminobenzyl derivative of the high-affinity receptor agonist IB-MECA ([¹²⁵I]-AB-MECA) is used as a model radioligand for A₃, however, it is not suitable for PET-imaging.

With the synthesis of the receptor antagonist [¹⁸F]FE@SUPPY Wadsak *et al.* introduced the first PET-tracer for the human A₃ receptor.⁽⁶⁸⁾ *In vitro* evaluation of [¹⁸F]FE@SUPPY highlighted the potential of the PET-tracer, whereas during *in vivo* evaluation a high tracer uptake was shown in fat-rich regions.^(69,70) However, the accumulation in fat tissue might be explained by the high lipophilicity of [¹⁸F]FE@SUPPY, which has a $\text{HPLClogP}_{\text{ow}}$ of 4.12.⁽⁷¹⁾

Moreover, with the conversion of the fluoroethyl ester group in [¹⁸F]FE@SUPPY to a fluoroethyl thioester, a second potential PET-tracer, called [¹⁸F]FE@SUPPY:2, for A₃

receptor imaging was introduced by Mitterhauser *et al.*⁽⁷²⁾ Likewise, ¹¹C- and ⁷⁶Br-labeled radiotracer are under discussion for A₃ receptor imaging.^(73,74)

Nevertheless, before clinical application further investigations are needed to guarantee specific A₃ receptor binding of [¹⁸F]FE@SUPPY.

1.4. Tumor imaging via PET

According to the World Health Organisation (WHO), tumor-related pathologies are one of the leading causes of morbidity and mortality worldwide. Therefore, early diagnosis and continuous therapy monitoring are important for increasing the chance of cure.

In comparison to anatomy-based imaging techniques, like computed tomography (CT), with positron emission tomography (PET) imaging of cellular events is possible, which allows early diagnosis as well as monitoring during pharmacological or radiation therapy. The principle behind PET-imaging is based on the use of radiolabelled molecules, the so-called tracers, which are injected in non-pharmacological doses and are capable of emitting a positron from their nuclei. As soon as the positron collides with an electron, they annihilate each other and consequently the produced energy, in the form of two 511 eV gamma-rays, is emitted in opposite directions and detected by a photomultiplier. Subsequent computer reconstruction of three-dimensional images allows following the distribution and concentration of the applied radiotracers within the body.⁽⁸²⁾

Since PET-imaging only provides metabolic information, modern PET-scanners are usually equipped with CT-scanners or combined with magnetic resonance imaging (MRI), in order to merge the physiological information with anatomical information.

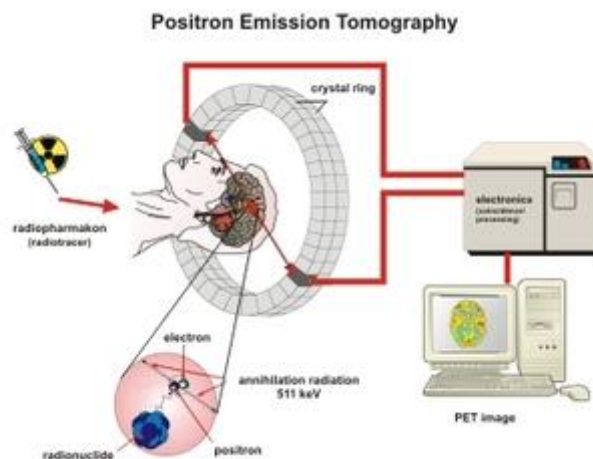


Figure 9: **Principle of positron emission tomography.** Image adapted from (84).

The currently used radionuclides ^{18}F , ^{11}C , ^{15}O and ^{13}N , are characterised by their relatively short half-lives and are incorporated into molecules, which are either normally metabolised by the body or bind to specific receptors. Since PET-tracers should trace biological pathways, radiolabelling should not alter biological properties of the parent molecule, like elimination, transport or target affinities. Moreover, dissociation of the linked radionuclide from the parent molecule must be avoided, otherwise the pathway of the radionuclide, instead of the molecule, is traced with PET.^(83,85)

Apart from above-named PET-tracers, which are used for specific purposes like hypoxia imaging, such as $[^{18}\text{F}]\text{FMISO}$, or receptor imaging, such as $[^{18}\text{F}]\text{FE@SUPPY}$, the glucose analogue $[^{18}\text{F}]\text{Fluorodeoxyglucose}$ (FDG) is the most frequently used radiotracer in oncology PET-scans. Like glucose, FDG is recognized by the GLUT-transporters and actively transported into the cells, where it gets phosphorylated to FDG-6-phosphate by hexokinase. Since FDG lacks a hydroxyl group in the second position, FDG is not available for further glycolysis. Subsequently FDG-6-phosphate gets intracellularly trapped.

Due to high mitotic rates, cancerous cells have a higher utilization of glucose, which makes them express a higher number of glucose transporters and display an up-regulated hexokinase activity. Consequently, the FDG accumulation increases within tumor tissue. Nevertheless, FDG is not specific for tumor cells, since all cells metabolize glucose. Therefore, fasting the patient for 4 to 6 hours before the PET-scan is necessary to minimize the competition with glucose.^(82,86,87)

2. Aim

In recent years, *in vitro* testing of chemotherapeutical agents and PET-tracers for tumor imaging is predominately performed using 2D tumor cell monolayers. However, 2D cell culture results are often not reflecting the *in vivo* situation, due to simulation deficiency of physiological tumor tissue.

Multicellular tumor spheroids (MTS) take an intermediate role between 2D cell monolayers and solid tumor tissue, since their morphology, microenvironment and functional features are more similar to human tumors. Therefore, with the use of multicellular tumor spheroids testing strategies for novel cytotoxic substances and PET-tracers can be refined and the number of animals needed could be subsequently reduced.

In the last decades, the efficiency testing of anti-cancer drugs in spheroids became highly popular, but the use of MTS for the evaluation of radiotracers used for tumor imaging is still very rare.

The aim of this work was the evaluation and establishment of a reliable method for different cell lines to form spheroids and further the characterization of the obtained multicellular tumor spheroids regarding stability, growth behavior and the appearance of necrotic regions. Moreover, 3D cell aggregates were compared to 2D cell monolayers regarding their ability of PET-tracer accumulation, depended on the temperature and incubation conditions. For this purpose accumulation experiments, internalization assays and LigandTracer® real-time accumulation experiments were performed, using [^{18}F]FDG, [^{18}F]NaF, [^{18}F]FMISO, [^{18}F]FE@SUPPY and [^{125}I]I-AB-MECA.

In order to ensure the expression of the adenosine 3 receptor (A3R), which represents the target for [^{18}F]FE@SUPPY and [^{125}I]I-AB-MECA, western blots were performed, using 2D cell lysate as well as spheroid lysate of the tested cell lines.

Since necrotic regions as well as hypoxic regions are described for MTS, in addition to accumulation assays using the hypoxia tracer [^{18}F]FMISO, the expression of the hypoxia inducible factor 2 α (Hif2 α) was examined by western blot analysis and immunohistochemically staining.

3. Materials and Methods

3.1. Cell lines and culture conditions

The human colorectal adenocarcinoma cell line HT-29 (HTB-38™), the human prostate cancer cell line PC3 (CRL-1435™) and the Chinese hamster ovary cell line CHO-K1 (CCL-61™) were purchased from ATCC® (Virginia, United States) and kindly provided by the Institute for Pathophysiology and Allergy Research (Medical University of Vienna, Austria). The CHO cell line, expressing human A3 receptor (CHO-hA3R) was obtained from PerkinElmer (Massachusetts, United States). HCT-116 (CCL-247™), a cell line derived from colon cancer, was a generous gift from the Institute of Inorganic Chemistry (University of Vienna, Austria) and identity was proven by Multiplex human Cell line Authentication Test (MCA). All cell lines were grown in 75 cm² cell culture flasks (Cellstar®, Greiner Bio-One, Frickenhausen, Germany) and maintained at 37°C in a cell incubator under humidified atmosphere with 5% CO₂.

All cell culture media and supplements, except from Non-Essential Amino Acids solution (Sigma Aldrich, Missouri, United States) were purchased from Gibco™ (Thermo Scientific, Massachusetts, United States). For cultivation of HT-29 and PC-3 cells RPMI medium, supplemented with 10% foetal bovine serum (FBS) and 2mM L-glutamine was used. HCT-116 cells were maintained in MEM medium supplemented with 10% FBS, 2 mM L-glutamine, 1% MEM non-essential amino acids solution (100x) and 1% sodium pyruvate, according to Schreiber-Brynzak *et al.*⁽²⁷⁾ For CHO-K1 cells Ham's F-12 nutrient mixture medium containing 10% FBS as well as 2 mM L-glutamine was used and for CHO-hA3R cells the medium was additionally supplemented with 0.4 mg/mL geneticin (G418).

Thawing of cells

Cells, frozen in FBS with 10% DMSO (Sigma Aldrich, Missouri, United States), were thawed quickly. Afterwards, cells were transferred into a Flacon™ conical centrifuge tube (Thermo Scientific, Massachusetts, United States) containing respective complete growth medium. Cells were centrifuged for 3 min at 1200 rpm. After supernatant was discarded, the remaining cell pellet was suspended in 1 mL growth medium and transferred into a cell culture flask already containing 9 mL of complete growth medium.

Splitting of cells

The confluency of the cells was checked daily under the microscope. As soon as they reached a confluency of approximately 90%, cells were splitted according to standard protocol. After removing the growth medium, adherent cells were washed once with Gibco® Dulbecco's phosphate buffered saline (DPBS). In order to detach cells from the flask, Gibco® trypsin-EDTA (0.05%) was used for 3 min at 37°C. For detaching HCT-116 cells Accutase® solution (Sigma Aldrich, Missouri, United States) was added to the flasks and incubated for 10 min at 37°C. After all cells were completely detached from the flask trypsinization and accutase reaction was stopped by adding twice the amount of complete growth medium. An aliquot of the cell suspension was transferred to a new flask and supplemented growth medium was added before incubation at 37°C.

3.2. Multicellular tumor spheroid formation (3D cell culture)

Formation of HT-29 spheroids

HT-29 tumor spheroids were obtained by using the hanging drop culture method according to Foty.⁽⁷⁵⁾

After detaching adherent cells with 0.05% trypsin-EDTA, cells were suspended in fully supplemented RPMI medium. Cells were counted using an automated cell counter (Scepter™, Merck Millipore, Darmstadt, Germany) and concentration was adjusted to 500 cells per 10 µL.

100 mm Petridishes (Cellstar®, Greiner Bio-One, Frickenhausen, Germany) were prepared by placing 5 mL of DPBS in the bottom of the dish, to create a hydration chamber. 10 µL drops of the cell suspension were deposited on the bottom of the inverted lid (Figure 10). After the lid was carefully placed onto the DPBS filled bottom, Petridishes were incubated in a cell incubator at 37°C for four days, until cells formed an aggregate.

Drops containing the spheroid were carefully transferred to a non-cell culture treated round bottom 96-well plate (polyethylene, Corning®, New York, United States) containing 100 µL of complete growth medium. Plates were incubated at 37°C and medium was changed every third day. Spheroid growth behaviour was observed daily by size measurement using Cell[^]B-Software (Olympus, Tokio, Japan).

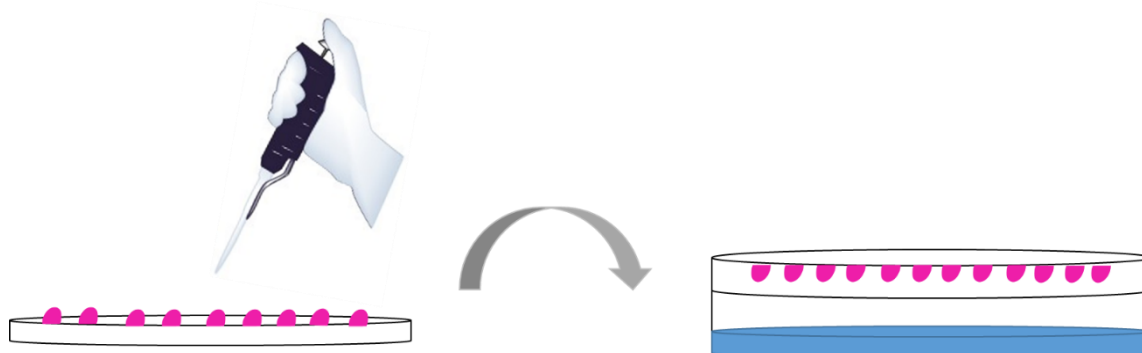


Figure 10: **Scheme of the hanging drop method**

Formation of HCT-116 spheroids

HCT-116 multicellular tumor spheroids were formed by using a modified pellet culture system.

Therefore, wells of round bottom 96-well plates were coated with a sterile filtered 1.5% aqueous agarose solution, to create an ultra-low attachment surface. HCT-116 cells were detached from the flask by using Accutase® as described in the splitting protocol above and counted with an automated cell counter. Cell concentration was adjusted to 3×10^3 respectively 5×10^3 cells per 10 μL .

A multichannel pipette (Eppendorf, Hamburg, Germany) was used to place 10 μL of each cell suspension into the wells of the agarose coated 96-well plate, prefilled with 100 μL fully supplemented growth medium. 96-well plates were incubated at 37°C , without moving the plates for three days, in order to let cells form stable aggregates.

From the fourth day on, spheroids were checked daily under the microscope and medium was changed every other day. Cell^B software (Olympus, Tokio, Japan) was used for taking photos and for growth behaviour analysis.

Formation of PC-3 and CHO-K1 spheroids

Hanging Drop method

To achieve spheroid formation hanging drop method was performed as described above. Cell concentrations of 250, 500, 750 and 1000 cells, respectively, per 10 μL drop were tested. To ease terms of aggregate building 5%, 10% and 20% methylcellulose, respectively, was added to the drops containing same cell concentrations as previously

described. Methylcellulose stock solution was prepared by dissolving 1.5% (w/v) methylcellulose (Sigma Aldrich, Missouri, United States) in 1000 mL high glucose (4.5 g/L) DMEM by continuous stirring at 60°C. After 100 mM L-glutamine were added, stirring was performed overnight at 4°C, followed by a centrifugation at 4700 g for 2 h. The obtained supernatant was used for the experiments.

Pellet culture system

For spheroid formation using the pellet culture method, 96-well round bottomed plates were coated with 1.5% agarose or 0.1% or 2% gelatine solution, in order to prevent cell adhesion. Cell concentration was adjusted to 250, 500, 750, 1×10^3 , 2×10^3 , 3×10^3 , 4×10^3 , 5×10^3 , 6×10^3 , 8×10^3 , and 1×10^4 cells per 10 μ L. An Eppendorf multichannel pipette was used to depose 10 μ L drops in the wells containing 100 μ L of the required growth medium. Spheroid formation was monitored under the microscope.

To help aggregates form, the same conditions as described above were performed with subsequently centrifuging the 96-well plates at 1200 rpm for 3 min, as soon as cell suspension was transferred.

In addition, suspensions of the same cell concentrations, were placed into ultra-low attachment (ULA) 96-well round bottomed plates (Corning®, New York, United States).

3.3. Embedding of multicellular tumor spheroids

Collagen Embedding

Collagen solution was prepared according to Naber *et al.*, by mixing 8 mL of type I rat tail collagen solution with 1 mL 10x DPBS. The pH needed to be adjusted to 7.4 using 1 mL sterile filtered 0.1 M NaOH.⁽⁷⁶⁾

For embedding cell aggregates, 50 μ L of the neutralized collagen solution were placed in the well of a round bottom 96-well plate and incubated at 37°C, until collagen solidified. Cell culture medium of multicellular tumor spheroids was removed and each spheroid was embedded in 200 μ L of the collagen solution. The dispensed spheroids were separately transferred to the collagen coated 96-well plate. For collagen solidifying the plate was incubated at 37°C before 50 μ L of required growth medium was added.

Growth behaviour of the embedded spheroids was checked daily by size measurement using Cell[^]B software.

Gelatine embedding

For real-time [^{18}F]FDG accumulation experiments, 500 μm spheroids were embedded in 10% gelatine solution, according to Dubiak-Szepietowska *et al.*⁽⁹⁾ Gelatine solution was prepared by dissolving gelatine powder in complete growth medium at 80°C, followed by a sterile filtration using a 0.2 μm sterile filter. A 500 μL drop of the freshly prepared solution was placed into the bottom of a Petridish. Right before gelatine polymerization the spheroid was carefully embedded in the drop and incubated at 37°C.

Matrigel embedding

Matrigel embedding was performed using a technique described by Puliafito *et al.*⁽⁷⁷⁾ For spheroid embedding, 25 μL of growth factor reduced Matrigel solution, in a concentration of 8 mg/mL was deposited on the bottom of a round-bottomed 96-well plate and incubated until it solidified. A second layer of 100 μL Matrigel, containing the cell aggregate, was placed on the top of the first layer. As soon as the layer polymerized, 50 μL of growth medium were added to each well. The microscope was used for spheroid growth monitoring.

3.4. Characterization of multicellular tumor spheroids

Cell count determination

For cell count determination spheroids of different sizes were harvested from the 96-well culture plates and transferred to a 1.5 mL Eppendorf tube. Cell aggregates were washed once with DPBS before 1 mL of trypsin, respectively 1 mL of Accutase® was added. After 15 min of incubation at 37°C, cells of the dissolved spheroids were well suspended using a 100 μL Eppendorf pipette. Cell counting was performed using a Neubauer hemocytometer.

Cell proliferation assay (MTT assay)

The proliferation ability of cells in the 3D aggregate was measured following MTT assay protocol. MTT solution was freshly prepared by dissolving MTT in DPBS to a final concentration of 5 mg/mL and filtered through a 0.2 μm sterile filter.

Multicellular tumor spheroids from sizes between 300 and 900 μm were harvested and transferred to a flat bottom 96-well culture plate (Corning®, New York, United States). Remaining medium was removed and replaced by 100 μL Gibco® RPMI medium. After 10 μL of MTT solution were added to each well, the plate was incubated for 6 hours at 37°C in a cell incubator. As soon as incubation time ended, RPMI medium was removed and spheroids were covered with 100 μL dimethyl sulfoxide (DMSO). Plates were carefully shaken until the tumor aggregates were completely solubilized.

The absorbance was recorded at 490 nm and a reference wavelength of 650 nm using a Synergy HTX Multi-Mode plate reader (BioTek Instruments, Vermont, United States). Received data were evaluated using BioTek's Gen 5 software.

Cell viability assay (Live-dead staining)

For live-dead staining HT-29 and HCT-116 spheroids from sizes between 300 and 900 μm were selected and transferred to a flat bottom 96-well microtiter plate. After removing the nutrient medium, the tumor spheroids were washed two times with DPBS. This important washing step was performed to remove unspecific esterase activity, which is generally present in growth medium supplemented with FBS, which cause high background signal. For detection of dead cells propidium iodide (PI), purchased from Sigma Aldrich, in a dilution of 1:1000 in complete growth medium was added to the spheroids. Plates were incubated for six hours at 37°C in a cell incubator.

Within the last 15 minutes of incubation time 100 μL of growth medium containing calcein acetoxymethylester (Calcein AM, Sigma Aldrich, Missouri, United States) in a dilution of 1:400 were added for detection of live cells.

After rinsing the cell aggregates twice with DPBS, they were covered with DPBS and green fluorescence emission for Calcein AM was measured at 517 nm (G filter) under an Olympus IMT-2 fluorescence microscope equipped with a XC50 camera. Red fluorescence emission for PI was measured at 617 nm (B filter).

Cell^B software was used for taking pictures as for size measurement of the necrotic regions within the multicellular tumor spheroid.

Haematoxylin and Eosin staining of spheroid cryosections

For the preparation of the cryosections HT-29 and HCT-116 spheroids were grown to a size of about 600 μm . As soon as they reached the required size, the spheroids were separately transferred to 1.5 mL Eppendorf tubes. The growth medium was discarded and

the 3D aggregates were fixed on ice using 4% paraformaldehyde (PFA) in DPBS for 1h. PFA was carefully removed and a 20% Sucrose solution was added to each spheroid, acting as a frost protection agent. The spheroids were stored in the fridge overnight, before they were embedded in Tissue-Tek® O.C.T.™ (Sakura Finetek, Alphen aan den Rijn, Netherlands). The embedded spheroids were cut into 10 µm slices on the cryotom and spread on Menzel-Gläser Superfrost® glass slides (Thermo Scientific, Massachusetts, United States). The slices were stored at -80°C until use.

Before the actual staining procedure, the spheroid slices were fixed with 96% ethanol. The slides were placed into pre-filtered haematoxylin staining solution for 3 min. The slides were washed with deionised water and put into tap water for 5 min for staining development. Then the slides were quickly dipped into acid alcohol for eight to twelve times. After rinsing the slides twice with tap water and deionised water, Eosin staining solution was dropped on the slices, followed by a five-minute wash in 70% ethanol. For dehydration of the slices 100% ethanol was used for 5 min. Before getting embedded in Histofluor slices were placed in n-butyl acetate for 10 min. The staining was checked under the microscope and pictures were taken.

3.5. Determination of intracellular calcium concentration

For quantification of intracellular calcium concentration, Fluo-8 No wash Calcium Assay kit (ab112129, Abcam, Cambridge, United Kingdom) was used, following the instructions provided in the product sheet.

The day before the assay, tumor cells, maintained in 75 cm² culture flasks, were splitted as described above. The concentration of the cell suspension was adjusted to 8×10^4 cells per 100 µL and transferred to a black-walled 96-well microtiter plate for fluorescence-based assays, purchased from Thermo Scientific.

Confluency of the cells was checked the following day, before Fluo-8 stock solution was prepared by adding 20 µL of DMSO to Fluo-8. For assay buffer preparation 9 mL of HBBS were mixed with 1 mL of 10x Pluronic® F127 Plus.

In order to minimize background fluorescence of FBS, cells were washed once with DPBS after growth medium was removed. 100 µL of Fluo-8 dye-loading solution, prepared by adding 5 µL of Fluo-8 stock solution to 2.5 mL of the assay buffer, were carefully pipetted to each well. The plate was at first incubated at 37°C for 30 min, then it was incubated at room temperature for another 30 min.

The calcium assay was run by monitoring the fluorescence intensity in BioTek's Synergy HTX multi-mode plate reader, using 490 nm for excitation and 525 nm for emission.

3.6. PET-tracer synthesis

All radiolabeled tracers were produced at the Department of Biomedical Imaging and Image-guided therapy of the General Hospital (AKH) in Vienna, Austria.

[¹⁸F]FDG synthesis

Radiosynthesis of [¹⁸F]FDG was routinely performed for patient's diagnostic scans in a fully automated synthesizer (TracerLab MX, GE Healthcare, Illinois, United States) with more than 99% radiochemical purity.

[¹⁸F]FMISO synthesis

The synthesis of the hypoxia tracer fluoromisonidazole (FMISO) radiolabeled with fluorine-18 was carried out using the same automated synthesizer as described above.

[¹⁸F]FE@SUPPY synthesis

The radiotracer [¹⁸F]FE@SUPPY was synthesized as described by Wadsak *et al.* with more than 99% radiochemical purity.⁽⁶⁸⁾

Receipt of [¹⁸F]NaF

[¹⁸F]NaF was directly received *via* cyclotron production.

3.7. Accumulation of PET-tracers ($[^{18}\text{F}]\text{FDG}$, $[^{18}\text{F}]\text{FMISO}$, $[^{18}\text{F}]\text{NaF}$, $[^{18}\text{F}]\text{FE@SUPPY}$) in multicellular tumor spheroids

Radiotracer uptake experiments in 3D cell aggregates

Cellular uptake experiments in tumor spheroids were performed according to Monazzam *et al.* at 37°C and 4°C, respectively, for downregulation of active transport mechanisms.⁽²⁵⁾ All experiments were performed in triplicates and repeated at least three times.

Multicellular tumor spheroids were selected by size between 500 and 600 μm and transferred to an 1.5 mL Eppendorf tube containing 500 μL of complete growth medium. Considering that some growth media containing high amount of glucose $[^{18}\text{F}]\text{FDG}$ uptake studies were also carried out with starved spheroids. Therefore, spheroids were transferred to Gibco® Minimal Essential Medium (MEM) and incubated for 1 h at 37°C. A triplicate of blank Eppendorf tubes containing only medium, treated exactly like the tubes containing the spheroids, served as background control for determination of unspecific radiotracer binding to the plastic or accumulation in medium. For the experimental setting requiring 4°C, tubes were put on ice 30 min before the experiment started.

After 500 μL of a 6 MBq/mL solution of respective radiotracer were added, tubes were incubated for 50 min at 37°C respectively 4°C. As soon as incubation time ended 20 μL of the incubation medium was transferred to new Eppendorf tubes, serving as reference. The tubes were carefully washed three times for 5 min with medium. After the last washing step, the spheroid was transferred to new tubes in 20 μL of medium and measured in a well calibrated γ -counter (Wizard 2, PerkinElmer, Massachusetts, United States). 20 μL of the last washing medium obtained from the blank tubes, acting as background control, were measured.

The uptake per spheroid was calculated as

$$\text{Total uptake in \%} = \frac{\text{Bq of Spheroid} - \text{Bq of Background control}}{\text{Bq of reference}} \times 100$$

To obtain an assessment of the uptake per cell, calculated values were divided by cell count, determined as described above.

2D cell culture uptake experiments

The uptake experiments for 2D cells were as well performed in triplicates at 37°C and 4°C, with blanks acting as background control. Compared to 3D uptake experiments the incubation of cells with the radiotracers was performed in 100 mm Petridishes (Cellstar®, Greiner Bio-One, Frickenhausen, Germany). Only the measurement in the γ-counter was carried out in Eppendorf tubes.

The day before the experiment, cells were detached from the flasks following the splitting protocol described above and counted using an automated cell counter. An aliquot of cell suspension, containing approximately 2.5×10^5 cells, was transferred to a petridish prefilled with growth medium. By the time cells settled down, medium was discarded and 3 mL of fresh medium was added to each dish.

The cells were incubated with 3 mL of a 6 MBq/mL concentrated solution of the radiolabelled tracer for 50 min at the required temperature. Immediately after incubation 200 µL of incubation medium were transferred to an Eppendorf tube, serving as reference solution. Cells were washed 5 min for two times using growth medium and detached from the plastic dish using a cell scraper. 500 µL of medium was added to the dish and cells were carefully dispensed, before 200 µL of cell suspension were transferred to an Eppendorf tube for measurement. The blank dishes were washed three times with medium before a sample of 200 µL was taken out for background measurement. Finally, cells, contained in the 200 µL of suspension, were counted using a Neubauer homocytometer.

The percentage cellular radiotracer uptake was calculated as

$$\text{Uptake per cell in \%} = \frac{\left(\frac{\text{Bq of cell suspension} - \text{Bq Background control}}{\text{Cell count}} \right)}{\text{Bq of reference}} \times 100$$

[¹⁸F]FE@SUPPY accumulation

The experimental set up basically corresponded to the settings described above. In accordance with previous experiments, [¹⁸F]FE@SUPPY was used in a final concentration of 100 kBq/mL. Regarding absolute amounts of activity, 50 kBq of [¹⁸F]FE@SUPPY were used for 3D cell aggregates (500 µL of 100 kBq/mL), while 2D monolayer cells were incubated with 300 kBq (3 mL of 100 kBq/mL).

3.8. Internalization assays

Internalization of [¹²⁵I]I-AB-MECA

For the determination of the actual intracellular uptake of the model human A3 receptor agonist [¹²⁵I]I-AB-MECA, internalization assays as described by Fischer *et al.*⁽⁷⁸⁾ were carried out using CHO-hA3R, CHO-K1, PC-3 and HT-29 cell monolayers. To determine non-specific binding, blocking of the A3 receptor was performed using non-radioactive solutions of FE@SUPPLY respectively I-AB-MECA in a final concentration of 1 μM (0,1% DMSO).

Suspensions of the cell lines mentioned above, containing approximately 5x10⁵ cells, were transferred to a 6-well cell culture plate and incubated, until they reached 90% of confluency. Growth medium was renewed and blocking solutions dissolved in DMSO were added to the wells. 0.1% DMSO, serving as vehicle control, was added to the remaining wells, where blocking was not performed.

Approximately 2.4 kBq [¹²⁵I]I-AB-MECA were added to each well and cells were incubated for 1 h at 37°C in a cell incubator. After incubation, supernatants were collected and cells were washed with DPBS once, both fractions representing the free radiotracer, which has not bound to the receptor. Cell culture plates were put on ice and cells were washed with acidic saline glycine buffer (100 mM NaCl, 50 mM glycine, pH 2.8) two times for 5 min, in order to collect the receptor-bound fractions. Cell lysis was performed at 37°C by incubating the cells for 10 min using 1 M NaOH, to determine the internalized fraction.

All collected fractions were transferred into 2 mL Eppendorf tubes and measured in the γ-counter.

Internalization of [¹⁸F]FDG

To determine the cell internalization of [¹⁸F]FDG, the same assay as described above was carried out, using HT-29 cell line.

Incubation of cells was performed with 100 kBq [¹⁸F]FDG either in growth medium, supplemented with FBS, or in serum free medium for 1 h at 37°C.

Furthermore, the effect of cell starving on the radiotracer internalization was determined by incubating cells for 1 h with DPBS before they were incubated with [¹⁸F]FDG for 1 h and 2 h, respectively.

Internalization of [¹⁸F]FE@SUPPY

Considering that [¹⁸F]FE@SUPPY is a very lipophilic radiotracer, the internalization assay, as described above, was not fully adaptable due to its high unspecific binding. Therefore, the washing procedure (washing agents and washing steps), had to be evaluated by testing four different methods. For method evaluation no receptor blocking was performed.

For the first method, the internalization assay was carried out as described for [¹²⁵I]I-AB-MECA internalization. With the difference that an additional washing step with DPBS, containing 0.1% Tween-20, was performed right after treating cells with acidic saline glycine buffer for two times.

For the second method, cells were washed with DPBS, supplemented with 0.1% Tween-20, after the supernatant was taken off.

The third method was comparable to the first with regard to the additional washing step after collecting the glycine buffer fractions. However, cell lysis was performed using RIPA-buffer.

For the fourth method, the experimental setting was comparable to the second method, with the difference that cell lysis was carried out using 1X RIPA-buffer for approximately 5 min.

3.9. Real-time [¹⁸F]FDG accumulation experiments

Real-time [¹⁸F]FDG accumulation experiments were performed using the LigandTracer®yellow (Ridgeview Instruments AB, Uppsala, Sweden).

Petridishes used for the experiments were prepared according to the instructions provided by Ridgeview Instruments. Tumor cells were detached from the flasks by trypsinization. After cell counting an aliquot of cell suspension, containing approximately one million cells, was transferred to a 100 mm cell culture dish. The cells were supplied with growth medium and incubated in a cell incubator, where dishes were tilt (Figure 11) in order to allow cell adherence only on the lower dish area. The following day cell confluency was checked under the microscope and growth medium was replaced by DPBS for cell starving. After starving for 1 h at 37°C, confluency was once more checked and DPBS was renewed. The

lid was removed before the cell culture dish was placed in the LigandTracer®, making sure that cells were placed in the target position (Figure 12).

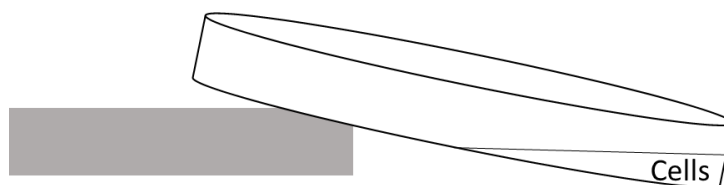


Figure 11: Preparation of the Petri dishes for LigandTracer® experiment

For the measurement LigandTracer® control software provided by Ridgeview was used. Following settings were chosen for the generation of the real-time kinetic: five seconds detection time and two seconds detection delay time. The experiment started with a background measurement for approximately 5 min followed by addition of 50, 100 or 500 kBq of [^{18}F]FDG to the dish. The measurement was continued, until an equilibrium has been reached. Then the run was paused in order to add additional [^{18}F]FDG to increase the concentration. As soon as a second equilibrium has been reached, DPBS was renewed and the run was continued to see whether there is an eventual [^{18}F]FDG dissociation from the cells. For data analysis TraceDrawer software was used.

For real-time accumulation experiments using multicellular tumor spheroids, spheroids needed to be embedded in a gel matrix. To determine unspecific [^{18}F]FDG uptake in matrices, 500 μL drops of solidified 10% gelatine solution, or 1.5% agarose solution were measured in the LigandTracer® yellow using the same settings as described above.

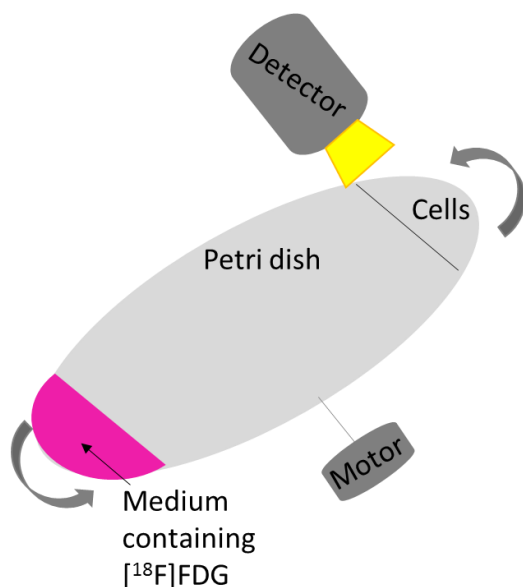


Figure 12: Principal of the LigandTracer®

3.10. Western Blot

Preparation of 3D cell lysate

For the preparation of the cell lysate HT-29 and HCT-116 multicellular tumor spheroids in the sizes of 300, 600 and 800 μm were collected classified by size and transferred in 5 mL Eppendorf tubes. After the growth medium was discarded, spheroids were carefully washed with precooled DPBS. 100 μL of ice-cold 1x RIPA lysis buffer (Merck Millipore, Massachusetts, United States) was added to each tube. Before tubes were gently shaken at 4°C for 30 min, protease inhibitor (Sigma Aldrich, Missouri, United States) in a dilution of 1:50 was added. Afterwards centrifugation was performed at 12000 rpm for 20min at 4°C. The received supernatant was transferred to a new Eppendorf tube and stored at -80°C.

Preparation of 2D cell lysate

The colon carcinoma cell lines HT-29 and HCT-116 were detached from the flasks as described above and cell suspension was diluted to the desired concentration of one million cells. Cell suspension was transferred to a 100 mm Petridish prefilled with complete growth medium. Cells were maintained at 37°C for approximately 24 h, until cells settled down.

The following day, the medium was discarded and cells were washed with ice-cold DPBS, before 750 μL of 1X RIPA lysis buffer were added to the dish. Cells were detached from the bottom of the petri dish by using a cell scraper and well dispensed in the lysis buffer. Suspension was transferred to 1.5 mL Eppendorf tubes, before protease inhibitor in a 1:50 dilution was added. Before centrifugation at 12000 rpm, tubes were shaken at 4°C for 30 min. The supernatant was taken off and transferred to new tubes, which were stored at -80°C.

Determination of protein concentration

The protein concentrations of the cell lysates were determined by using Pierce™ BCA Kit, purchased from Thermo Scientific. The dilutions of the bovine serum albumin standards (125-2000 $\mu\text{g/mL}$) were prepared according to the enclosed manual using RIPA buffer. 10 μL of the diluted standard solutions as well as 10 μL of the protein lysates were mixed with 200 μL of the freshly prepared developing solution in the wells of a flat bottom 96-well plate. Plates were incubated at 37°C for 30 min before absorption was measured at 562

nm using a multi-mode plate reader. Data analysis was performed using Gen 5 software provided by BioTek Instruments (Vermont, United States).

SDS Page

In order to prepare the samples for the gel electrophoresis, the respective protein concentrations obtained by the BCA Kit were used for calculation.

To load the desired amount of 20 µg protein per sample, cell lysates were mixed with 4x sample buffer, containing mercaptoethanol to achieve reducing conditions and filled up with DPBS to a final volume of 30 µL. The samples were cooked for 5 min at 95°C on a thermomixer, while the electrophoresis chamber was assembled and filled with electrophoresis buffer, containing 24.7 mM Tris, 193.3 mM glycine and 3.5 mM SDS in distilled water.

30 µL of the samples as well as 6 µL of PageRuler™ Prestained Protein Ladder, obtained from Thermo Scientific, were loaded into the pockets of the gel and electrophoresis run was carried out for 30 min at 200 V.

For semidry blotting of the membrane, semidry transfer buffer (5.82g Tris, 2.93 g glycine, 0.375 g SDS, 200 mL methanol, 800 mL distilled water) was freshly prepared.

As the gel was taken out of the chamber, the blotting sandwich was built using a nitrocellulose membrane (Amersham Protran™ Premium 0.2 µm NC, GE Healthcare Lifesciences, Wisconsin, United States). After making sure that all air bubbles were removed, the gel was blotted at 80 mA for 1 h in the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, California, United States).

Western Blot HIF2α and A3 receptor detection

After semidry blotting was finished, the membrane was washed with TBST (49.59 mM Tris, 137.9 mM NaCl in distilled water, pH 7.4 supplemented with 0.1% Tween-20). The membrane was blocked with 5% dry milk powder (DMP) in TBST for 2 h at room temperature. The blocked membrane was cut into two pieces. One part of the membrane was incubated with the primary monoclonal mouse antibody against HIF2α, purchased from abcam (ab8365, Abcam, Cambridge, United Kingdom) using a dilution of 1:500 in TBST with 5% DMP, while the other part was incubated with a polyclonal rabbit antibody against human A3 receptor in a dilution of 1:750 (Inc. sc-13938, Santa Cruz Biotechnology, Texas, United States). Incubation was performed at 4°C overnight.

On the next day, membranes were washed three times for 10 min with TBST, before they were incubated with the second antibodies conjugated to horse radish peroxidase (HRP). Goat-anti-mouse antibody was added in a dilution of 1:1000 in TBST containing 5% DMP to the HIF2 α membrane. A 1:2500 dilution of the second goat-anti-rabbit in DMP-TBST was added to the A3 receptor membrane. The membranes were incubated at room temperature for 1 h, followed by washing three times for 10 min with TBST.

As soon as washing procedure was finished, membranes were developed using the Super Signal West Pico Chemiluminescence substrate detection kit, obtained from Thermo Scientific.

VersaDoc™ Imaging system (Bio-Rad, California, United States) was used for detection of chemiluminescence. White light pictures were taken, in order to detect the position of the protein ladder.

Detection of Beta-Actin

Before incubation with beta-actin antibody was possible, the membranes needed to be stripped using Restore™ Western Blot Stripping Buffer, purchased from Thermo Scientific, for 20 min.

Membranes were carefully washed with TBST for 10 min, before they were blocked overnight using a freshly prepared 2% solution of bovine serum albumin. Blocked membranes were incubated with Beta Actin antibody, already conjugated to HRP (ab197277, Abcam, Cambridge, United Kingdom), in a dilution of 1:500 in albumin for 1h at room temperature.

As incubation ended, the membranes were washed three times with TBST for 10 min. Detection was carried out as described above.

3.11. Immunohistochemistry staining of HIF2 α

Immunohistochemistry staining of HT-29 tumor spheroid cryoslices was performed following the instructions on the product sheet provided by Abcam.

Glass slides were washed twice with DPBS, before slices were permeabilized by incubation in 0.2% Triton X-100 in DPBS for 10 min at room temperature. After slides were rinsed with DPBS, slices were incubated with the same primary mouse antibody against HIF2 α , as used for the western blot, in a dilution of 1:1000 for 1 h at room temperature. After a 5 min washing in DPBS, second goat-anti-mouse antibody, conjugated to HRP was added in a dilution of 1:1000 for 10 min. Binding was detected using 3',3'-diamino

benzidine for 1min, followed by another DPBS wash. Slides were mounted using the aqueous fixant Fluoromount-G® (SouthernBiotech, Alabama, United States).

4. Results

4.1. Spheroid formation

The formation of a three-dimensional structure for established cell lines is highly dependent on a cell lines morphology. In this thesis, four different mammalian cell lines were tested on their ability to form multicellular tumor spheroids, namely the human cell lines HT-29, PC-3, and HCT116, as well as the hamster cell line CHO-K1. Several established methods for spheroid formation were tested for each cell line including hanging-drop and pellet cell culture methods (Table 1).

The human colorectal adenocarcinoma cell line HT-29 was developing tight cell aggregates (Table 2) using the hanging drop culture method with a cell concentration of 500 cells per 10 μ L drop. This method was highly reproducible for this cell line to obtain stable and uniform multicellular tumor spheroids. After spheroid formation was observed within four days, cell aggregates were transferred carefully to non-cell culture treated 96-well plates, containing growth medium, in order to guarantee sufficient nutrient supply. Since it was not possible to take pictures of the aggregates within the drop, without disturbing spheroid formation, spheroid growth was monitored from the first day on after transfer. Day 1 in the table refers to the respective transfer date.

The best results for HCT-116 cells were observed using pellet culture in 96-well plates coated with 1.5 % agarose. Spheroid formation of the colon carcinoma cell line HCT-116 occurred within 24 hours, using 3000 or 5000 cells per well, respectively. Initially loose cell aggregates were formed, which solidified within two days if the plate was free of concussions. The pellet culture was a highly reproducible and fast method for HCT-116 cell line to obtain tight spheroids, which were uniform in size and shape. On the other hand, loose cell aggregates were obtained performing this method using CHO-K1 and PC-3 cells in concentrations between 250 up to 10000 cells per well.

Furthermore, for the prostate carcinoma cell line PC-3 and the chinese hamster ovary cell line CHO-K1 the hanging drop method was also not suitable. There was no spheroid formation obtained within five days using cell concentrations between 250 and 1000 cells per drop. Subsequently, three more methods, including methylcellulose as stabilizing scaffold, pellet culture including centrifugation, as well as commercially available ultra-low-attachment (ULA) plates were tested.

First of all, different concentrations of methylcellulose ranging from 5% to 20% were added to the cell suspension, prior to drop pipetting. However, no tense spheroids were achieved, especially at higher concentrations of methylcellulose it seemed to exacerbate the aggregate building.

Secondly, even centrifugation of the 96-well plates after cell seeding did not increase the stability of the spheroids. However, the obtained aggregates kept in shape within the well, but harvesting was not possible, because of their missing stability.

To obtain tight aggregates of CHO-K1 and PC-3 cell line, cell concentrations between 250 to 10000 cell were seeded in commercially available ULA plates. Loose aggregate formation was observed within one hour under the microscope. Even after six days, the formed platelets were not able to withstand the transfer using a pipette. Therefore, they were not beneficial for further experiments.

In conclusion, HT-29 and HCT-116 form highly stable and reproducible spheroids within at least 4 days, whereas PC-3 and CHO-K1 cells only build up loose aggregates, which are not stable due to mechanical strain, like pipetting and transferring.

	Pellet Culture- ULA plates	Hanging- Drop- Method	Hanging- Drop- Method +MC	Pellet Culture- Agarose coated plate	Pellet Culture Gelatine coated plate	Pellet Culture Centrifugation
HT-29	+	+	-	+	-	-
HCT-116	+	-	~	+	-	-
PC-3	~	-	-	-	-	-
CHO-K1	~	-	-	-	-	-

Table 1: Results obtained through testing of established spheroid formation methods. + tight spheroid formation, ~ loose aggregate building, and – no aggregate.

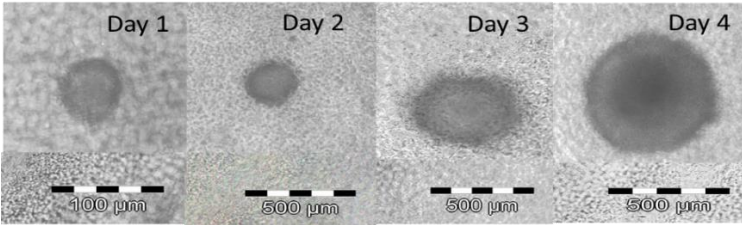
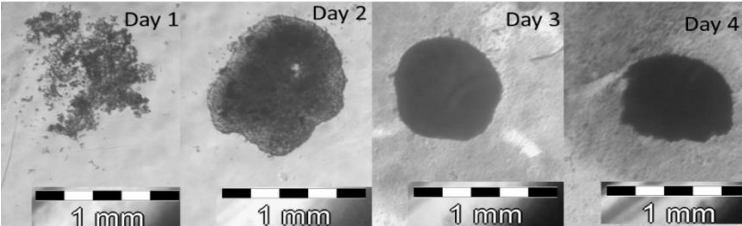
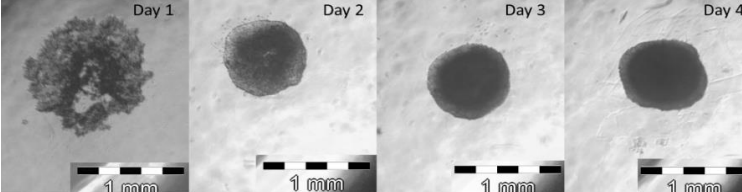
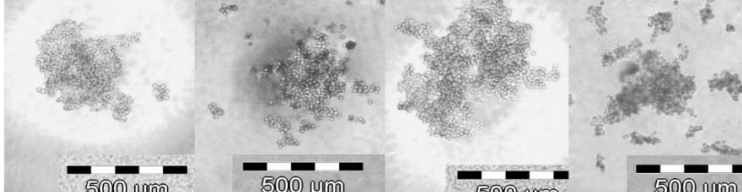
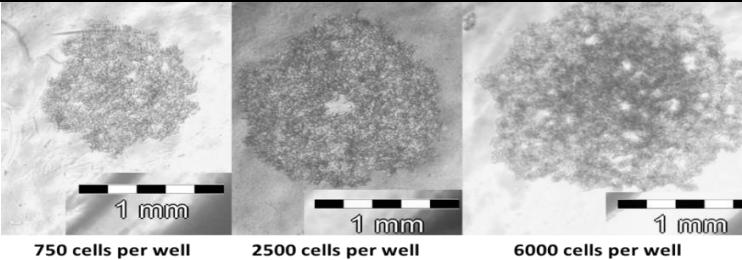
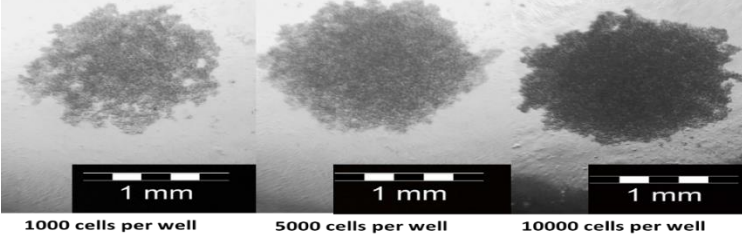
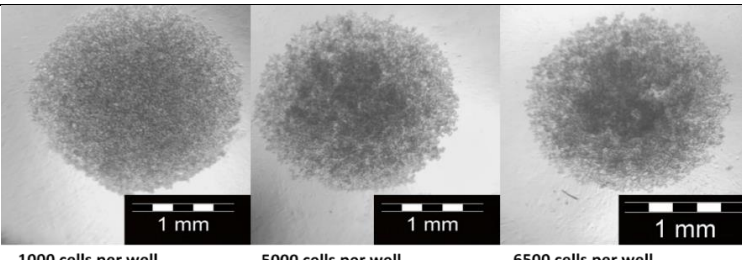
Cell line	Applied method	Spheroid formation	Spheroid morphology
HT-29	Hanging drop method		Tight cell aggregates
HCT-116	Pellet culture 3000 cells per well		Tight cell aggregates
HCT-116	Pellet culture 5000 cells per well		Tight cell aggregates
PC-3	Hanging drop	 250 cells per drop + 20% MC 500 cells per drop + 5% MC 500 cells per drop + 10% MC 500 cells per drop + 20% MC	No Spheroid formation
PC-3	Pellet culture agarose plates	 750 cells per well 2500 cells per well 6000 cells per well	Loose cell aggregate
PC-3	Pellet culture ULA-plates	 1000 cells per well 5000 cells per well 10000 cells per well	Loose cell aggregate
CHO-K1	Pellet culture ULA-plates	 1000 cells per well 5000 cells per well 6500 cells per well	Loose cell aggregate

Table 2: Formation of multicellular tumor spheroids. Spheroid formation for HT-29 and HCT-116 was obtained performing established procedures like hanging drop and pellet culture method, whereas PC-3 and CHO-K1 cells barely form spheroids.

4.2. Spheroid growth behavior

The growth behavior of the dense multicellular tumor spheroids derived from HT-29 and HCT-116 was observed under the microscope. Diameter of the spheroids was measured with an Olympus IMT-2 microscope, equipped with a XC50 camera, using Cell[^]B software.

Once HT-29 multicellular tumor spheroids were transferred to 96-well plates, their growth (Figure 13) was monitored every 2-3 days under the microscope. The increase in size was approximately linear, starting with a size of 200 μm on the first day after transfer, up to 930 μm on day 21.

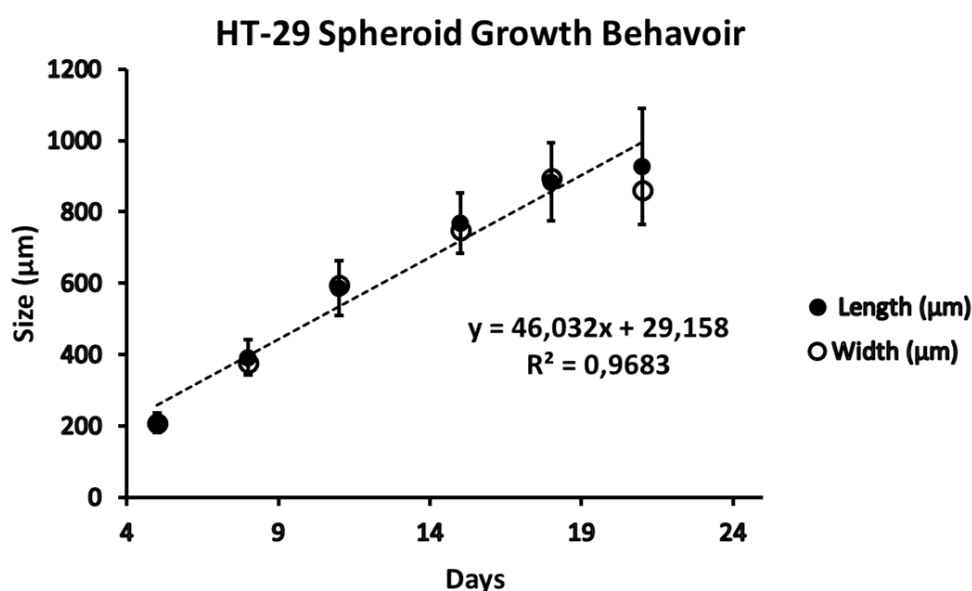


Figure 13: **Growth behavior of HT-29 multicellular tumor spheroids (mean $\pm$ SD).** Spheroids were transferred to round-bottom 96-well plates on day 4. Growth measurement started one day after transfer.

As even the tense spheroids of HT-29 cells are instable at a size bigger than 1 mm, embedding in a stabilizing scaffold, such as collagen, gelatine, or Matrigel was tested.

Spheroid embedding in collagen led to an increase in size of average 350 μm within the first day. A few of the cell aggregates could be grown to a size of nearly 1500 μm , before they started spreading within the collagen matrix.

Matrigel embedding did not have an advantage on the growth behavior of HT-29 multicellular tumor spheroids (MTS), because of the missing matrix stability.

Moreover, 10 % gelatine solution was not suitable for use as a matrix, because the dissolved gelatine powder in growth medium offered a breeding ground for contaminations.

Growth measurement of HCT-116 cell aggregates was carried out from the third day on, in order to not disturbing spheroid formation.

Seeding 3000 cells per well, produced spheroids in the size of approximately 700 μm (Figure 14), which maintained their size for one week, before they started to disaggregate. Whereas with seeding 5000 cells per well, spheroids in the size of 700 μm could be obtained, which grew to 800 μm on day 8.

Due to the moderate growth of HCT-116 spheroids, a cell proliferation assay was performed using MTT solution. The assay revealed that the spheroids of all tested sizes and cell lines were still proliferating (Figure 15).

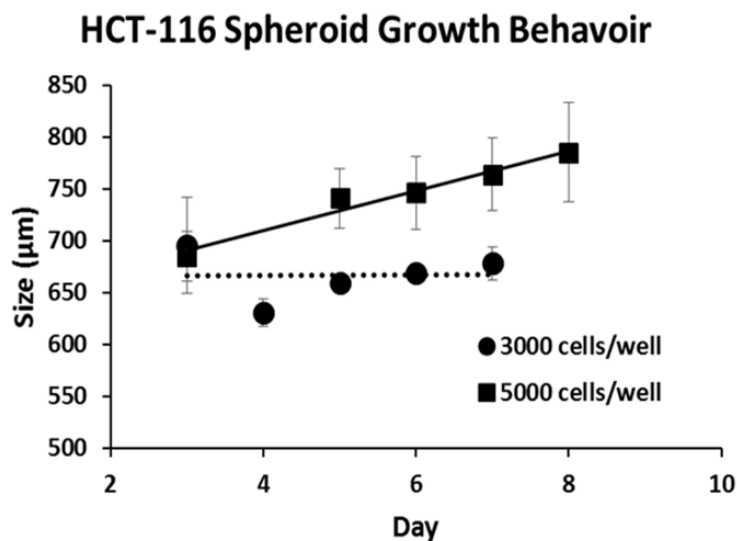


Figure 14: Growth behavior of HCT-116 multicellular tumor spheroids cultivated in agarose coated 96-well plates (Mean $\pm$ SD). Growth measurement was performed from the third day on.

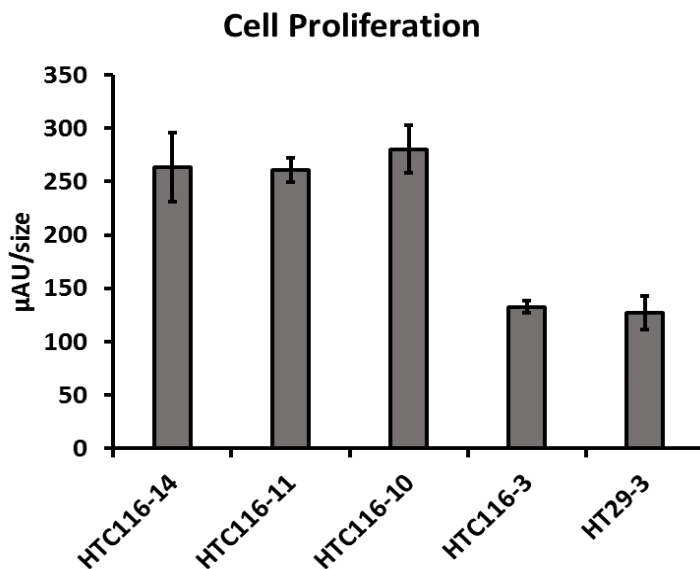


Figure 15: **Cell proliferation of HCT-116 and HT-29 spheroids (Mean $\pm$ SD).** HCT-116 spheroids were measured at day 3,10,11 and 14. HT-29 spheroids were measured at day 3.

Cell count determination

For determination of the cells containing in spheroids of defined size, multicellular tumor spheroids from different sizes were disaggregated using trypsin-EDTA or accutase®, respectively and cells were counted using a Neubauer hemocytometer.

As shown in figure 16, doubling of the diameter of HT-29 spheroids led to a triplication of the cells counted. In comparison to HCT-116 spheroids, an increase in size of 200 μ m led to a triplication of the cell count.

For accumulation experiments, cell aggregates in the size between 500 and 600 μ m were used, which corresponds to approximately 8000 cells for HT-29 cell line and 13000 cells for HCT-116 cell line.

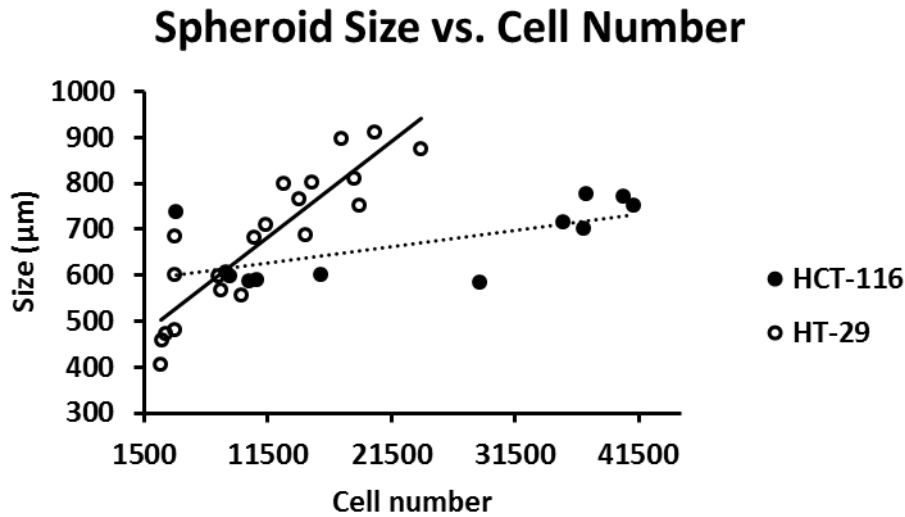


Figure 16: **Diagram shows the spheroid size in comparison to the cell number.** HCT-116 spheroids in the size of approximately 750 μm show a twice as high cell number compared to HT-29 spheroids from equal dimension.

4.3. Visualization of necrotic regions within spheroids

For visualization of necrotic and proliferating regions within HT-29 and HCT-116 multicellular tumor spheroids, calcein AM was used for staining of the living cells and PI for imaging of the dead cells. As shown in figure 17 a necrotic core is formed within the spheroid for both cell lines.

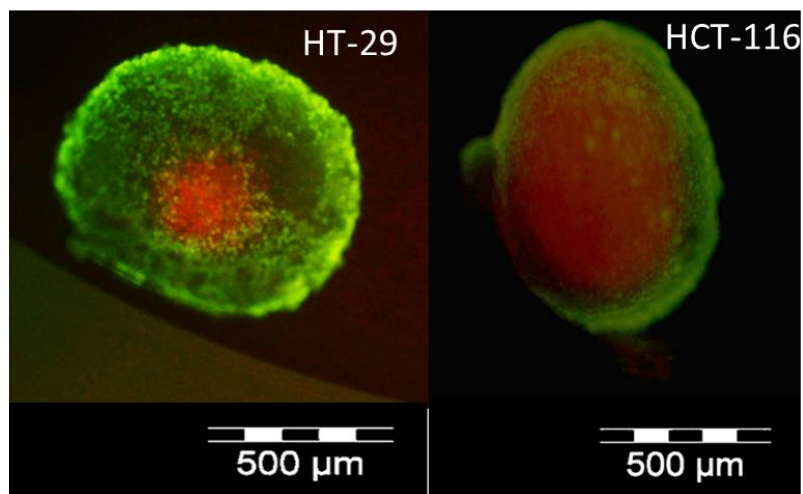


Figure 17: **Live-dead staining of HT-29 and HCT-116 spheroids using calcein AM and PI.** Live cells appear as green, while necrotic cells appear as red.

Subsequently, the size of the necrotic core was measured and compared with the spheroid size (Figure 18). For HT-29 cell line necrotic regions initially occurred in spheroids in the size of approximately 500 μm . The size of the necrotic core increased simultaneously with spheroid growth. Considering spheroids bigger 1 mm, the whole aggregate, apart from the outermost layer, consisted of necrotic cells.

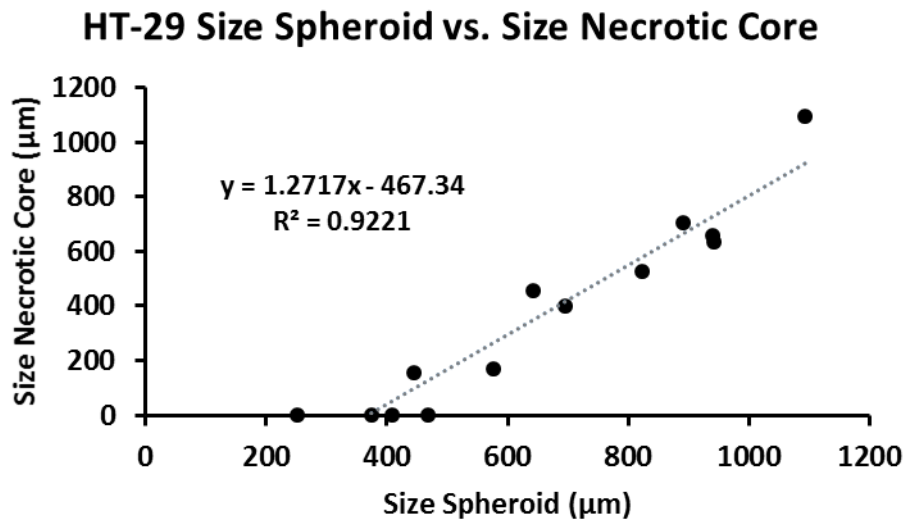


Figure 18: **Size of the necrotic regions within the HT-29 spheroids compared to their size.** Necrotic regions were observed in spheroids bigger than 500 μm .

Compared to HT-29 spheroids, the sizes of the necrotic regions within HCT-116 were approximately comparable (Figure 19). Due to different spheroid production processes, no spheroids smaller than 500 μm could be obtained. Live-dead staining of all the aggregates showed necrotic cores.

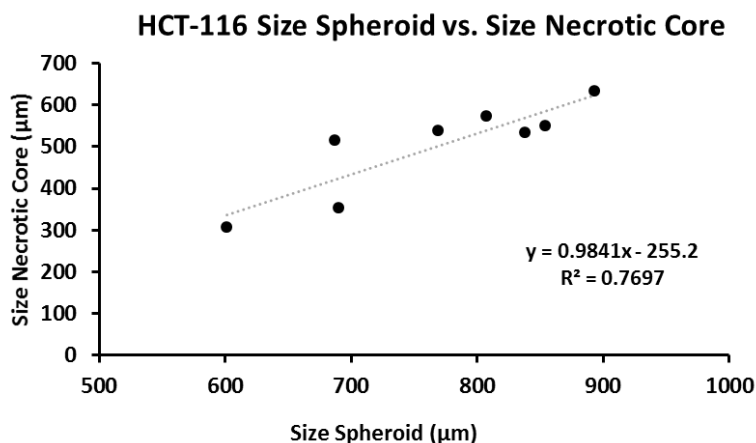


Figure 19: **Size of the necrotic regions within HCT-116 multicellular tumor spheroids compared to their size.** Necrotic regions were observed in all stained spheroids.

4.4. PET-tracer accumulation experiments

For radiotracer accumulation experiments, multicellular tumor spheroids as well as 2D cell monolayers were incubated at 37°C and 4°C with [^{18}F]FDG, [^{18}F]FMISO, [^{18}F]FE@SUPPY and [^{18}F]NaF. Whereas [^{18}F]FDG and [^{18}F]FMISO accumulation is correlating with tumor metabolism and hypoxia, [^{18}F]FE@SUPPY accumulation is indicating an A3 receptor binding and [^{18}F]NaF serves as negative control. For comparison of the PET-tracer accumulation, the percentage of dose accumulated in the cells was analysed.

In both cell lines and in 2D- and 3D-cell culture, [^{18}F]FDG was accumulated the most, whereas [^{18}F]FMISO was only minor and [^{18}F]NaF was not accumulated.

The results show, that the accumulation of [^{18}F]FDG is highly temperature-dependent, as incubation at 37°C or 4°C, had significant influence on the accumulated dose. In case of [^{18}F]FDG, down regulation of temperature led to an around 8-fold decreased accumulation, which is mainly attributed to the down-regulation of active transportation as previously described in literature.⁽⁷⁹⁾ Also the accumulation of [^{18}F]FE@SUPPY at 4°C was distinctly lower than at 37°C. Whereas [^{18}F]NaF accumulation was not significantly changed in HT-29 and HCT-116 spheroids in 2D cell monolayers as well as spheroids. Moreover, incubation at 4°C increased the accumulation of [^{18}F]FMISO except from HCT-116 spheroids.

Considering the radiotracer accumulation in multicellular tumor spheroids (Figure 20), the percentage of [^{18}F]FDG accumulated dose in HT-29 cell aggregates was twice as high as in HCT-116 spheroids for both temperatures. For starved HT-29 spheroids the [^{18}F]FDG accumulation was fivefold lower, compared to non-starved cells.

The [^{18}F]NaF accumulation at 37°C was approximately twice as high, whereas eight fold higher at 4°C in HCT-116 spheroids compared to HT-29 spheroids.

For [^{18}F]FMISO, the accumulation at 37°C in HCT-116 aggregates was slightly higher than in HT-29, whereas at 4°C HT-116 spheroids accumulated nearly twice as much as at 37°C.

Although [^{18}F]FE@SUPPY accumulation experiments were performed with relatively low amounts of activity (100 kBq/mL), the highest percentage of accumulated dose was observed for this PET-tracer, indicating either unspecific binding or a high A3 receptor expression in HT-29 spheroids.

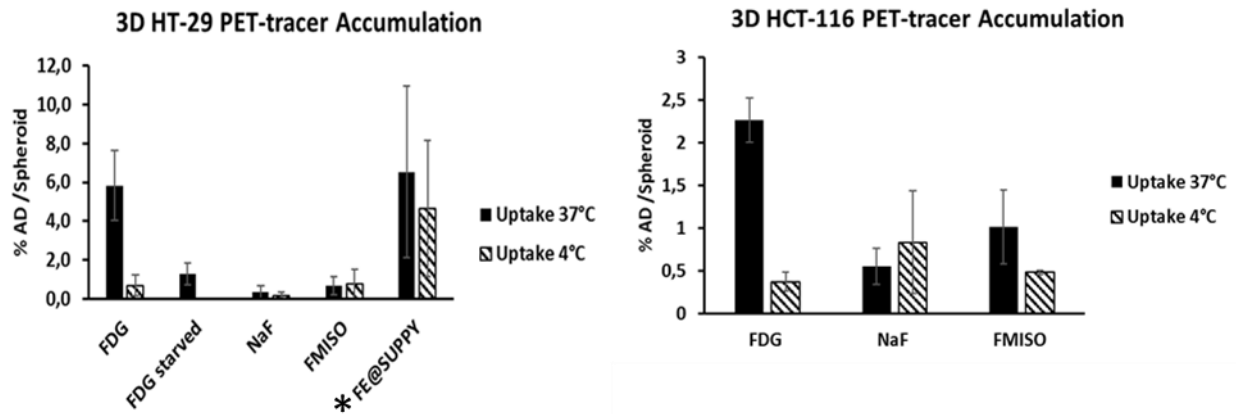


Figure 20: Accumulation of common radiotracers in multicellular tumor spheroids ($n=3$, mean $\pm$ SD). Spheroids were incubated with a 6 MBq/mL radiotracer solution for 50 min at 37°C and 4°C. Results were obtained by measurement in a γ -counter after a threefold medium wash. * [^{18}F]FE@SUPPY incubation was performed with a 100 kBq/mL solution.

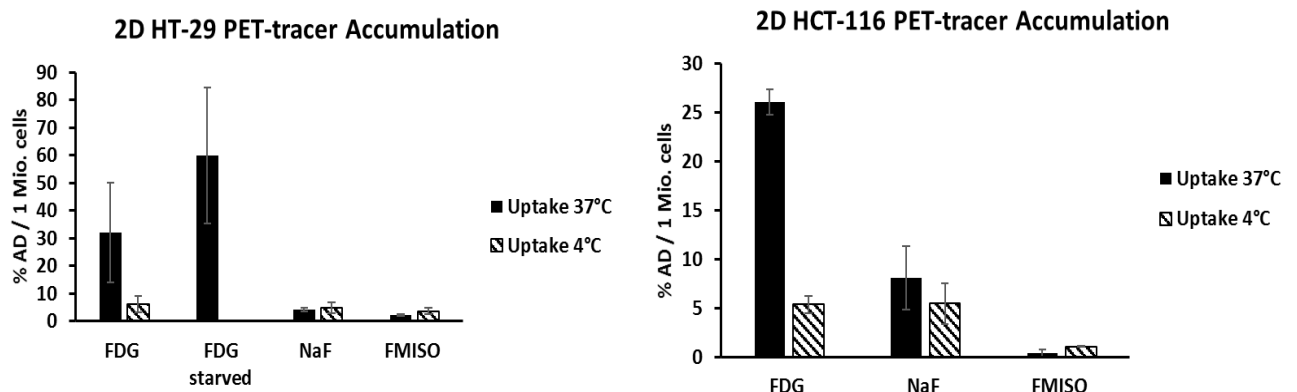


Figure 21: Accumulation of PET-tracers in 2D monolayers of HT-29 and HCT-116 tumor cell line ($n=3$, mean $\pm$ SD) Cells were incubated with a 6 MBq/mL solution of the respective radiotracer for 50 min at 37°C and 4°C. After sufficient wash cells were transferred to an Eppendorf tube and measured in a γ -counter.

In 2D cell monolayers the [^{18}F]FDG accumulation in HCT-116 and HT-29 cell line was comparable (Figure 21). Considering the decreased [^{18}F]FDG accumulation of starved spheroids, the accumulation in starved 2D cell monolayers was approximately twofold higher than in cells, incubated with supplemented growth medium.

While [^{18}F]NaF accumulation in HT-29 was slightly comparable for 37°C and 4°C, in HCT-116 cells twice as much radiotracer was accumulated at 37°C, whereas the accumulation at 4°C was comparable to HT-29 2D cell monolayers. Since, NaF is known for its high affinity to calcium, an intracellular calcium fluorescence assay was performed using HT-

29 and HCT-116 2D cell monolayers, in order to explain the slight differences of [^{18}F]NaF accumulation between both cell lines. The assay revealed that the intracellular calcium concentration in HT-29 cells was by one third higher compared to HCT-116 cells (Figure 22).

The accumulation of the hypoxia tracer [^{18}F]FMISO in HT-29 tumor cells was twice as high at 37°C, respectively threefold higher at 4°C compared with the HCT-116 cell line.

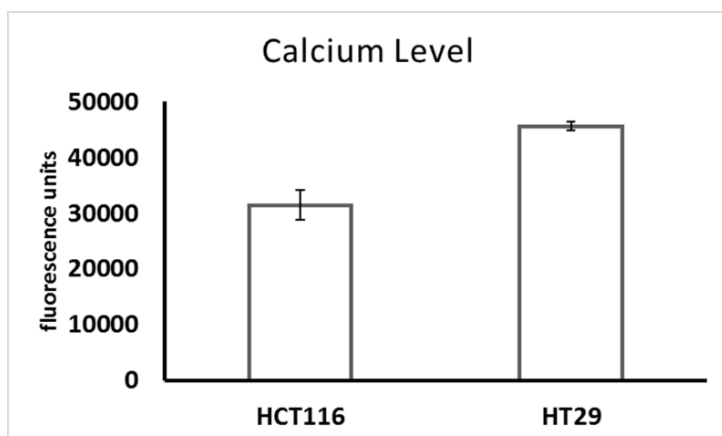


Figure 22: Comparison of the intracellular calcium concentrations in HT-29 and HCT-116 cell monolayers. (n=6, mean $\pm$ SD). Intracellular calcium concentration was determined using a Fluo-8 based fluorescence assay.

For the determination of [^{18}F]FE@SUPPY accumulation in a 2D cell culture model, HT-29 and CHO-K1 cell monolayers were incubated with a 100 kBq/mL solution of the A₃ receptor radioligand.

With regard to both incubation temperatures, the percentage of accumulated [^{18}F]FE@SUPPY in HT-29 2D cell monolayers was approximately twice as high, compared with CHO-K1 cells.

The Chinese hamster ovary cell line CHO-K1, which is not expressing human A₃ receptors, showed a comparatively small accumulation of [^{18}F]FE@SUPPY, calculated as percentage of applied dose per 10000 cells (0.3 % $\pm$ 0.1 % at 37°C, 0.2 % $\pm$ 0.1 % at 4°C).

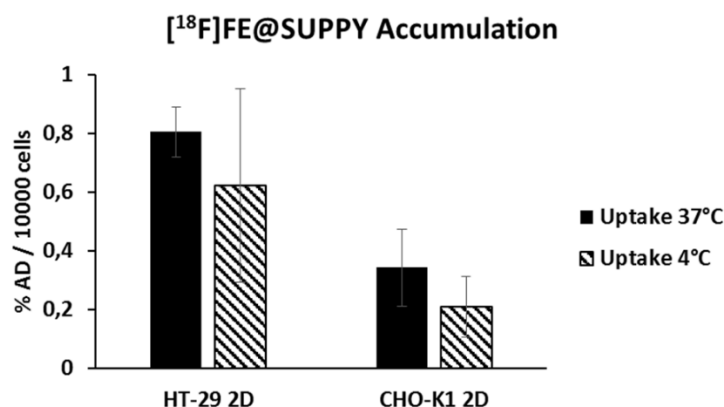


Figure 23: Accumulation of $[^{18}\text{F}]\text{FE@SUPPY}$ in colorectal carcinoma cell line HT-29 and CHO-K1 2D cell culture ($n=3$; mean $\pm$ SD). 2D cell layers were incubated with 3 mL of a 100 kBq/mL radiotracer solution for 50 min. Calculated for 10000 cells, HT-29 cell monolayers showed higher accumulation at 37°C and 4°C.

4.5. Internalization assays

Internalization of $[^{18}\text{F}]\text{FDG}$

Since the performed accumulation assays provided no information on the intracellular $[^{18}\text{F}]\text{FDG}$ uptake within HT-29 cell monolayers, internalization assays under different conditions were carried out.

In tumor cells treated with serum free RPMI medium, the smallest proportion of internalized $[^{18}\text{F}]\text{FDG}$ was determined ($0.49\% \pm 0.25\%$), as shown in Figure 24. Incubating cells with supplemented growth medium increased the internalized fraction by approximately 50 % compared to the cell incubated with medium, containing no FBS.

The effect of cell starving using DPBS is clearly recognizable, since a nine fold higher intracellular uptake was observed for starved cells, which were incubated for 1 h. Whereas in starved cells incubated with $[^{18}\text{F}]\text{FDG}$ for 2 h, a twenty-four times higher internalization, compared to non-starved cells, was observed.

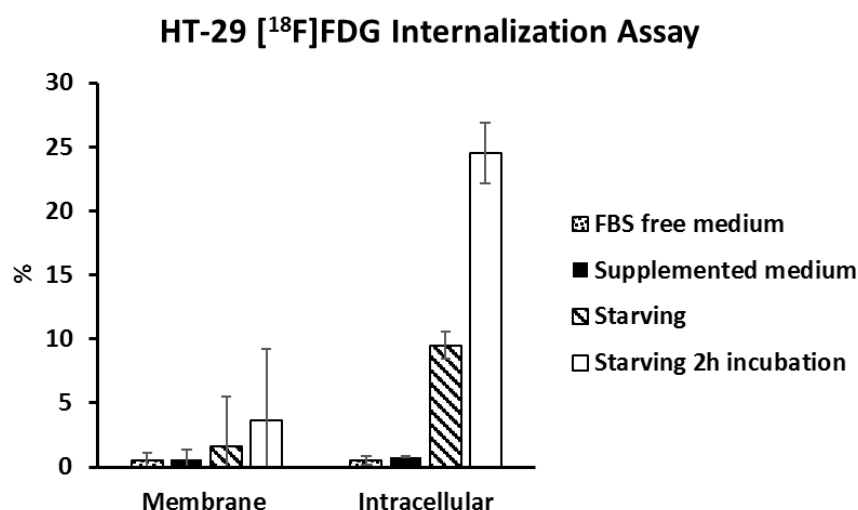


Figure 24: **Internalization of a 100 kBq/mL solution of [¹⁸F]FDG within HT-29 2D cell monolayers ($n=2$, mean $\pm$ SD).** Two hours of incubation using starved cells showed the highest internalisation rate.

Internalization of [¹²⁵I]I-AB-MECA

In order to determine the membrane binding and internalization of the A₃ receptor model ligand [¹²⁵I]I-AB-MECA in CHO-hA3R cells, HT-29 and PC-3 cells were incubated with a 2.4 kBq/mL radiotracer solution. The CHO-K1 cell line, which demonstrably is not expressing a human A₃ receptor, was serving as a negative control.

Compared to the CHO-K1 cell line, significantly higher membrane binding as well as internalized fraction was found in the CHO-hA3R cell line (Figure 25). Moreover, it was examined, that doubling the incubation time did not significantly increased radioligand binding or internalization.

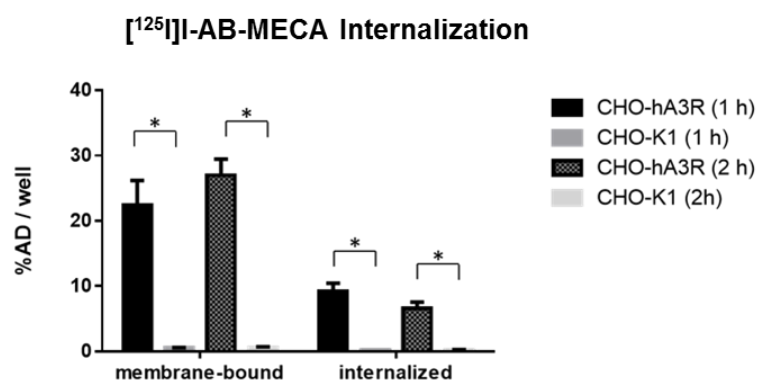


Figure 25: **Internalization assay performed with a 2.4 kBq/mL solution of the A₃R model ligand [¹²⁵I]I-AB-MECA on CHO-hA3R and CHO-K1 cell line.** Cells were incubated for 1 h and 2 h, respectively. The CHO-hA3R cell line shows a significantly higher membrane binding and internalization of the radioligand.

In the following experiments solutions of FE@SUPPY and I-AB-MECA, dissolved in DMSO, were used for receptor blocking. Therefore, dimethylsulfoxide was used as vehicle control (VC) to consider potential effects of DMSO on radioligand binding. The comparison of untreated CHO-hA3R cell monolayers with cells, treated with 0,1 % DMSO shows a significant decrease in the membrane bound fraction of approximately 14% as well as a significant increase in the internalized fraction (Figure 26). No such effects were observed for the negative control cell line (CHO-K1).

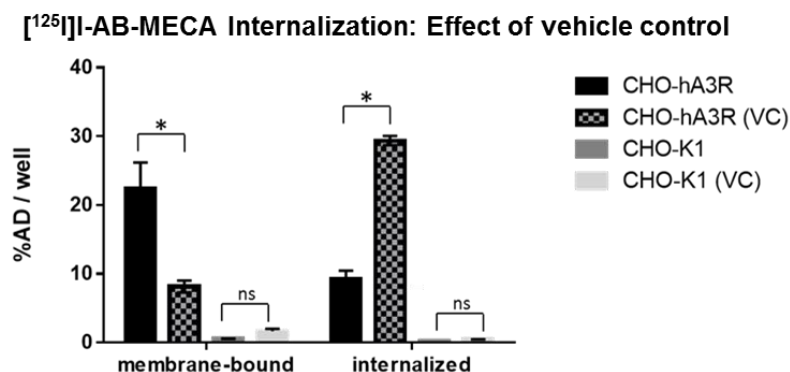


Figure 26: Determination of the effect of DMSO on membrane binding and internalization. CHO-hA3R and CHO-K1 cell monolayers were incubated with a 2.4 kBq/mL solution of the radiotracer. 0.1 % DMSO was added to one group of each cell line, serving as vehicle control. In CHO-hA3R cell monolayers the addition of DMSO had a significant effect on the membrane binding and internalization of the A₃ model ligand.

FE@SUPPY, added as a blocking agent, significantly decreased radioligand binding and internalisation of [125 I]-AB-MECA, suggesting specific binding of the radiotracer on the A₃ receptor (figure 27).

[125 I]-AB-MECA Internalization Blocking FE@SUPPY

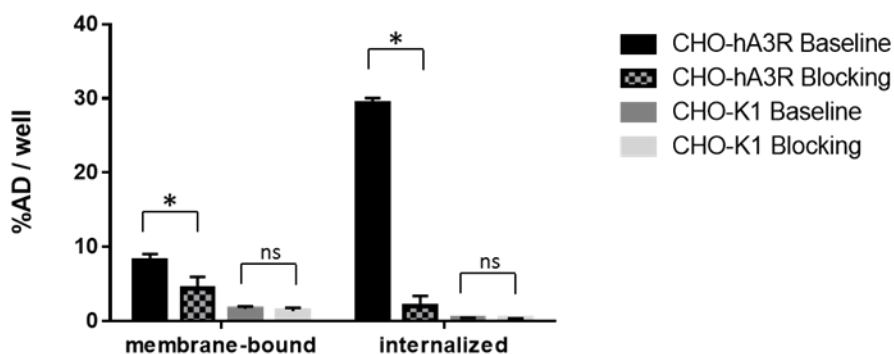


Figure 27: **Comparison of [125 I]-AB-MECA Internalization in CHO-hA3R and CHO-K1 cell line.** Blocking was carried out using 1 μ L of a 1mM solution of FE@SUPPY, dissolved in DMSO (final concentration 1 μ M, concentration of DMSO 0.1 %). For baseline measurements, 0.1 % DMSO was added, acting as vehicle control. The addition of FE@SUPPY showed a significant effect on the membrane binding and internalization of [125 I]-AB-MECA in CHO-hA3R cells.

In a repeated internalization experiment, performed with CHO-hA3R cell monolayers, shown in figure 28, the reduction of the membrane bound fraction by the addition of FE@SUPPY, could be confirmed. Moreover, a decrease in the membrane bound fraction was achieved by adding I-AB-MECA (final concentration 1 μ M, 0.1 % DMSO). However, in the repetition, the internalized fraction of [125 I]-I-AB-MECA was lower than 1%. Consequently, the internalized fraction was not significantly reduced by the addition of blocking agents.

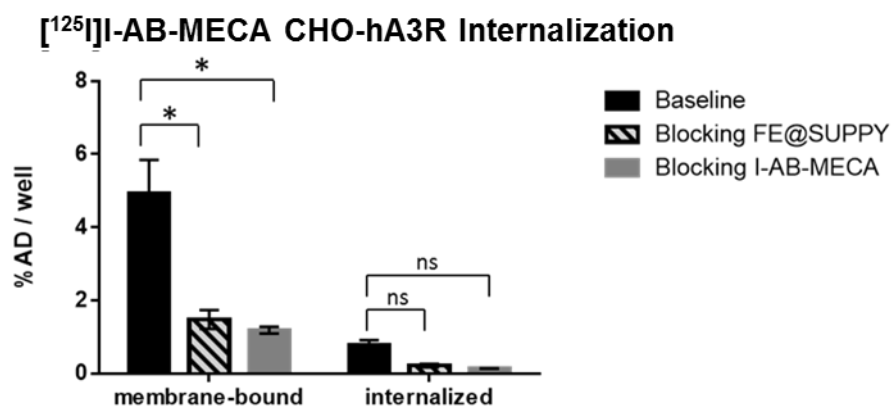


Figure 28: **Internalization of [¹²⁵I]I-AB-MECA in CHO-hA3R cell line.** Blocking was performed using 1 μ M FE@SUPPY and I-AB-MECA respectively, dissolved in DMSO. Baseline measurements were carried out using 0.1 % DMSO as vehicle control. FE@SUPPY as well as I-AB-MECA significantly decreased the membrane bound fraction.

As expected, in CHO-K1 cell monolayers neither the membrane bound fraction nor the internalized fraction could be significantly reduced by the addition of FE@SUPPY or I-AB-MECA, suggesting non-specific binding of the radioligand in this non-target cell line (Figure 27 and 29).

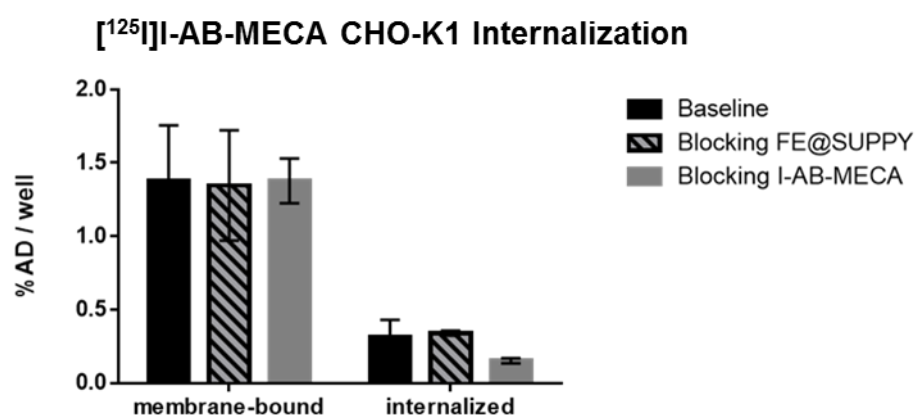


Figure 29: **Internalization assay performed with CHO-K1 cell monolayers.** Blocking was performed by adding 1 μ M solutions of FE@SUPPY and I-AB-MECA. DMSO served as vehicle control in baseline measurements. The addition of the blocking agents barely influenced the membrane bound fraction and the internalized fraction.

In both evaluated tumor cell lines (HT-29 and PC-3), [¹²⁵I]I-AB-MECA binding and internalization was lower than in the positive control cell line (CHO-hA3R). Similar to the observations made in internalization experiments using CHO-K1 cell monolayers, in PC-3 cells neither the membrane binding nor the internalization of the radioligand could be

significantly blocked by the addition of FE@SUPPY or I-AB-MECA, respectively (Figure 30).

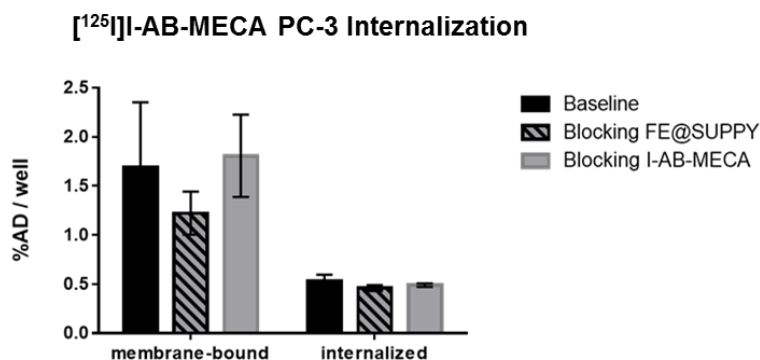


Figure 30: **Effects of receptor blocking by the addition of FE@SUPPY and I-AB-MECA on the membrane binding and internalization of [¹²⁵I]I-AB-MECA in PC-3 cells.** 0.1 % DMSO served as vehicle control in baseline measurements. No significant alterations were observed when adding the blocking agents.

While in internalization experiments, using CHO-hA3R cell monolayers, an addition of blocking agents resulted in a decrease of the membrane binding, figure 31 shows, that the addition of FE@SUPPY significantly enhanced the membrane bound fraction in HT-29 cells, without influencing the internalized fraction. Whereas I-AB-MECA showed no significant effect on the membrane bound fraction as well as on the internalized fraction.

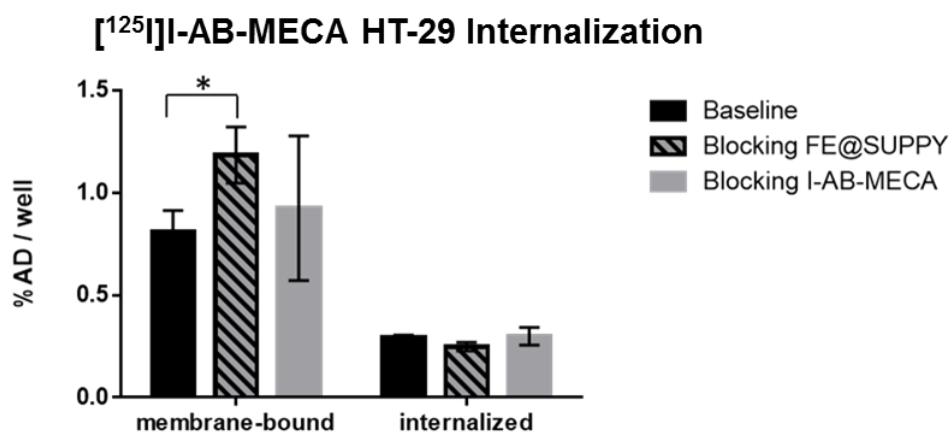


Figure 31: **[¹²⁵I]I-AB-MECA Internalization in HT-29 cell monolayers.** Receptor blocking was carried out using 1 μ M I-AB-MECA or FE@SUPPY, respectively. Cells for the baseline measurement were treated with 0.1 % DMSO. Addition of FE@SUPPY enhanced the membrane binding of [¹²⁵I]I-AB-MECA.

Method evaluation of [^{18}F]FE@SUPPLY internalization

Compared to the assays performed for [^{125}I]I-AB-MECA and [^{18}F]FDG internalization, an additional washing step was carried out using DPBS containing 0.1 % Tween-20. The tested methods distinguish themselves by washing steps in varying order as well as using 1 M NaOH or RIPA-buffer, respectively, for cell lysis. While 1 M NaOH was used for cell lysis in the first and second method, RIPA-buffer was used in Method 3 and 4, with the advantage that a cell pellet could be obtained through centrifugation, which provided information on the remaining percentage bound to the cell membrane after washing.

In Figure 32, all tested methods are compared, showing that cell washing with DPBS containing 0.1 % Tween-20 was efficient. The fractions obtained through washing with the supplemented DPBS before the acid glycine washing, are comparable with them in the first and third method, where washing with the emulsifier was performed after the acid wash. Especially for CHO-hA3R cell line approximately 29 % of radiotracer were found in the DPBS fraction, supplemented with Tween-20.

In fractions, where washing with non-supplemented DPBS was performed, in average 5 % of radiotracer were determined, indicating that cell washing without an emulsifier is insufficient for lipophilic radiotracers.

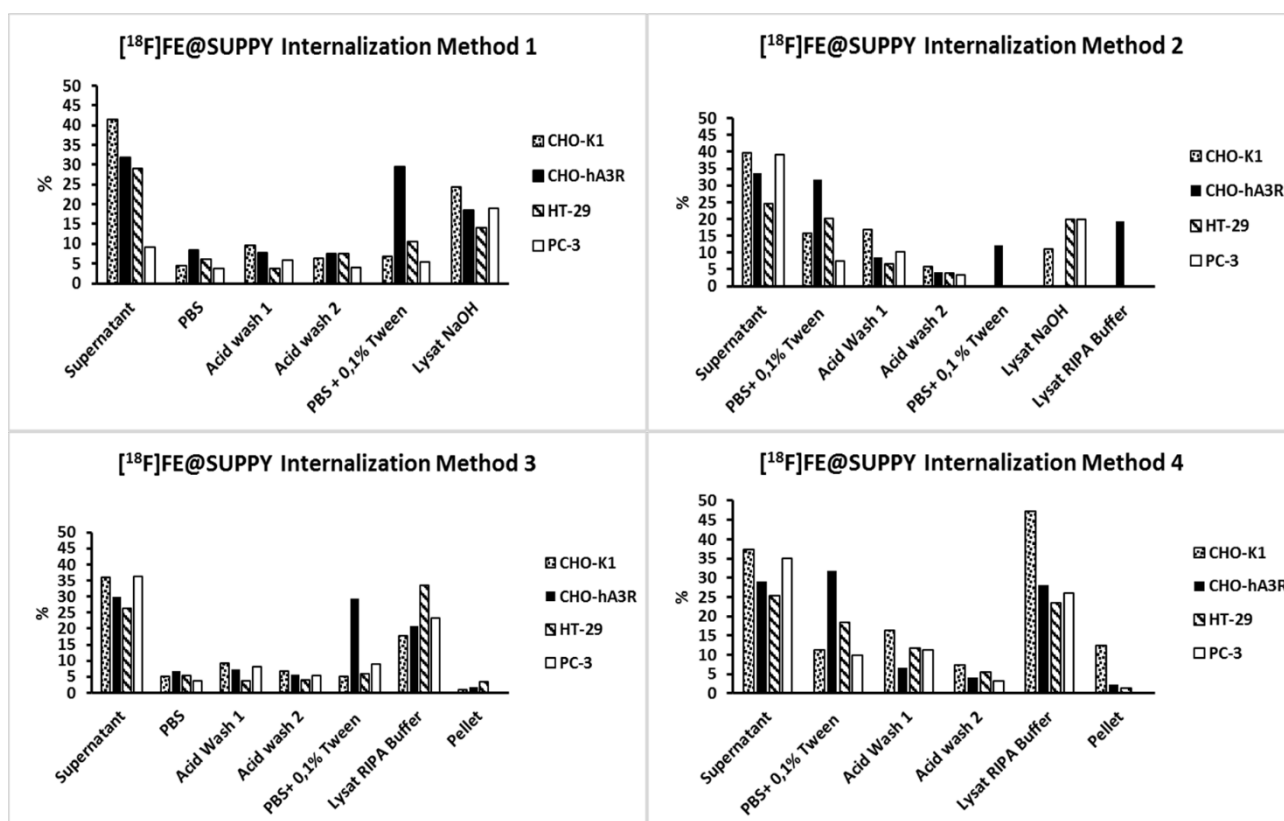


Figure 32: **Evaluation of $[^{18}\text{F}]\text{FE@SUPPY}$ internalization methods (n=1)** CHO-hA3R, CHO-K1, HT-29 and PC-3 cell monolayers were incubated with a 100 kBq/mL solution of $[^{18}\text{F}]\text{FE@SUPPY}$ for 1 h, cell washing was performed using DPBS, DPBS supplemented with 0.1 % Tween-20 and acid glycine buffer. Cell lysis was performed using RIPA-buffer

4.6. Real-time $[^{18}\text{F}]\text{FDG}$ accumulation experiments

For visualization of real time $[^{18}\text{F}]\text{FDG}$ accumulation kinetics the LigandTracer® yellow was used. In the frame of the experiment starved cells of HCT-116, HT-29 and PC-3 cell line, as well as different matrices were treated with solutions of 50 kBq, 100 kBq or 500 kBq $[^{18}\text{F}]\text{FDG}$.

In case of the human prostate carcinoma cell line an addition of a 50 kBq $[^{18}\text{F}]\text{FDG}$ solution was sufficient, to obtain an increase in the signal, shown in figure 33 as target-background curve. After an equilibrium was reached at 30 min, further addition of a 50 kBq $[^{18}\text{F}]\text{FDG}$ solution led to a linear increase in the curve for another 50 min, before the second equilibrium was reached. Changing the incubation medium was not followed by a decrease in the signal, which indicates that the accumulated $[^{18}\text{F}]\text{FDG}$ was trapped inside the cell. The obtained results were reproducible three times.

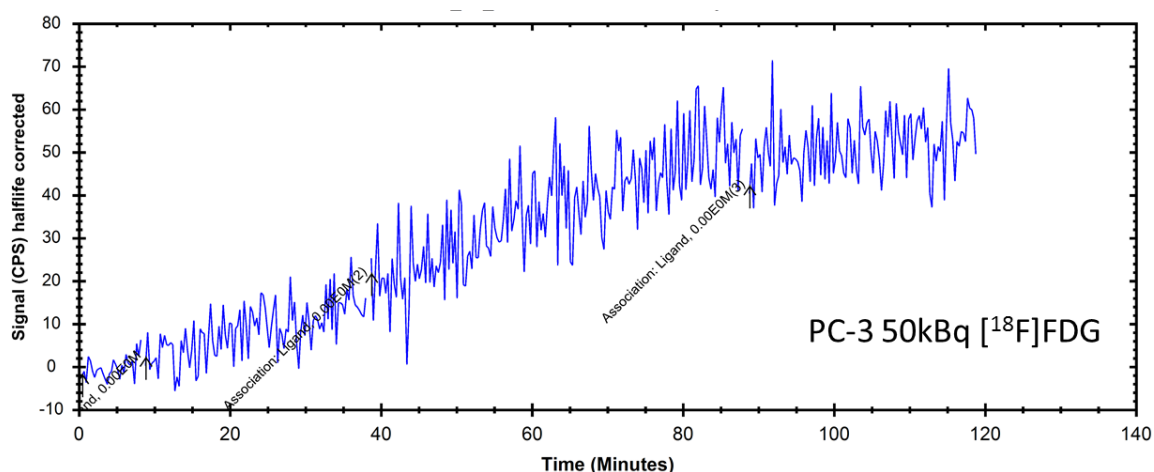


Figure 33: **Real-time accumulation curve of a 50 kBq $[^{18}\text{F}]$ FDG solution in PC-3 cell monolayers.** The curve shows the accumulated fraction.

While performing this experiment using HCT-116 cells neither with addition 50 kBq, 100 kBq nor 500 kBq $[^{18}\text{F}]$ FDG a significant accumulation within the cells was observed. As an example for this the obtained accumulation curve of a 100 kBq $[^{18}\text{F}]$ FDG solution is shown in figure 34.

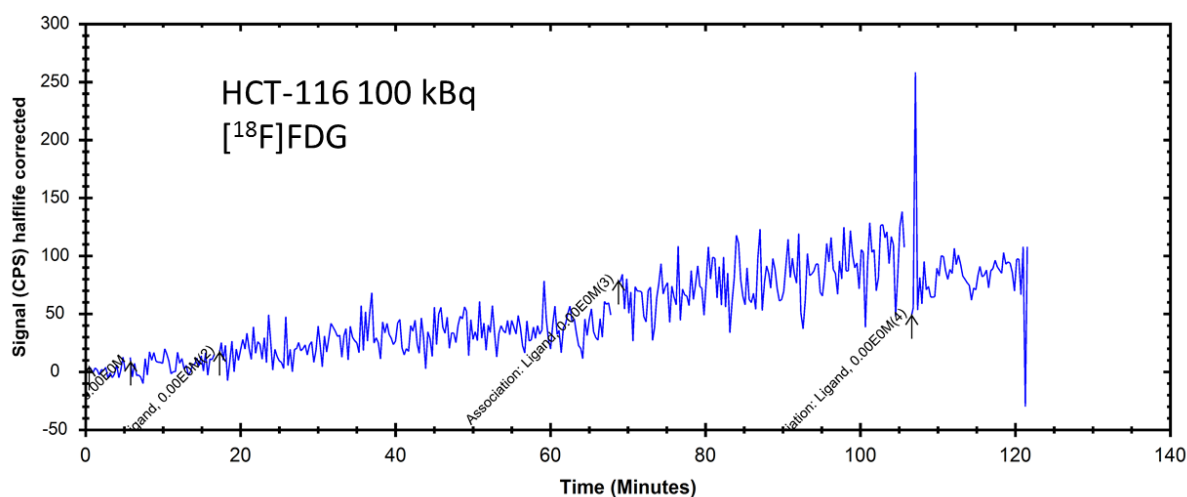


Figure 34: **Real-time accumulation of $[^{18}\text{F}]$ FDG in HCT-116 cells.** Cells were incubated with a 100 kBq solution of the radiotracer, but showed no significant accumulation.

In HT-29 cell line no accumulation was observed using incubation solutions containing 50 kBq or 100 kBq $[^{18}\text{F}]$ FDG, respectively. Using a solution of 500 kBq resulted in an increase in the curve, reaching an equilibrium at approximately 10 min after addition of $[^{18}\text{F}]$ FDG (Figure 35). The second equilibrium was reached 15 min after further addition of

radiotracer solution. DPBS exchange did not lead to a decrease in the signal. However, although the obtained result seemed promising, it was not reproducible.

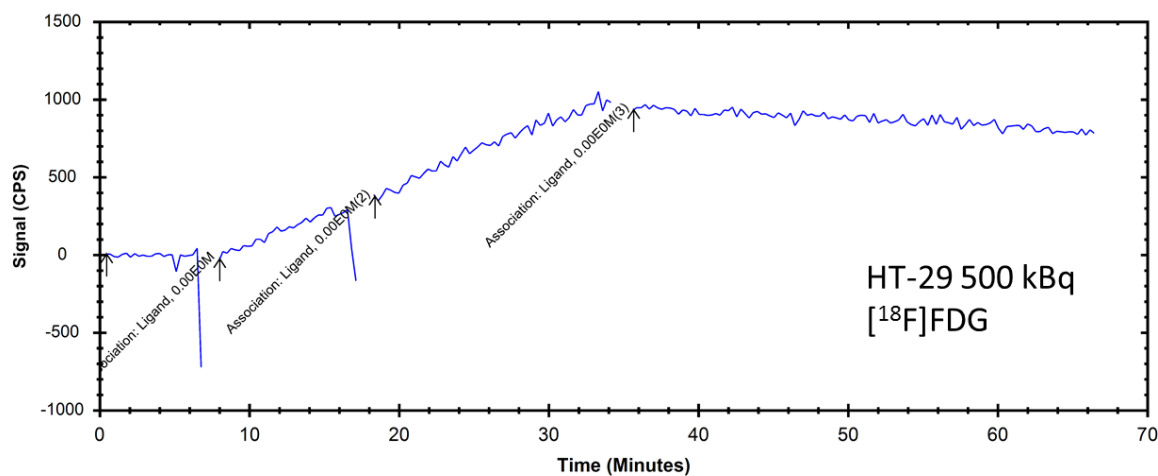


Figure 35: Real-time accumulation of $[^{18}\text{F}]\text{FDG}$ in HT-29 2D cell monolayers. Cells were incubated with 500 kBq $[^{18}\text{F}]\text{FDG}$. HT-29 cells showed an accumulation of the tracer, which was, however, not reproducible in further experiments.

In order to obtain real-time accumulation kinetics of multicellular tumor spheroids, an appropriate matrix for spheroid embedding needed to be found. Therefore accumulation of $[^{18}\text{F}]\text{FDG}$ within 1.5 % agarose solution, 10 % gelatine solution and Matrigel, respectively, was measured in the LigandTracer®.

The results of the tests revealed, that neither 1.5 % agarose solution, nor Matrigel could withstand the continuous rotation of the LigandTracer®.

Only 10 % gelatine solution remained at the bottom of the Petri dish, but had the disadvantage that the increase in $[^{18}\text{F}]\text{FDG}$ accumulation (Figure 36) was much higher, than observed within 2D cell monolayers.

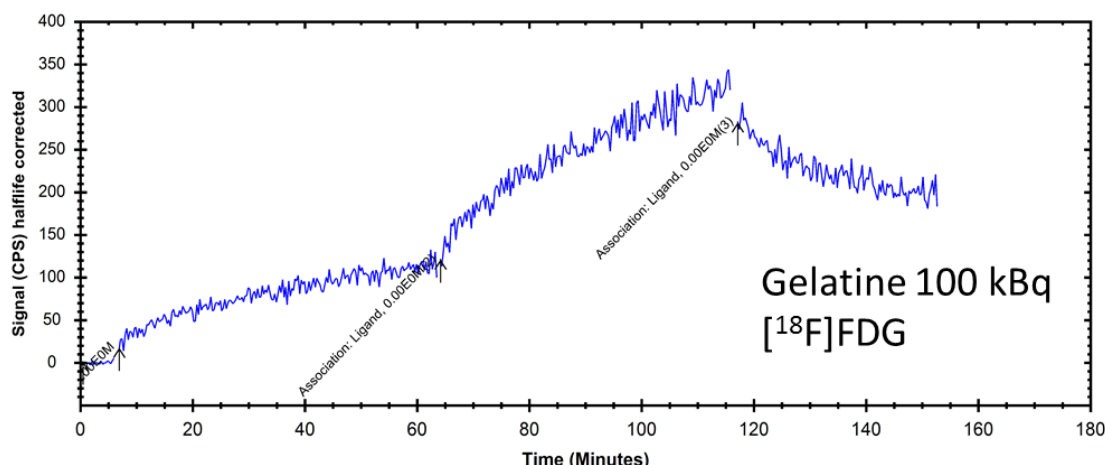


Figure 36: **$[^{18}\text{F}]\text{FDG}$ accumulation observed in a 10 % gelatine solution, using 100 kBq for incubation.** Gelatine could withstand the rotation of the LigandTracer®, however, with regard to the high accumulation of the radiotracer, it is not suitable for spheroid embedding.

4.7. Western blot

For the determination of Hif2 α and human A3 receptor expression, western blots were performed using lysates of spheroids with different size, namely 300, 500, and 800 μm as well as 2D cells, serving as negative control for Hif2 α expression.

Figure 37 shows the western blot for HT-29 cell line, using a monoclonal antibody against Hif2 α , which has a predicted molecular weight of 100 kDa. According to the PageRuler™ protein ladder (lane 5) there were no bands detected at 100 kDa. However unspecific protein bands in various intensity were detected below the 55 kDa mark. Thus, HT-29 spheroids of all sizes did not express HIF2 α .

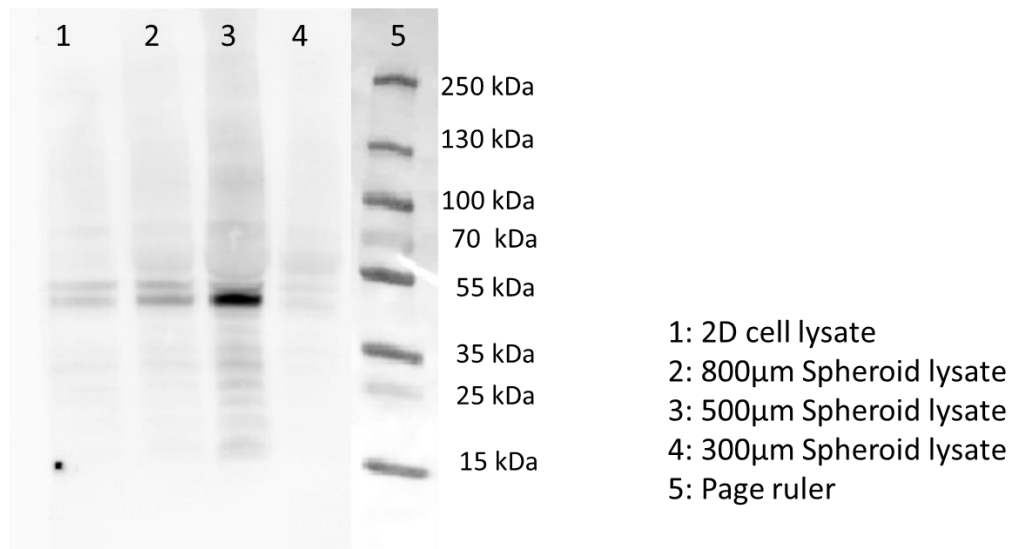


Figure 37: Detection of Hif2α (molecular weight 100 kDa) in HT-29 2D cell lysate and spheroid lysate. Antibody shows no expression of Hif2α in the cell lysates.

In order to confirm that the same amount of protein was loaded, the stripped membrane was incubated with an antibody against beta-actin, serving as loading control. Figure 38 shows the stripped Hif2α membrane, incubated with the beta-actin antibody. Beta-actin has a predicted molecular weight of 42 kDa and this band is clearly observable. The detected protein bands in lanes 1-3 did not varied in strength, indicating that the same amount of protein was loaded. Whereas the lysate, obtained from 300 µm spheroids (lane 4), showed a less intensive protein bond, indicating that the loaded protein concentration was lower compared to the other ones. Nevertheless, there is an enormous amount of unspecified protein lanes, subsequently the quality of the antibody is questionable.

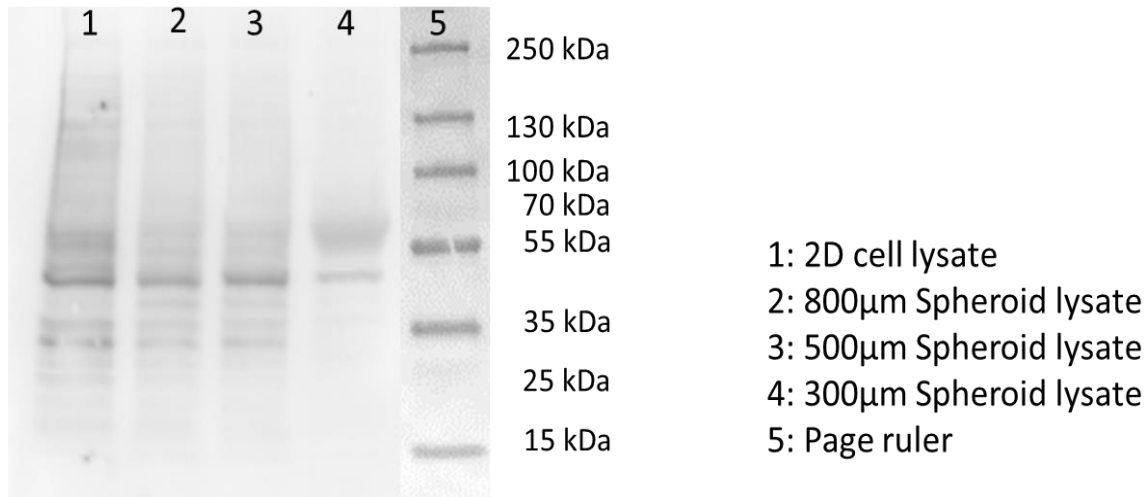


Figure 38: Detection of beta-actin (predicted molecular weight 42 kDa). Gel electrophoresis was performed under reducing conditions. Membrane was incubated with an antibody dilution of 1:500 for 1h at room temperature.

Western blot detection using a monoclonal antibody against Hif2 α did not show any protein expression in HCT-116 cell lysates. Especially in 2D cell lysate (lane 5) many unspecific protein bonds, as shown in figure 39, were detected.

In order to exclude unspecific-binding of the second antibody, the stripped membrane was again incubated with the goat-anti-mouse antibody conjugated to HRP. However, repeated detection did not show any protein bonds.

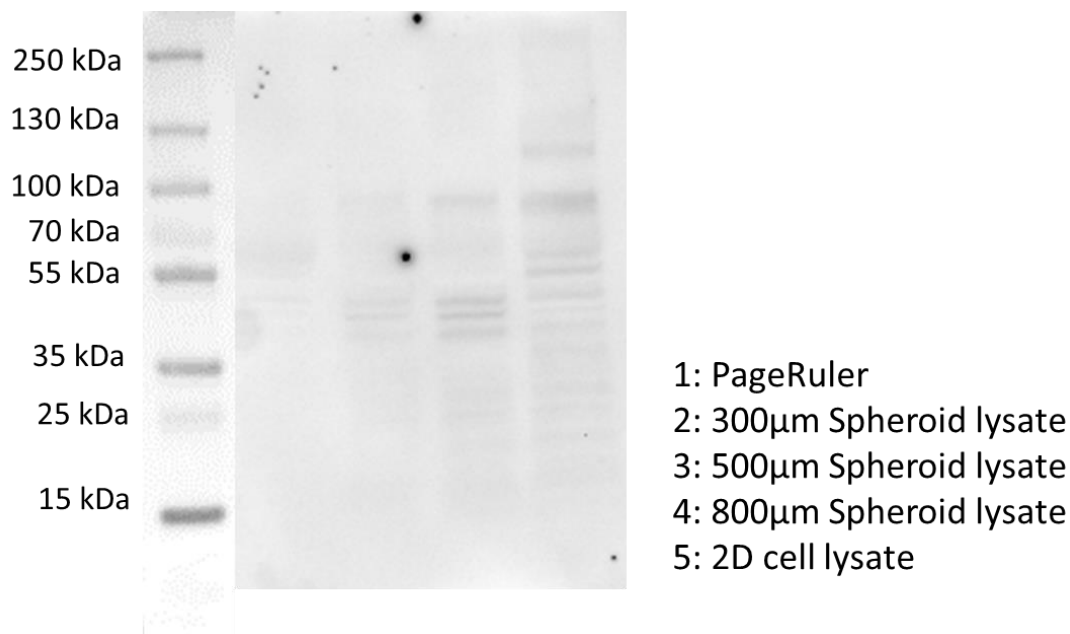


Figure 39: Detection of Hif2 α (molecular weight 100 kDa) in HCT-116 2D cell and spheroid lysate. Gel electrophoresis was performed under reducing conditions. Hif2 α antibody shows no expression of the transcription factor.

Figure 40 and 41, respectively, shows the expression of human A3 receptor (molecular weight 36 kDa) in HT-29 and HCT-116 2D cell and spheroid lysate using a polyclonal antibody.

For HT-29 cells, every sample expressed A3 receptors, which is visible due to the protein bands at around 35 kDa. The protein band in lane 4 varied in intensity, indicating either lower expression of the A3 receptor in multicellular tumor spheroids in the size of 300 μm (lane 4) or is a result of unequal membrane loading, which can not be excluded since the equality of protein loading was not examined. Whereas the expression of the A3 receptor in 2D cell lysate (lane 1) was comparable to the expression in 500 μm and 800 μm HT-29 cell aggregates (lanes 2 & 3). Close to the 55 kDa mark of the PageRuler™ an additional protein band was detected in lane 1, 2, and 3, suggesting that 2D cell lysate, as well as tumor spheroids in the size of 500 μm and 800 μm may express a modified version of the A3 receptor.

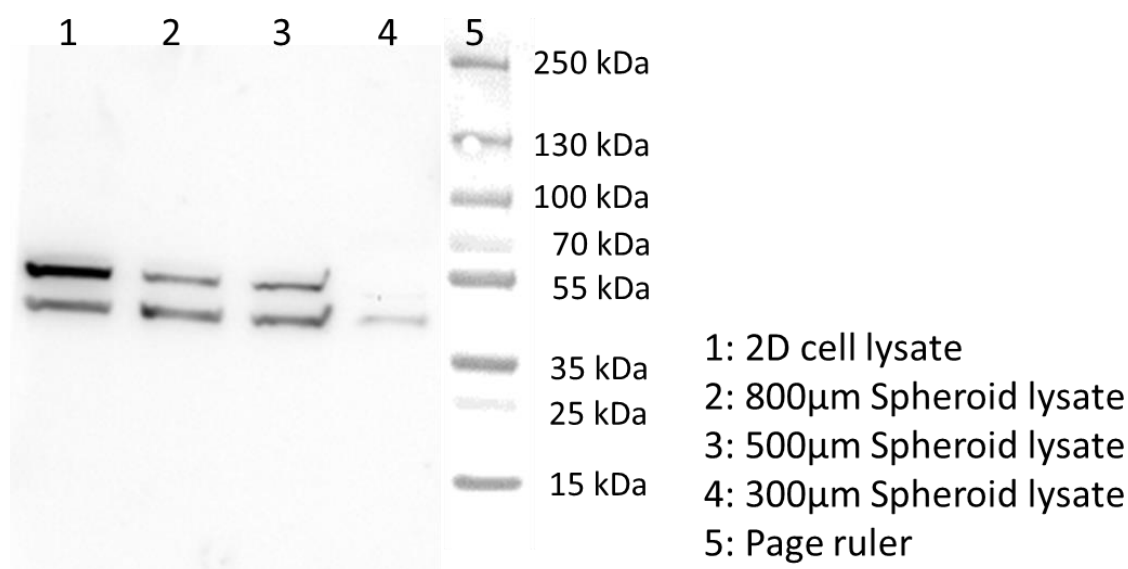


Figure 40: Western blot detection of A3 receptor expression in HT-29 cell and spheroid lysate. Gel electrophoresis was performed under reducing conditions. A₃R antibody shows the A₃ receptor expression in all lysate. The detected protein bands in lane 1,2 and 3 suggest an expression of a modified version of the receptor.

A slightly different picture was found for the HCT-116 cell line. Here, the expression of the A3 receptor increases with increasing size of the spheroids, whereas only a minor amount of A3 receptor is expressed in 2D cells. As already observed on the HT-29 A3 membrane, a second protein bond was detected in the lysate of 500 μm and 800 μm cell aggregates, suggesting the expression of a glycosylated A3 receptor.

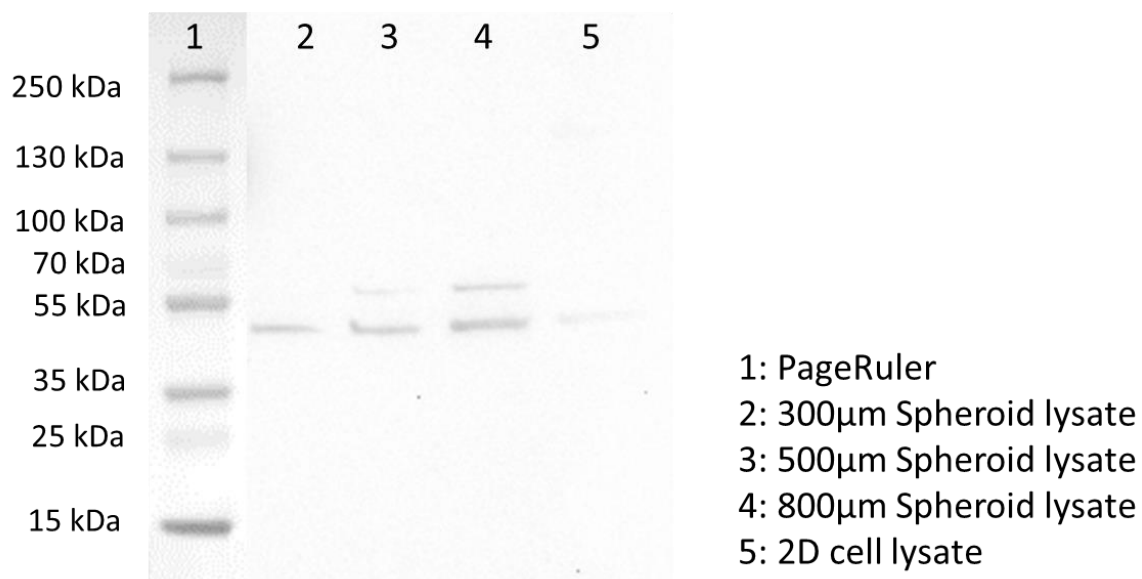


Figure 41: Western blot shows A3 receptor expression (predicted molecular weight 36 kDa) in HCT-166 cell and spheroid lysate. The additional bands at 55 kDa indicate an expression of a modified receptor in from 500 µm and 800 µm multicellular tumor spheroids.

4.8. Detection of Hif2α via immunohistochemical staining

Immunohistochemical staining was performed on 10 µm cryo-slices obtained from HT-29 multicellular tumor spheroids, using a monoclonal antibody against Hif2α. Since Hif2α is a transcription factor, specific staining was expected in cytoplasm and nuclei, especially within the inner core of the spheroid.

The obtained staining is shown in Figure 42. Hif2α was not detected within the spheroid, since the low intensity of the antibody signal was not relatable to specific regions.

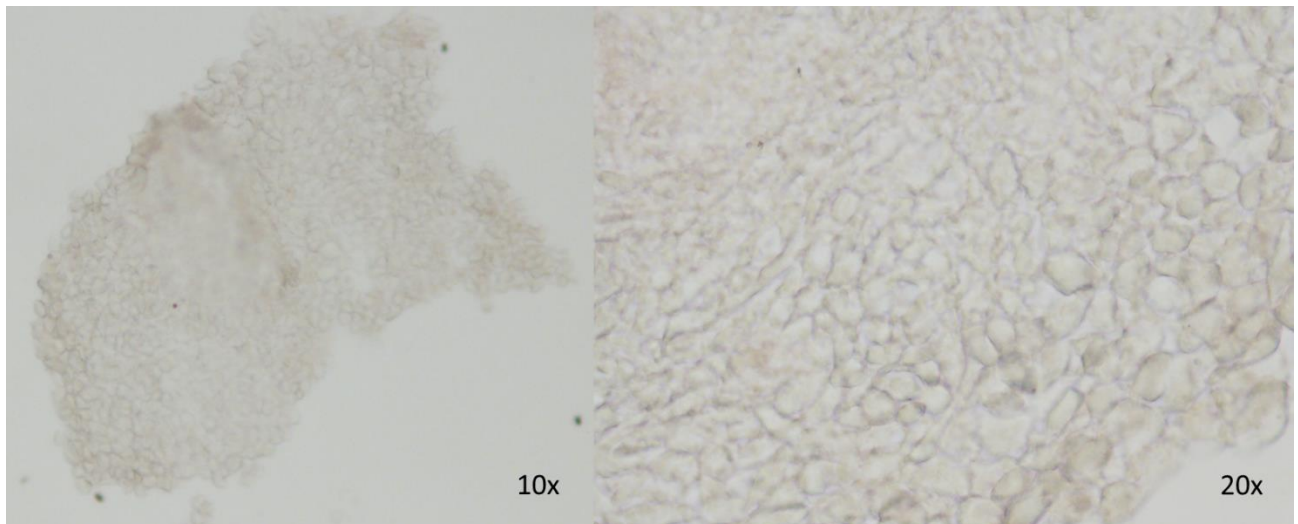
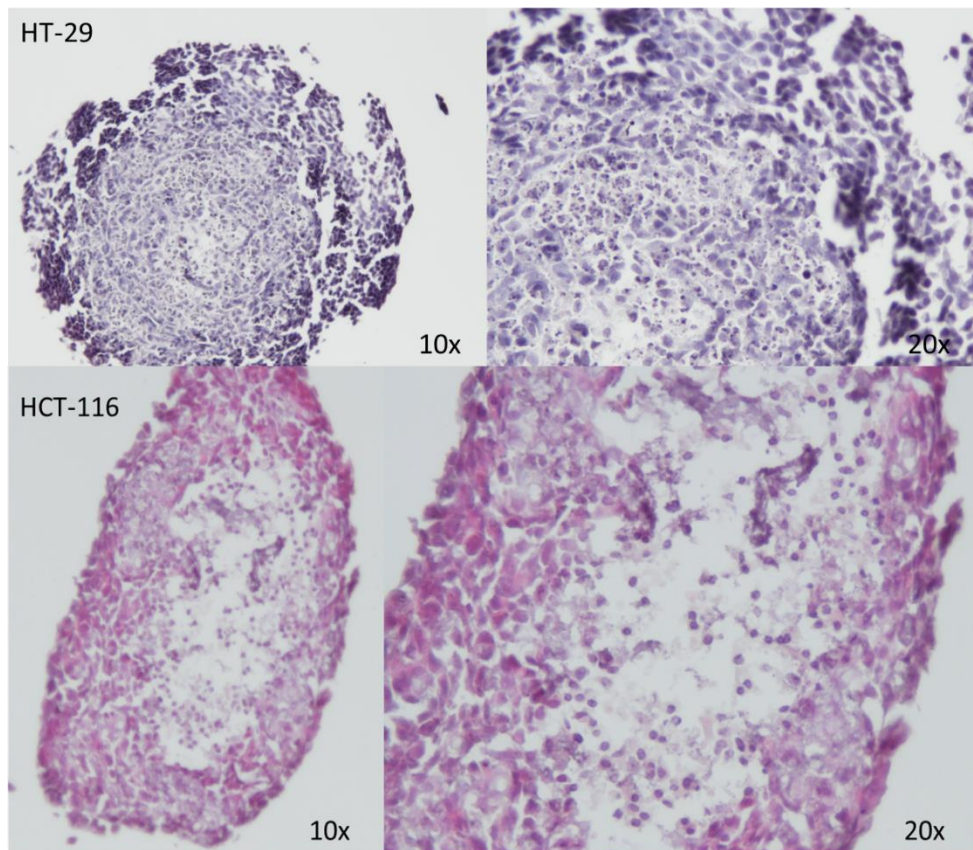


Figure 42: Immunohistochemical staining of Hif2 α in multicellular tumor spheroids. Hif2 α was not detected within the spheroid.

For counterstaining of the spheroid cryo-slices haematoxylin and eosin was used. The H&E staining of HT-29 and HCT-116 slices showed an intense staining in the outermost layer, whereas a pale coloration was observed in the inner core region of the spheroid (Figure 43). Moreover, the staining showed a change in the morphology of the nuclei, appearing as blue to purple, from spread to spherical towards the centre, indicating that cells of the inner spheroid region are already damaged.

It is also striking, that the pale coloured regions observed in H&E stained cryo-slices, closely resembled the necrotic spheroid regions imaged with live-dead staining.



*Figure 43: **H&E staining of HT-29 and HCT-116 spheroid cryo-slices.** Nuclei appear as blue to purple, while cytoplasm appears in various shades of pink.*

5. Discussion

Establishment and characterization of multicellular tumor spheroids

The results for the formation of MTS clearly indicate that there is a high dependency on the cell line, cell morphology, and passage of the cells in order to receive well-shaped spheroids. On the one hand, cells with an elongated morphology, like CHO-K1 and PC-3, did not form tight and spheroidic cell clusters as this morphology might aggravated cell aggregation. On the other hand, both colon cell lines, HT-29 and HCT-116, which show an insular (island-like) growth pattern formed highly symmetrically and tense spheroids. Nevertheless, for HT-29 cells the obtained spheroids were misshapen and not stable enough for transfer, if the cells have passed the 50th passage.

However, spheroid formation was not only cell line dependent, but also reliant on the selected production method and cell concentration, which had to be evaluated for each cell line. Most notably the hanging drop culture method turned out to be highly reproducible for HT-29 colon carcinoma cells. Unfortunately, this method was not applicable for all other cell lines as most of the time no spheroid formation was obtained, and if aggregate building was observed, they were not able to withstand the transfer to 96-well plates. Surprisingly, with the addition of gel forming agents like methylcellulose, which was expected to ease terms of spheroid formation, aggregate building was even exaggerated. Superior quality of spheroids for HT-29 as well as HCT-116 cells could be obtained using the pellet culture method. Particularly ULA-plates proved to be the most efficient tool for spheroid formation, referring to equality in shape and minimal effort in time and pipetting steps. Nevertheless, ULA-plates are very cost-intensive, hence as an alternative round-bottomed 96-well plates, coated with 1.5 % agarose solution, turned out to be the most economic method for reproducible aggregate formation.

Although the selection of the cell line and the culture method was decisive for spheroid formation, another crucial role played the selection of the cell concentration. Whereas on the one hand tight cell aggregates were obtained using concentrations between 3000 and 5000 cells per well in pellet culture system, on the other hand concentrations higher than 500 cells were insufficient at performing the hanging drop method for HT-29 cells.

Once spheroids were obtained, their growth behaviour was analysed to elucidate the ideal size for further experiments. While HT-29 spheroids, received through the hanging drop method, showed an increase in size of approximately 700 μm within 21 days, HCT-116

cell aggregates, produced with the pellet culture method, did show vanishingly low spheroid growth. This result was mainly based on the fact, that approximately a tenfold higher cell concentration, compared to the hanging drop method, was used in the pellet culture. The cells formed loose aggregates in short time, before they got condensed into compact spheroids. The proliferation of the cells was subsequently checked to ensure that the cells are still proliferating. The MTT-assay clearly point out, that there are proliferating cells, although the spheroids are not increasing in size. Subsequently, there could be stated, that depending on the size of the spheroids there is a formation of a steady-state between the proliferating cells in the outer layer and the necrotic ones in the inner core.

What both production methods had in common was, that spheroids were maintained to a size of approximately 900 μm , before they disaggregated.

A method, which increased the spheroid size to approximately 1 mm was to embed the MTS in a collagen matrix. The diameter of 1 mm is crucial, as this size is necessary for future μPET -experiments, due to the limits of resolution. However, on the second day after transfer, the cells started spreading within the matrix, which is also described in literature.⁽²²⁾

MTS are able to build out a microenvironment comparable to small solid tumors, including oxygen and nutrient gradients, as well as necrotic cores. The appearance of a necrotic core within multicellular tumor spheroids was described by McMahon *et al.* among others.⁽¹³⁾ In order to identify potential necrotic cores, live-dead staining was performed using Calcein AM and PI. Live-dead staining of HT-29 and HCT-116 spheroids showed necrotic regions in both cell lines, which were comparable for the respective size. In HT-29 spheroids, necrotic cores were initially observed in aggregates bigger than 500 μm . Whereas necrotic regions were found in all HCT-116 spheroids, used for staining, due to the fact, that obtaining aggregates smaller than 500 μm was not possible using the pellet culture method. However, it should be kept in mind that it took 11 days to obtain HT-29 cell aggregates in the size of 600 μm , whereas HCT-116 cell line built spheroids bigger than 600 μm within three days. This observation indicates, that the appearance of a necrotic core depends on the spheroid size and not on their lifespan. This results correlate very well with data from the literature.⁽¹⁴⁾

Even H&E staining of spheroid cryo-slices illustrate the existence of necrotic regions. The pale coloration of the staining within the inner spheroid core as well as the altered nuclei

morphology indicate that the cells are not alive. The size of the light-coloured regions was comparable with the necrotic regions shown in cell viability experiments.

PET-tracer accumulation

MTS can act as model for tumor environment and subsequently the accumulation of different PET tracers for oncological issues ($[^{18}\text{F}]\text{FDG}$, $[^{18}\text{F}]\text{FMISO}$, $[^{18}\text{F}]\text{FE@SUPPY}$ and $[^{18}\text{F}]\text{NaF}$) were tested at two different temperatures, namely 37°C and 4°C. Accumulation was compared with results obtained from common 2D cell culture experiments.

As expected, the highest accumulation was measured for $[^{18}\text{F}]\text{FDG}$ in spheroids as well as 2D cell culture. 2D accumulation experiments also revealed that in HT-29 cells, normally cultivated in high glucose RPMI medium, $[^{18}\text{F}]\text{FDG}$ accumulation could be increased with starving the cells for at least 1 hour before incubation. Although an increase of accumulation in HT-29 multicellular tumor spheroids was expected as well, accumulation turned out to be fivefold lower compared to non-starved spheroids. This phenomenon mainly occurred because spheroid starving, using unsupplemented minimal essential medium, led to disaggregation of the spheroids.

As it was described by Monazzam *et al.* the results, obtained through radio-tracer accumulation experiments, verified that temperature down-regulation to 4°C decreased the accumulation of tracers, which depend on mechanisms of active transport, such as $[^{18}\text{F}]\text{FDG}$, which is dependent on glut-transporters.^(25,82) This temperature-dependent reduction in accumulation was found for both cell lines, HT-29 and HCT-116, as well as in 3D and 2D experiments for $[^{18}\text{F}]\text{FDG}$, whereas $[^{18}\text{F}]\text{NaF}$ and $[^{18}\text{F}]\text{FMISO}$ accumulation was not significantly altered. Consequently, this results underline the mechanism described in literature, that $[^{18}\text{F}]\text{FMISO}$ and $[^{18}\text{F}]\text{NaF}$ are not actively transported.^(49,82)

Moreover, it was observed that incubation at 4°C slightly increased the accumulation of the hypoxia tracer $[^{18}\text{F}]\text{FMISO}$ in HT-29 multicellular tumor spheroids and 2D cell monolayers of the HT-29 and HCT-116 cell line.

Accumulation of $[^{18}\text{F}]\text{FE@SUPPY}$ did not significantly decrease when incubation was performed at 4°C. $[^{18}\text{F}]\text{FE@SUPPY}$, as an A3R receptor antagonist, binds the A3 receptor, located on the cell membrane.⁽⁹⁴⁾ In this case, accumulation is not reliant to temperature sensitive transport mechanisms. The formation of radioligand – receptor complexes (radioligand binding) is reversible and based on the law of mass action. Reduction of

temperature may prolong the establishment of equilibrium and less radioligand-receptor complexes may be formed.

Furthermore, the results of [^{18}F]FE@SUPPY accumulation experiments showed, that the accumulation in HT-29 multicellular tumor spheroids was approximately fifteenfold higher compared to 2D cell monolayers. However, higher accumulation in spheroids compared to 2D cell monolayers is not explainable by A₃R over-expression in spheroids, as it was shown by Western Blot analysis.

Three-dimensional distribution of [^{18}F]FE@SUPPY within the spheroid caused by passive diffusion, which is facilitated by the high lipophilicity, may lead to increased non-specific binding and therefore, higher accumulated doses. In addition, internalization assays indicate, that the use of emulsifying agents, as Tween-20, is effective for reducing the amount of non-specific bound radiotracers. Therefore, insufficient washing might additionally have increased the percentage of non-specific bound [^{18}F]FE@SUPPY, since spheroid washing during accumulation experiments was only performed using growth medium.

Accumulation of [^{18}F]FE@SUPPY found in CHO-K1 2D cell monolayers accounts for non-specific binding, as this cell line does not express the respective human target.

Moreover, the question raised whether A₃ receptors are internalized upon [^{18}F]FE@SUPPY binding, as it is described for the A₃R agonist I-AB-MECA.⁽⁸⁸⁾ However, the internalized fraction of [^{18}F]FE@SUPPY in CHO-hA3R cells was comparable to the internalized fraction of CHO-K1 cells. Therefore, it can be concluded that A₃ receptors are not internalized upon antagonist exposure. In contrast, internalization of the agonist [^{125}I]I-AB-MECA was found to be up to 29% in one experiment, which is in accordance with literature.⁽⁸⁸⁾

The results obtained through internalization experiments using the A₃ model ligand [^{125}I]I-AB-MECA, confirm the hypothesis of non-specific radioligand binding in the non-target cell line CHO-K1. This is supported by the observation, that barely any membrane binding or internalization of the radioligand took place. In addition to this, neither the membrane bound fraction nor the internalization of [^{125}I]I-AB-MECA was decreased by the addition of blocking agents as unlabelled I-AB-MECA or FE@SUPPY.

The same observations, however, were made during internalization experiments using PC-3 cell monolayers, which are supposed to express the human adenosine A₃ receptor.⁽⁹¹⁾

Nevertheless, the observed membrane binding and internalization was comparable to the CHO-K1 cell line, which is demonstrably not expressing the human target. Moreover, neither the addition of FE@SUPPY nor I-AB-MECA led to a significant decrease in the membrane bound fraction or the internalized fraction, which unexpectedly suggests non-specific binding of the radioligand on PC-3 cells.

When using CHO cells, transfected with the human A₃ receptor, a significant membrane binding of [¹²⁵I]-AB-MECA was expected, which should be blocked by the addition of FE@SUPPY and I-AB-MECA. Since the membrane bound fraction could be reduced through the addition of both blocking agents, the binding specificity and therewith the receptor expression could be demonstrated. Moreover, the internalization of the A₃ receptor after ligand binding, which was described by Trincavelli *et al.*, could be confirmed in one internalization experiment.⁽⁸⁸⁾ Although the assay was carried out in the same way, in the repetition of the experiment hardly any internalization of the radioligand was observed, which might give an indication, that the A₃ receptor internalization depends either on the confluency or the vitality of the selected cells.

Also the colon carcinoma cell line HT-29 is supposed to express human A₃ receptors.⁽⁸⁹⁾ Through the addition of FE@SUPPY and I-AB-MECA the receptor binding specificity of [¹²⁵I]-AB-MECA should be confirmed, like it was shown for the CHO-hA3R cell line. In contrast to the results of the CHO-hA3R cell line, the addition of the A₃ receptor antagonist FE@SUPPY enhanced the membrane binding of the radioligand, whereas the addition of the A₃ agonist I-AB-MECA showed no significant alterations in the membrane bound fraction compared to the baseline measurements. The internalized fraction of [¹²⁵I]-AB-MECA was generally low in HT-29 cells and similar to those observed in CHO-K1 cells.

Moreover, it was observed, that the addition of DMSO, serving as vehicle control in baseline measurements decreased the membrane binding of [¹²⁵I]-AB-MECA, whereas the internalization was significantly increased, suggesting, that dimethylsulfoxide permeabilizes the cell membrane and therewith facilitates the transport of molecules. However, neither permeabilizing effects nor toxic effects on cells are described for the use of DMSO in low concentrations, as 0.1 %.⁽⁹²⁾ Since this observation was made in CHO-hA3R cell monolayers, but not in CHO-K1 cells, it could be an A₃ receptor dependent phenomenon. Shukla *et al.*, however, showed that even concentrations up to 2 % DMSO had no adverse effect on the binding affinity of mammalian G-protein coupled receptors.⁽⁹³⁾

Referring to the results obtained through HT-29 [^{18}F]FDG internalization assays, a dependence from incubation medium and time was determined. Referring to the high observed [^{18}F]FDG accumulation in starved cells, it was confirmed, that cell starving and doubling the incubation time to 2 h, led to a significant increase in the intracellular uptake compared to non-starved cells. However, it should be noticed, that incubating cell in DPBS led to a detachment of some cells, which were washed off during the acid glycine washing step. This might have led to a slightly increase in the membrane bound fraction. Anyhow, it can be concluded that with cell starving more striking results could be achieved, especially for cells cultivated in growth medium, containing high amounts of glucose.

To sum up, the information gained with the internalization assays confirmed the functionality of the method. However, the evaluation of [^{18}F]FE@SUPPY internalization methods revealed, that cell washing with DPBS is insufficient for the use of highly lipophilic radiotracers. The results of washing with DPBS, containing 0.1 % Tween-20, indicate that washing with emulsifying agents is necessary for decreasing unspecific binding. Despite the good washing efficiency of Tween-20, a high percentage was found in the intracellular fraction, which gave rise to the question whether the emulsifying properties cause a damage in the membrane, which would lead to an increase in the internalized fraction.

Nevertheless, during the method evaluation it could be markedly shown, that cell lysis using RIPA-buffer appeared more appropriate compared to the use of NaOH, since it was possible to obtain a cell pellet through centrifugation. Hence a differentiation can be made between the internalized fraction and the unspecific membrane binding. However, before commitments are made on one specific method, outstanding issues deserve clarification.

Real-time kinetic measurements for [^{18}F]FDG accumulation

In order to gain further insights into the accumulation kinetics of [^{18}F]FDG, LigandTracer® experiments were performed, using HT-29, HCT-116, and PC-3 cell monolayers.

Since both colon carcinoma cell lines, HT-29 and HCT-116, showed a significant accumulation of the radiotracer in accumulation experiments as well as in internalization assays, similar results were expected in real-time accumulation measurements.

However, this measurements showed no significant [^{18}F]FDG accumulation, neither in HT-29 nor in HCT-116 cell monolayers. Therefore, following assumptions can be made.

The experimental conditions as cell starving, using DPBS, may lead to a reduced cell viability and subsequently lower accumulation. Internalization experiments as well as accumulation experiments using HT-29 cell monolayers, however, showed, that cell starving significantly increases the accumulation of the glucose analogue. Since the cells were not mechanically stressed within these assays, it could be concluded, that the combination of cell starving and the rotation of the LigandTracer® device may negatively affect the radiotracer accumulation in both tested colon carcinoma cell lines.

Moreover, we have found, that the cell passage has major impact on the functionality of cells. Especially HT-29 cell line has shown, that high numbers of cell passages aggravate spheroid formation. It seems reasonable, that this factor might have influence on radiotracer accumulations too. However, this assumption does not apply for the HCT-116 cell line, since accumulation experiments as well as LigandTracer® measurements were performed with cells of same age.

By contrast, LigandTracer® experiments, using the prostate carcinoma cell line PC-3 showed promising results. In this measurements significant and reproducible [¹⁸F]FDG accumulation was observed, suggesting, that this cell line is more resistant to cell starving and shear forces. In addition, the experiments verified, that the accumulated [¹⁸F]FDG was trapped in PC-3 cells, since the equilibrium maintained in spite of the DPBS change.

In order to establish an adequate method to fix multicellular tumor spheroids within the Petri dish for further real-time accumulation measurements, different embedding matrices such as agarose, Matrigel and gelatine were tested.

Agarose and Matrigel could not withstand the rotation of the LigandTracer® device. Although gelatine was promising in this respect, it turned out to be insufficient for spheroid embedding, due to high non-specific [¹⁸F]FDG accumulation.

Nevertheless, gelatine measurements brought the recognition, that [¹⁸F]FDG trapping is a cellular phenomenon, since the measured counts in the target region decreased as soon as incubation medium was changed.

Hif2α expression

Since Menrad *et al.* reported Hif2α expression in Hep G2 spheroid cryo-slices, immunohistochemical staining was performed using HT-29 slices.⁽⁴⁶⁾ As it might be

expected, considering the results obtained through western blot analysis, the staining showed no expression of the transcription factor Hif2 α .

In western blots using an antibody against Hif2 α on the HT-29 membrane no bands at 100 kDa were detected, whereas unspecific bands in varying intensities were observed below the 55 kDa mark. Referring to the specific references provided by abcam, this bands also occur in normoxic cell lysate. In HCT-116 lysate, however, an observation of this bands was not made, although the detection showed many unspecific bands. Moreover, in 800 μ m spheroid lysate as well as in 2D cell lysate, a faint line approximately at the 100 kDa mark was detected, which would indicate an expression of Hif2 α . However, this thesis should be unlikely, since 2D cell monolayers were not cultivated in a hypoxia chamber, before the cell lysate was produced. Therefore, they should be serving as a negative control. In 800 μ m spheroids the expression of Hif2 α is more likely, since the appearance of necrotic regions was observed in live-dead staining and therefore hypoxia was suggested. However, the fact that a lot of unspecific bands were detected, combined with the missing bands below the 55 kDa mark, which could serve as a control band, since its appearance in normoxic cell lysate is described, raise doubts. Since unspecific binding of the second antibody could be excluded, the evaluation of the obtained results proves to be difficult. Therefore, a repetition of the experiment would be reasonable, whereby cell lysate, obtained from hypoxic cell monolayers, should be serving as positive control.

Moreover, it is worth noting that the appearance of hypoxic regions must not be excluded based on the achieved results, since Hif2 α expression is described as a result of long-term hypoxia, whereas Hif1 α expression occurs early.⁽⁴³⁾ Hence, a second blot could be performed using an antibody against Hif1 α . Furthermore, the performance of fluorescence hypoxia assays using whole spheroids instead of lysate should be considered. Consequently, imaging of hypoxic regions could be obtained on one hand and on the other hand an eventual destruction of transcription factors due to cell lysis could be excluded.

A3 receptor expression

Despite the fact, that the desired expression of Hif2 α was not observed in HT-29 and HCT-116 cell lysates (derived from MTS), the expression of the A₃ receptor in HT-29 and HCT-116 cell monolayers, which was described by Gessi *et al.* and Ohana *et al.*, respectively, could be confirmed with western blot analysis.^(89,90) Moreover, the detected protein bands suggested the expression of the A₃ receptor in spheroid lysate, obtained from both cell

lines. Especially in HCT-116 lysate, it was observed, that the receptor expression might increase with spheroid size, since in the 2D cell lysate the detected bands were less intensive.

In addition, further bands were detected on both membranes, suggesting that a modified version of the receptor is expressed in HT-29 spheroids, as well as in HCT-116 aggregates from a size of 500 μm .

6. Conclusion

The aim of this work was the evaluation and establishment of different spheroid formation methods for the efficiency and reliability used for different cell lines and consequently, the characterization of multicellular tumor spheroids regarding to growth behavior, PET-tracer accumulation ($[^{18}\text{F}]\text{FDG}$, $[^{18}\text{F}]\text{NaF}$, $[^{18}\text{F}]\text{FMISO}$, $[^{18}\text{F}]\text{FE@SUPPY}$ and $[^{125}\text{I}]\text{I-AB-MECA}$) and target expression (Hif2 α , A3R).

The performed experiments revealed that formation of stable multicellular tumor spheroids was obtained in the human colorectal adenocarcinoma cell lines HT-29 and HCT-116, whereas formation of stable cell aggregates could not be established for the Chinese hamster ovary cells CHO-K1 and the human prostate carcinoma cell line PC-3. Referring to the observations, it can be concluded that spheroid formation as well as their growth behavior depends on multiple factors, such as the selection of the cell line, cell concentration, cell morphology, number of the cell passage and production method.

Whereas HT-29 multicellular tumor spheroids were produced using the hanging drop method, spheroid formation in HCT-116 cell line was obtained using the pellet culture method. This indicates, that the universal applicability of one specific manufacturing process cannot be confirmed. Therefore, to obtain reproducible and stable aggregates an evaluation of all factors, eligible for spheroid formation, is necessary for each cell line.

In addition, with the performance of live-dead staining experiments, it could be confirmed that the appearance of necrotic regions within multicellular tumor spheroids depends on the spheroid size, since dead cells could not be visualized in spheroids smaller than 500 μm .

In accumulation experiments the expected temperature dependence of radiotracers, which are linked to active transport mechanisms, like $[^{18}\text{F}]\text{FDG}$, could be shown for 2D as well as 3D cell culture. While temperature down-regulation during incubation with $[^{18}\text{F}]\text{NaF}$ and $[^{18}\text{F}]\text{FMISO}$ scarcely made a difference in the accumulation, the percentage of accumulated $[^{18}\text{F}]\text{FDG}$ significantly decreased when cells were incubated at 4°C. The temperature reduction to 4°C resulted in a decrease of $[^{18}\text{F}]\text{FE@SUPPY}$ accumulation as well, which could allow the conclusion that also receptor binding mechanisms are temperature sensitive. Moreover, the importance of cell starving for increasing the

accumulation of [^{18}F]FDG could be pointed out in accumulation and internalization experiments using HT-29 cell monolayers.

For interpreting the results of the accumulation experiments in more detail, the performance of internalization assays appeared to be helpful. The therewith collected data made it possible to differentiate between membrane-bound and internalized fraction.

However, considering the results obtained through [^{18}F]FE@SUPPLY accumulation experiments, a detailed method evaluation, especially for the use of lipophilic radiotracers, proved to be necessary to avoid false-positive results caused by unspecific binding.

Summarized it can be highlighted that the advantages of multicellular tumor spheroids by far outweigh the negative aspects, like time-consuming cultivation and the need of precise evaluation of spheroid formation methods. Due to the similarity to human tumor tissue, multicellular tumor spheroids could replace 2D cell monolayers in preclinical PET-tracer evaluation experiments. It should be noted that *in vitro* experiments, using 3D cell aggregates, would implicate detailed method evaluation for each cell line and tested radiotracer, the application of multicellular tumor spheroids, however, offers a savings potential for animal experiments. Due to the fact, that 3D cell culture has the potential to close the information gap between 2D cell culture and animal tests, subsequent animal testing could possibly be refined and reduced.

7. Abbreviations

A₃R	Adenosine A ₃ receptor
BSA	Bovine serum albumin
BME	Basement membrane extract
CA IX	Carbonic anhydrase IX
Calcein AM	Calcein acetoxymethylester
DMSO	Dimethyl sulfoxide
EPO	Erythropoietin
FDG	2-Fluor-2-deoxy-D-glucose
FE@SUPPY	5-(2-fluoroethyl) 2,4-diethyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate
FMISO	Fluoromisonidazole
H&E	Haematoxylin and eosin
HAF	Hypoxia associated factor
Hif	Hypoxia inducible factor
HRE	Hypoxia-response element
I-AB-MECA	4-Aminobenzyl-5'-N-methyl-carboxamidoadenosine
MTS	Multicellular tumor spheroid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
NaF	Sodium fluoride
DPBS	Dulbecco's Phosphate-Buffered Saline
PET	Positron emission tomography
PI	Propidium iodide
pVHL	von Hippel-Lindau protein
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris-buffered saline with Tween20
Tris	Tris(hydroxymethyl)aminomethane
ULA plates	Ultra-Low Attachment Plates
VEGF	Vascular Endothelial Growth Factor

8. References

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