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Toxizität und Effektivität der Kombinationstherapie mit dem mTOR-Inhibitor Everolimus und [^{177}Lu]Lu-DOTA-TATE im Vergleich mit den Einzeltherapien im Kleintiermodell

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Johannes Anton Josef Zellmer

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Berichterstatter:	Prof. Dr. Peter Bartenstein
Mitberichterstatter:	Prof. Dr. Markus Guba
	Prof. Dr. Marily Theodoropoulou
Mitbetreuung durch den Promovierten Mitarbeiter:	PD Dr. Harun Ilhan
Dekan:	Prof. Dr. med. Thomas Gudermann
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Eidesstattliche Versicherung

Zellmer, Johannes Anton Josef

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema:

Toxizität und Effektivität der Kombinationstherapie mit dem mTOR-Inhibitor Everolimus und [^{177}Lu]Lu-DOTA-TATE im Vergleich mit den Einzeltherapien im Kleintiermodell

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München, den 14.10.2024 Johannes Anton Josef Zellmer

Inhaltsverzeichnis

Abkürzungsverzeichnis

ANOVA	analysis of variance (Varianzanalyse)
CTCAE	common terminology criteria for adverse events (Allgemeine Terminologiekriterien von unerwünschten Ereignissen)
FUR	fractional uptake rate (beschreibt den Anteil der Aktivität in einer ROI an der Aktivität insgesamt)
G	Grad
HE	Hämatoxylin-Eosin
HPLC	high preformance liquid chromatography (Hochleistungsflüssigkeitschromatographie)
mTOR	mechanistic Target of Rapamycin
NEC	Neuroendokrines Karzinom
NEN	Neuroendokrine Neoplasie
NET	Neuroendokriner Tumor
PAS	periodic acid–Schiff
PET	Positronen-Emissions-Tomographie
pNET	Pankreatischer neuroendokriner Tumor
PRRT	Peptidrezeptor-Radiotherapie
RBC	red blood cell (Erythrozyt)
RDS	renal damage score (Score der histomorphologischen Nierenschädigung)
ROI	region of interest (definierter Bereich innerhalb eines Messdatensatzes)
SPECT	single photon emission computed tomography (Einzelphotonen-Emissions-Computertomographie)
SRH	Scheirer–Ray–Hare
SSA	Somatostatinanalogon
SSTR	Somatostatinrezeptor
SSTR2	Somatostatinrezeptor Subtyp 2
WBC	white blood cell (Leukozyt)

Publikationsliste

Die vorliegende kumulative Dissertation umfasst gemäß § 4a der Promotionsordnung zwei bereits publizierte Manuskripte:

Zellmer J, Yen HY, Kaiser L, Mille E, Gildehaus FJ, Böning G, Steiger K, Hacker M, Bartenstein P, Todica A, Haug AR, Ilhan H, 2020. **Toxicity of a combined therapy using the mTOR-inhibitor everolimus and PRRT with [^{177}Lu]Lu-DOTA-TATE in Lewis rats.** EJNMMI Res. 2020 Apr 25;10(1):41.

Zellmer J, Yen HY, Kaiser L, Gildehaus FJ, Böning G, Steiger K, Hacker M, Bartenstein P, Todica A, Haug AR, Ilhan H. **Evaluation of the Efficacy of a Combined Treatment Using the mTOR-Inhibitor Everolimus and [^{177}Lu]Lu-DOTA-TATE in Nude CD1 Mice with SSTR-Expressing Pancreatic AR42J Xenograft Tumors.** Biomedicines. 2022 Dec 1;10(12):3102.

1. Einführung

1.1. Pankreatische neuroendokrine Tumoren

Neuroendokrine Tumoren (NET) stellen eine heterogene Entität maligner Neoplasien dar. Die Primärlokalisation ist häufig der Gastrointestinaltrakt, aber auch die Lunge und das Pankreas sowie weitere endokrine Drüsen können betroffen sein (1). Innerhalb der Gruppe neuroendokriner Neoplasien (NEN) werden gut differenzierte NET von schlecht differenzierten neuroendokrinen Karzinomen (NEC) abgegrenzt (2). Nach Malignitätsgrad (G) eingestuft werden die NET abhängig von der Expression von Ki-67 oder der Mitoserate als G1, G2 oder G3. Mehr als zwei Drittel der NEN weisen eine relativ niedrige Teilungsrate auf (entsprechend G1- oder G2-Tumoren), wobei der Anteil dieser niedrig malignen Neoplasien an allen neudiagnostizierten NEN zunimmt (3). In den letzten Jahrzehnten ist darüber hinaus eine deutliche Zunahme der Inzidenz von NEN insgesamt beobachtet worden (1, 3). Häufig erfolgt die Diagnose jedoch erst in fortgeschrittenen Stadien, in denen eine vollständige Resektion nicht mehr möglich ist. Systemische Therapieansätze spielen dann eine wichtige Rolle im Management (2). Aktuelle Leitlinien empfehlen die Auswahl der Therapie in Abhängigkeit der Primärlokalisation sowie des Malignitätsgrades und biologischer Tumoreigenschaften (2, 4). Bei pankreatischen neuroendokrinen Tumoren (pNET) kommen neben der seit Langem etablierten Chemotherapie mit Streptozotocin und 5-Flourouracil nach positiven randomisierten kontrollierten Studien nunmehr weitere Substanzen wie die Somatostatin-Analoga (SSA) Octreotid und Lanreotid sowie Everolimus oder Sunitinib zum Einsatz (5-9).

Daneben konnte mit der NETTER-1-Studie auch für die Peptidrezeptor-Radiotherapie (PRRT) mit [^{177}Lu]Lu-DOTA-TATE eine signifikante Verlängerung des mittleren progressionsfreien Überlebens bei Patienten mit metastasierten NET des Mitteldarms gegenüber einer Monotherapie mit Octreotid gezeigt werden (10). Für pNET liegen dagegen keine Daten aus einer Phase-III-Studie für [^{177}Lu]Lu-DOTA-TATE vor. In retrospektiven Analysen von Patienten, die mit [^{177}Lu]Lu-DOTA-TATE behandelt wurden, konnte in dieser Subgruppe jedoch ein gutes Ansprechen beobachtet werden (11, 12).

Die Verfügbarkeit einer größeren Anzahl systemischer Therapieoptionen stellt zwar einen großen Fortschritt dar, eine dauerhafte Remission kann jedoch in der Regel nicht erreicht werden. Die simultane Behandlung mit verschiedenen Therapieoptionen

könnte daher ein Ansatz sein, die Wirksamkeit für die betroffenen Patienten weiter zu verbessern. In der vorliegenden Arbeit wurde die Kombination von Everolimus und [^{177}Lu]Lu-DOTA-TATE bei pNETs im Kleintiermodell hinsichtlich Toxizität und Effektivität evaluiert.

1.2. Somatostatin-Rezeptor-Expression als Grundlage von Bildgebung und Therapie

Seit etwa 1985 ist bekannt, dass neuroendokrine Tumorzellen Somatostatin-Rezeptoren (SSTR) in hoher Dichte exprimieren (13, 14). Durch diese Erkenntnis ließ sich die Wirkung des wenige Jahre zuvor vorgestellten synthetischen Somatostatin-Analogons Octreotid erklären (15). Schnell wurde begonnen, sich dieses Wissen für die nuklearmedizinische Diagnostik und Therapie zu Nutze zu machen. Es gelang, mit [^{111}In]In-Pentreotid eine chemische Verbindungen aus einem Somatostatin-Analogon und einem Radionuklid zu entwickeln, die sich klinisch zur Bildgebung oder Therapie von NET einsetzen ließ (16, 17).

Seither wurde mit verschiedenen Ansätzen versucht, die Bildqualität und die Sensitivität nuklearmedizinischer Verfahren zur Diagnostik SSTR-exprimierender Tumoren zu verbessern. Erste vielversprechende Ergebnisse mit dem Chelator 1,4,7,10-Tetraazacyclododecan-1,4,7,10-tetraessigsäure (DOTA) in Verbindung mit Octreotid (DOTATOC) wurden 1997 veröffentlicht. Im Rattenmodel konnte in den Zielgeweben eine höhere Aktivität beobachtet werden, wenn mit dem Betastrahler ^{90}Y oder mit ^{111}In markiertes DOTATOC zum Einsatz kam als beim bisherigen Standard ^{111}In-Pentreotid (18). Darüber hinaus wurde ebenso bereits 1997 eine erfolgreiche Behandlung eines Patienten mit [^{90}Y]Y-DOTATOC beschrieben, sowie der erfolgreiche diagnostische Einsatz von [^{111}In]In-DOTATOC bei im Vergleich zu [^{111}In]In-Pentreotid geringerer unerwünschter Strahlenexposition der Nieren (19).

Eine weitere Neuerung stellte die Einführung des Radiopharmakons DOTA-TATE dar. In Verbindung mit dem Betastrahlers ^{177}Lu ([^{177}Lu]Lu-DOTA-TATE) zeigte es im Rattenmodell einen besseren therapeutischen Effekt als [^{177}Lu]Lu-DOTATOC und wies insbesondere eine hohe Affinität zum SSTR Typ 2 (SSTR2) auf (20).

Es folgte der experimentelle klinische Einsatz dieser Substanz an einer großen Zahl von Patienten mit NET. 2017 wurde von der Gruppe um Eric Krenning in Rotterdam eine entsprechende umfangreiche Fallserie publiziert. Die Ansprechrate in der Subgruppe von 112 Patienten mit nicht funktionell aktiven Tumoren pankreatischen Ursprungs betrug 59 % (12).

Etwa gleichzeitig konnten Deppen et al. zeigen, dass eine Bildgebung mit kombinierter Positronen-Emissions-Tomographie und Computer-Tomographie (PET/CT) unter Verwendung von [^{68}Ga]Ga-DOTA-TATE eine höhere Sensitivität in der Detektion von Tumorläsion bei Patienten mit NET aufweist als die kombinierte Single-Photon-Emissions-Computertomographie und Computer-Tomographie (SPECT/CT) mit [^{111}In]In-Pentreotid, was bei 36 % der analysierten Patienten zu einer Änderung der Therapiestrategie führte (21). Mittlerweile werden in der klinischen Routine zur PET-Bildgebung daher mit ^{68}Ga beladene Radiopharmaka empfohlen (2). Neue Konzepte zur weiteren Optimierung der Somatostatinrezeptor-Bildgebung unter Verwendung des Radioisotops ^{18}F werden derzeit klinisch und präklinisch getestet und haben erste erfolgsversprechende Ergebnisse geliefert (22, 23).

Naheliegend erscheint der parallele Einsatz von [^{68}Ga]Ga-DOTA-TATE und [^{177}Lu]Lu-DOTA-TATE. So soll durch die Verwendung des gleichen SSA anhand des [^{68}Ga]Ga-DOTA-TATE-PET eine möglichst adäquate Vorhersage des Uptakes des therapeutischen Radiopharmakons durch das Tumorgewebe gewährleistet werden. Aus diesem Grund stellt die vorherige SSTR-PET/CT Bildgebung auch eine obligate Voraussetzung vor Durchführung einer Peptid-Rezeptor-Radionuklidtherapie (PRRT) dar.

In die einzige derzeit vorliegende Phase-III-Studie, in der die Effektivität von [^{177}Lu]Lu-DOTA-TATE evaluiert wurde (NETTER-1), wurden ausschließlich Patienten mit intestinalen NET eingeschlossen. Auch wenn mit einem signifikant höheren progressionsfreien Überleben der primäre Endpunkt erreicht wurde, konnte kein statistischer Vorteil beim Gesamtüberleben gezeigt werden. Es fand sich lediglich eine tendenzielle Überlegenheit von 48,0 gegenüber 36,3 Monaten (10, 24). Dennoch wird [177Lu]Lu-DOTA-TATE bei intestinalen NET als Zweitlinientherapie Leitlinien-konform eingesetzt, sofern für alle Tumorläsionen eine SSTR-Expression in der [^{68}Ga]Ga-DOTA-TATE-PET nachweisbar ist. Für pNET ist die Empfehlung schwächer, denn es liegen wie oben dargestellt bisher lediglich retrospektive Daten vor (2, 25).

1.3. Stellenwert des mTOR-Inhibitors Everolimus

Everolimus ist ein Inhibitor von mTOR (mechanistic Target of Rapamycin), einer Serin/Threonin-Kinase, die in der Regulation von Zellwachstum, -teilung und Überleben eine wichtige Rolle spielt. Die Substanz ist in verschiedenen Indikationen in der medikamentösen Tumortherapie zugelassen (26). Die randomisierte kontrollierte Phase-III-Studie „RAD001 in Advanced Neuroendocrine Tumors, Third Trial“ (RADIANT-3) konnte zeigen, dass bei Patienten mit nicht resektablen oder metastasierten pNET (G1/G2) nach einer Tumorprogression eine Verlängerung des medianen progressionsfreien Überlebens auf 11 Monate unter Everolimus gegenüber 4,6 Monaten unter Placebo erreicht werden kann (6). Das Gesamtüberleben war tendenziell, jedoch nicht statistisch signifikant verbessert (27). Dennoch wird Everolimus gemäß den Empfehlungen der ENETS von 2023 in dieser Indikation leitliniengerecht als Zweitlinientherapie eingesetzt, wobei die Empfehlungen der ESMO von 2020 bei G2-Tumoren ab einem Ki-67 von 10 % die Anwendung sogar in der ersten Linie als gerechtfertigt ansahen (2, 4).

Everolimus wurde darüber hinaus als therapeutische Möglichkeit untersucht, Tumoren gegenüber ionisierender Strahlung zu sensibilisieren. Eine solche Sensibilisierung ist in vivo für Modelle von Mamma-, Kolon-, Pankreas- sowie oralen und zervikalen Plattenepithelkarzinomen beschrieben (28-31). Zusätzlich konnte ebenso in vivo an Mäusen gezeigt werden, dass ein strahlungsresistenter Klon einer Zelllinie eines oralen Plattenepithelkarzinoms durch die Behandlung mit Everolimus erneut gegenüber therapeutischer Gamma-Strahlung sensibilisiert werden kann (32).

1.4. Kombinationstherapie als mögliche Option zur Erhöhung der Effektivität

Wie in den vorangegangenen Abschnitten dargelegt, zeigen sowohl Everolimus als auch [^{177}Lu]Lu-DOTA-TATE eine therapeutische Wirkung bei Patienten mit fortgeschrittenen pNET niedrigen oder intermediären Malignitätsgrades. Dabei fehlen für [^{177}Lu]Lu-DOTA-TATE derzeit noch prospektive Daten.

Das berichtete objektive Therapieansprechen ist mit 5 % für Everolimus (RADIANT-3) gering. Die bereits erwähnten Ergebnisse aus Rotterdam [^{177}Lu]Lu-DOTA-TATE schei-

nen mit 59 % objektivem Ansprechen nummerisch deutlich überlegen zu sein, aufgrund der retrospektiven Untersuchung und der unterschiedlichen Einschlusskriterien sind die Daten jedoch kaum vergleichbar.

Somit kann bei einem großen Teil der Erkrankten mit den bisher verfügbaren Therapieoptionen keine relevante Reduktion der Tumorlast erreicht werden. Eine weitere Optimierung der Therapiestrategien erscheint wünschenswert.

Aufgrund der bereits dargestellten Sensibilisierung von Tumorgewebe gegenüber ionisierender Strahlung durch die Behandlung mit Everolimus ist ein synergistischer Effekt bei der gleichzeitigen Anwendung von [^{177}Lu]Lu-DOTA-TATE denkbar. Eine eventuelle klinische Evaluierung dieser Strategie sollte im vorliegenden Promotionsprojekt durch Daten aus einem Tiermodel unterstützt werden.

1.5. Toxizität

Die Nieren haben sich als besonders vulnerabel gegenüber der Toxizität der PRRT von erwiesen. Dies triff auch für die Behandlung mit [^{177}Lu]Lu-DOTA-TATE zu. Die toxischen Effekte können in der klinischen Anwendung durch die gleichzeitige Verabreichung von Aminosäurelösungen weitgehend vermieden werden (10, 11). Auch das Knochenmark ist von der Toxizität der PRRT betroffen. Die Häufigkeit des Auftretens von schwerer Lymphozytopenie (Common Terminology Criteria for Adverse Events, CTCAE Grad 3 oder 4) als unerwünschter Arzneimittelwirkung beträgt in der retrospektiven Analyse von Brabander et al. 50 %, in der prospektiven NETTER-1-Studie wird sie dagegen lediglich mit 9 % beziffert (10, 12). Everolimus weist ebenso eine erhebliche Hämatotoxizität auf. So traten in der RADIANT-3-Studie schwere Fälle von Anämie oder Thrombozytopenie (CTCAE Grad 3 oder 4) bei 6 % beziehungsweise 4 % der Patienten die Everolimus erhielten auf, während solche Ereignisse in der Placebogruppe nicht beobachtet wurden (6).

Durch die gleichzeitige Anwendung beider Therapeutika ist daher auch vor dem Hintergrund der Sensibilisierung gegenüber strahleninduzierten Schäden durch Everolimus eine (über-)additive Zunahme dieser unerwünschten Wirkungen denkbar. Aus diesem Grund stellte die Untersuchung von Nephro- und Myelotoxizität einen Schwerpunkt des vorliegenden Projekts dar.

1.6. Evaluation der Nephrotoxizität und funktionelle Bildgebung der Nieren mit [^{99m}Tc]Tc-MAG3-Szintigraphien

Die Jaffé-Reaktion zur Bestimmung der Kreatinin-Konzentration ist seit langem als Standardmethode zur Überwachung der Nierenfunktion klinisch etabliert. Sie beruht auf der Komplexbildung zwischen Kreatinin und Picrinsäure und liefert beim Menschen eine sehr gute Übereinstimmung mit Referenzmethoden wie der Hochleistungsflüssigkeitschromatographie (HPLC). Bei Mäusen und Ratten scheint das Blut jedoch einen Farbstoff zu enthalten, der bei der notwendigen kolorimetrischen Messung eine systematische Abweichung verursacht (33).

Neuere Ergebnisse deuten zumindest darauf hin, dass in diesen Modellorganismen wenigstens ein linearer Zusammenhang zwischen den Messergebnissen mittels Essays auf Basis der Picrinsäure und der HPLC besteht. Jedoch können die Ergebnisse bei der Verwendung von Picrinsäure bereits durch minimale nicht sichtbare Hämolyse weiter verfälscht werden (34).

Ähnlich häufig zur Evaluation der Nierenfunktion wird in der klinischen Routine die Messung der Harnstoffkonzentration im Plasma bzw. des im Harnstoff gebundenen Stickstoffs (blood urea nitrogen, BUN) verwendet. Sie ist ebenso einfach verfügbar, weist jedoch eine geringe Sensitivität auf und lässt bei Anstieg keine Rückschlüsse auf die geschädigte anatomische Struktur zu (35).

Die szintigraphische Bildgebung der Nieren mit dem Tracer [^{99m}Tc]Tc-MAG3 hat sich für die Messung geringer Veränderungen der Nierenfunktion im Mausmodell als geeigneter als die Messung von Kreatinin und Harnstoff erwiesen. Mit hoher Sensitivität kann sie Veränderungen der tubulären Funktion detektieren (36).

Radioaktiv markierte Peptide werden in den proximalen Tubuli der Nieren über den Megalin/Cubilin-Rezeptorkomplex reabsorbiert. Entsprechend führte die Verabreichung von [^{177}Lu]Lu-DOTA-TATE bei zunehmender absorbierter Dosis in den Nieren im Mausmodell zu einer selektiven Reduktion des Anteils der proximalen Tubuli, wohingegen Glomeruli und distale Tubuli nicht betroffen waren (37). Daher kann angenommen werden, dass die Nierenszintigraphie mit [^{99m}Tc]Tc-MAG3 eine sehr sensitive Methode darstellt, auch eine diskrete Schädigung der Nieren durch [^{177}Lu]Lu-DOTA-TATE frühzeitig erfassen zu können.

1.7. Zielsetzung der Arbeit

Ziel des vorliegenden Forschungsprojekts war es, zunächst zu evaluieren, ob durch eine Kombination der etablierten Pharmaka Everolimus und [^{177}Lu]Lu-DOTA-TATE die Toxizität der Therapie inakzeptabel gesteigert wird. In einem zweiten Schritt sollte die mögliche synergistische therapeutische Wirkung untersucht werden.

2. Zusammenfassung

2.1. Versuchsablauf

In der vorliegenden Arbeit wurden die Toxizität sowie die Effektivität einer möglichen Kombinationstherapie von Everolimus und [^{177}Lu]Lu-DOTA-TATE in zwei aufeinander aufbauenden Versuchsreihen untersucht. Die detaillierten Versuchspläne sind in den beiden entsprechenden Veröffentlichungen dargestellt (38, 39) und seien im Folgenden noch einmal kurz zusammengefasst:

Für die Toxizitätsuntersuchung wurden weibliche Lewis-Ratten als Modellorganismus genutzt. Es erfolgte eine Einteilung in vier Gruppen, wovon die ersten beiden keine PRRT erhielten, Gruppe 1 ein vom Hersteller von Everolimus (Novartis®) zur Verfügung gestelltes Placebo, Gruppe 2 Everolimus. Die Gruppen 3 und 4 wurden mit [^{177}Lu]Lu-DOTA-TATE behandelt, Gruppe 3 zusätzlich mit dem Placebo, Gruppe 4 mit Everolimus. An der Hälfte der Ratten jeder Gruppe wurden bis zum Ende des Beobachtungszeitraums ausschließlich laborchemische Untersuchungen durchgeführt, nämlich die Bestimmung von Kreatinin und BUN zunächst einmal wöchentlich und zwischen Woche 8 und Woche 16 alle 14 Tage. Zusätzlich erfolgte die Erstellung eines Differentialblutbilds nach 16 Wochen bei Euthanasie. Die andere Hälfte der Ratten wurde für sequenzielle planare [^{99m}Tc]Tc-MAG3-Szintigraphien eingesetzt um die tubuläre Funktion im Zeitverlauf zu untersuchen. Eine Autopsie mit Fixierung der Nieren erfolgte bei allen Tieren am Ende des sechszehnwöchigen Beobachtungszeitraums.

Für die Planung der Untersuchung der Effektivität wurde zunächst ein Pilotversuch mit dem bereits bekannten Modellorganismus (weibliche Lewis-Ratten) durchgeführt. Hier zeigte sich leider kein Wachstum der pankreatischen, SSTR-2-positiven, aus Ratten stammenden Zellinie AR42J. Im nächsten Schritt wurden immundefiziente Nacktratten verwendet (Rowett Nude, RNU). Es kam zwar zu einem Tumorwachstum, dieses unterschied sich aber in seiner Dynamik erheblich zwischen den Versuchstieren, sodass die Zeitpunkte der geplanten [^{177}Lu]Lu-DOTA-TATE-PET-Scans nicht sinnvoll im Voraus festzulegen gewesen wären.

Aus diesem Grund erfolgte der Wechsel hin zu Nacktmäusen (CD-1). Die Dynamik des Tumorwachstums war hier hoch, jedoch ausreichend homogen in den untersuchten Tieren, sodass ein Versuchsplan erstellt werden konnte. Dieser beinhaltete die Impfung mit Tumorzellen und einen Ausgangs-[^{68}Ga]Ga-DOTA-TATE-PET-Scan fünf Tage

später. Die Tiere wurden analog zu den Toxizitäts-Versuchen in vier Gruppen eingeteilt. Unmittelbar nach dem Ausgangs-Scan erhielten die Mäuse aus den Gruppen 3 und 4 [^{177}Lu]Lu-DOTA-TATE in einer Dosis von 200 MBq, die Behandlung mit Everolimus beziehungsweise dem Placebo wurde bei allen Tieren begonnen. Mit einem kalibrierten Messschieber wurde das Tumorwachstum perkutan überwacht und zudem nach einer, zwei und vier Wochen erneut jeweils ein [^{68}Ga]Ga-DOTA-TATE-PET durchgeführt. Bei übermäßigem Tumorwachstum erfolgte die Euthanasie der Tiere und es wurden zum Toxizitätsmonitoring ein Blutbild angefertigt und die Nieren histopathologisch untersucht.

2.2. Ergebnisse und Interpretation

Die Tiere in den Gruppen, die Everolimus erhielten, nahmen langsamer an Gewicht zu. Die Kreatininkonzentration als Marker der Nierenfunktion stieg in diesen Gruppen im Beobachtungszeitraum auch weniger stark an. Kreatinin ist ein Abbauprodukt im Stoffwechsel der Muskulatur und die Kreatininkonzentration im Allgemeinen abhängig von der Muskelmasse. Deswegen erscheint eine Interpretation des geringeren Kreatininwerts nach Everolimustherapie als Folge einer geringeren Muskelmasse plausibler als eine tatsächlich bessere Nierenfunktion bei Tieren, die Everolimus statt Placebo erhalten haben. Auch das BUN zeigt keine signifikanten Unterschiede zwischen den Gruppen. Dies ist in erster Linie bei intravasalem Volumenmangel erhöht. Unter der Annahme, dass alle ansonsten gesunden Versuchstiere, die stets freien Zugang zu Wasser hatten, während des gesamten Versuchszeitraums in der Regel euvoläm waren, ist auch hieraus kein Rückschluss auf eine unterschiedliche Nierenfunktion in den verschiedenen Gruppen möglich.

Everolimus führte zu einer signifikanten Zunahme von Erythrozytenzahl, Hämoglobin und Hämatokrit und der Neutrophilenzahl, jedoch zu einer signifikanten Abnahme sowohl der Thromobozytenzahl als auch der Leukozyten- und Lymphozytenzahlen. Die massive Auswirkung auf die Lymphozytenzahlen sowie die Thrombopenie als unerwünschte Wirkung sind aus der klinischen Anwendung von Everolimus bekannt. In klinischen Studien bewirkte Everolimus jedoch eine Tendenz zur Anämie. Eine mögliche Erklärung ist der wesentliche höhere Anteil, den die lymphoide Zellreihe bei der Ratte im Vergleich zum Menschen an der Hämatopoese einnimmt.

In den [^{99m}Tc]Tc-MAG3-Szintigraphien konnte in Woche 16 in den Gruppen mit Everolimus ein höherer und späterer Peak der spezifischen Aktivität in den Nieren beobachtet werden. Allerdings war die Abnahme der relativen Aktivität in den Nieren während der weiteren Untersuchungszeit also die sogenannte „late excretion“ vergleichbar. Da [^{99m}Tc]Tc-MAG3 in erster Linie tubulär sezerniert wird, lässt sich hieraus eine bessere Funktion der Tubuli nach der Behandlung mit Everolimus ableiten. Einleuchtend erscheint diese Interpretation zumindest für den Vergleich der Gruppen 3 und 4, die beide PRRT erhalten hatten. [^{177}Lu]Lu-DOTA-TATE wird tubulär in Abhängigkeit vom Megalin-Rezeptor resorbiert. Es ist bekannt, dass die Expression von Megalin durch Everolimus inhibiert wird. Somit könnte in den Tieren, die die Kombinationstherapie erhalten haben, die in den Tubuli absorbierte Strahlendosis geringer gewesen sein als in den Tieren mit PRRT und Placebo. Leider wurde im Rahmen der hier dargestellten Versuche keine Autoradiographie oder renale Dosimetrie durchgeführt, die diese Erklärung stützen könnte.

Demgegenüber zeigten die Nieren in Gruppe mit der Kombinationtherapie in der histopathologischen Untersuchung stärkere Schädigungen als in der Gruppe, die das Placebo und die PRRT erhalten hatte. Auch in den Gruppen, die keine PRRT erhalten hatten, erschienen die Nieren nach Everolimus stärker morphologisch verändert als nach dem Placebo. Jedoch waren diese Unterschiede insgesamt nicht statistisch signifikant. Insgesamt waren die Unterschiede in den gemessenen Parametern gering ausgeprägt. Wir folgern daraus, dass zumindest in diesem Modell die Nephrotoxizität der PRRT mit [^{177}Lu]Lu-DOTA-TATE nicht wesentlich durch die gleichzeitige Therapie mit Everolimus gesteigert war.

Auch wenn das Versuchsziel in der Untersuchung mit Tumor-tragenden Mäusen primär die Untersuchung der Effektivität der Kombinationstherapie war, wurden zum Toxizitätsmonitoring auch hier das Blutbild quantitativ und die Nieren histopathologisch untersucht. Die durch Everolimus hervorgerufenen Veränderungen waren nicht statistisch signifikant. Allerdings konnte nach der Therapie mit PRRT eine signifikante Zunahme sowohl der Neutrophilen- als auch der Lymphozytenzahl beobachtet werden. Ein solcher Effekt ist in der Untersuchung an Ratten nicht aufgefallen und möglicherweise eine temporäre Veränderung, da der Abstand zwischen Therapie und Versuchsabbruch in der Untersuchung an Mäusen wesentlich geringer war.

In der Histologie wurde wie erwartet eine signifikante Nephrotoxizität nach PRRT beobachtet. Diese wurde durch die Kombination mit Everolimus nicht zusätzlich gesteigert.

Die AR42J-Tumoren wuchsen in den Tieren, die mit PRRT behandelt wurden, zunächst deutlich langsamer. Dadurch konnten diese Tiere vom Therapiebeginn an insgesamt 4 Wochen nachbeobachtet werden, während die Beobachtungsdauer bei Mäusen ohne PRRT bis zur Beendigung des Versuches bei maximal 2 Wochen lag. Die in dieser Zeit sowohl mittels Messschieber als auch durch Auswertung der [^{68}Ga]Ga-DOTA-TATE-PET bestimmten Tumorvolumina zeigten eine gute Übereinstimmung und waren an Tag 19 in den Tieren mit PRRT signifikant geringer als in den Tieren ohne PRRT. Zwischen Everolimus und Placebo bestand kein signifikanter Unterschied.

Die Tumor-to-Background-Ratio war unter der Behandlung mit Everolimus höher als unter Placebo, auch wenn dies wie erwähnt keinen zusätzlichen therapeutischen Nutzen brachte.

Insgesamt lässt sich aus der vorliegenden Untersuchung kein klarer Hinweis auf eine verbesserte Effektivität durch die Kombination von Everolimus und [^{177}Lu]Lu-DOTA-TATE ableiten. Vielmehr ist auch ein gewisses Signal in Richtung einer erhöhten Toxizität zu beachten, auch wenn dieses nicht signifikant ist. Zur Optimierung der systemischen Therapie bei neuroendokrinen Tumoren ist daher aus unserer Sicht dieser Ansatz leider nicht sehr vielversprechend.

3. Summary

3.1. Experimental procedure

In the present work, the toxicity and efficacy of a possible combination therapy of everolimus and [^{177}Lu]Lu-DOTA-TATE were examined in two successive trials. The detailed experimental plans are presented in the two corresponding publications (35, 36) and are briefly summarized below: Female Lewis rats were used as a model organism for the toxicity study. They were divided into four groups, of which the first two received no PRRT, group 1 received a placebo provided by the manufacturer of everolimus, and group 2 received everolimus. Groups 3 and 4 were treated with [^{177}Lu]Lu-DOTA-TATE, group 3 additionally with the placebo, group 4 with everolimus. Until the end of the observation period, only laboratory chemical tests were carried out on half of the rats in each group, namely the determination of creatinine and BUN initially once a week and between weeks 8 and 16 every 14 days. In addition, a differential blood count was performed after 16 weeks at euthanasia. The other half of the rats were used for sequential planar [^{99m}Tc]Tc-MAG3 scintigraphies to examine tubular function over time. An autopsy with fixation of the kidneys was carried out in all animals at the end of the sixteen-week observation period. To plan the study of efficacy, a pilot test was first carried out with the already known model organism (female Lewis rats). Unfortunately, there was no growth of the pancreatic, SSTR-2-positive, rat-derived cell line AR42J. In the next step, immunodeficient nude rats were used (Rowett Nude, RNU). Although tumor growth was observed, its dynamics differed significantly between the test animals. Therefore, it would not have been sensible to determine the dates of the planned [^{177}Lu]Lu-DOTA-TATE PET scans in advance. For this reason, the switch to nude mice (CD-1) was made. The dynamics of tumor growth was high, but sufficiently homogeneous in the examined animals so that an experimental plan could be designed. This included vaccination with tumor cells and a baseline [^{68}Ga]Ga-DOTA-TATE PET scan five days later. The animals were divided into four groups as in the toxicity experiments. Immediately after the initial scan, the mice from groups 3 and 4 received [^{177}Lu]Lu-DOTA-TATE at a dose of 200 MBq, and treatment with everolimus or placebo was started in all animals. The tumor growth was monitored percutaneously using a caliper and a [^{68}Ga]Ga-DOTA-TATE PET was carried out again after one, two and four weeks. If tumor growth was excessive, the animals were removed from the experiment,

a blood count was taken to monitor toxicity and the kidneys were histopathologically examined.

3.2. Results and interpretation

The animals in the groups that received everolimus gained weight more slowly. The creatinine concentration, a marker of kidney function, also increased less in these groups during the observation period. Creatinine is a breakdown product in muscle metabolism and the creatinine concentration generally depends on muscle mass. Therefore, an interpretation of the lower creatinine value after everolimus therapy resulting from lower muscle mass seems more plausible than an actual improved kidney function in animals that received everolimus instead of placebo. The BUN also showed no significant differences between the groups. This is primarily increased due to intravascular volume deficiency. Assuming that all otherwise healthy test animals that always had free access to water were generally euvolemic throughout the entire experimental period, it is not feasible to draw any conclusions about different kidney function in the different groups.

Everolimus resulted in a significant increase in erythrocyte counts, hemoglobin and hematocrit, and neutrophil counts, but a significant decrease in both platelet counts and leukocyte and lymphocyte counts. The massive impact on lymphocyte counts and thrombocytopenia as an undesirable effect are known from the clinical use of everolimus. However, in clinical trials, everolimus caused a tendency toward anemia. A possible explanation is the significantly higher proportion that the lymphoid cell line plays in hematopoiesis in rats compared to humans.

In the [^{99m}Tc]Tc-MAG3 scintigraphs, a higher and later peak of specific activity in the kidneys was observed at week 16 in the everolimus groups. However, the decrease in relative activity in the kidneys during the further examination period, the so-called “late excretion”, was comparable. Since [^{99m}Tc]Tc-MAG3 is primarily secreted in the tubules, this suggests that the tubules function better after treatment with everolimus. This interpretation seems plausible, at least for the comparison of groups 3 and 4, both of which had received PRRT. [^{177}Lu]Lu-DOTA-TATE is absorbed tubularly depending on the receptor megalin. Megalin expression is known to be inhibited by everolimus. Thus, in the animals that received the combination therapy, the radiation dose absorbed in

the tubules may have been lower than in the animals that received PRRT and placebo. Unfortunately, no autoradiography or renal dosimetry was performed in the experiments presented here that could support this explanation.

In contrast, the kidneys in the group that received the combination therapy showed greater damage in the histopathological examination than in the group that received the placebo and PRRT. Even in the groups that did not receive PRRT, the kidneys appeared more morphologically changed after everolimus than after placebo. However, these differences were not statistically significant. Overall, the differences in the measured parameters were minor. We conclude that, at least in this model, the nephrotoxicity of PRRT with [^{177}Lu]Lu-DOTA-TATE was not significantly increased by concurrent therapy with everolimus.

Even though the aim of the experiment in the study with tumor-bearing mice was primarily to investigate the effectiveness of the combination therapy, the blood count was also examined quantitatively and the kidneys histopathologically examined to monitor toxicity. The changes induced by everolimus were not statistically significant. However, after therapy with PRRT, a significant increase in both neutrophil and lymphocyte counts was observed. Such an effect was not noticed in the study on rats and may be a temporary change, as the interval between therapy and termination of the experiment was significantly smaller in the study on mice.

As expected, significant nephrotoxicity after PRRT was observed in histology. This was not further increased by the combination with everolimus.

The AR42J tumors initially grew significantly slower in the animals treated with PRRT. This meant that these animals could be followed up for a total of 4 weeks from the start of therapy, while for mice without PRRT it was a maximum of 2 weeks. The tumor volumes determined during this time both using calipers and by evaluating the [^{68}Ga]Ga-DOTA-TATE-PET showed good agreement and were significantly lower on day 19 in the animals with PRRT than in the animals without PRRT. There was no significant difference between everolimus and placebo.

The tumor-to-background ratio in mice treated with Everolimus was higher than in those receiving placebo, although as mentioned this did not provide any additional therapeutic benefit.

Overall, no clear indication of improved efficacy through the combination of everolimus and [^{177}Lu]Lu-DOTA-TATE can be derived from the present study. Rather, a certain signal towards increased toxicity should be considered, even if it is not significant. In our opinion, the investigated approach is unfortunately not very promising for optimizing systemic therapy for neuroendocrine tumors.

4. Beitrag zu den Publikationen

4.1. Beitrag zur Publikation „Toxicity of a combined therapy using the mTOR-inhibitor everolimus and PRRT with [^{177}Lu]Lu-DOTA-TATE in Lewis rats”

Erstellung eines Zeitplans für die Versuchsdurchführung. Organisation des Zugangs zu erforderlichen Ressourcen (Platz im Tierstall, Zeitliche Verfügbarkeit technischer Assistenten und der Gammakamera). Koordinierung der Herstellung der Radiopharmaka. Kontaktherstellung zur Arbeitsgruppe für vergleichende experimentelle Pathologie der Technischen Universität München (PD Dr. med. vet. Katja Steiger und Dr. Hsi-Yu Yen).

Schulungen und Qualifikationsnachweis in der Versuchstierkunde und im Strahlenschutz. Bestellung und Betreuung der Versuchstiere. Verabreichung der Pharmaka und Radiopharmaka, Blutabnahmen, Narkotisierung der Versuchstiere, Durchführung der Szintigraphien. Prozessierung und Auswertung der Rohdaten. Erstellung von Matlab-Code zur Datenextraktion und Berechnung der Fractional Uptake Rate (FUR).

Euthanasie der Ratten am Versuchsende, Organentnahme und Konservierung, Einbettung.

Dateneingabe, Visualisierung und statistische Auswertung sowie Interpretation der erhobenen Daten.

Verfassung des Manuskripts und Bearbeitung der Kommentare im Review-Prozess.

4.2. Beitrag zur Publikation „Evaluation of the Efficacy of a Combined Treatment Using the mTOR-Inhibitor Everolimus and [^{177}Lu]Lu-DOTA-TATE in Nude CD1 Mice with SSTR-Expressing Pancreatic AR42J Xenograft Tumors”

Versuchsdurchführung wie im ersten Teilversuch. Erstellung eines konkreten Zeitplans, Organisation der Versuche. Bearbeitung laufend anfallender Aufgaben im Umgang mit den Versuchstieren.

Inokulation der Versuchstiere mit den AR42J-Tumorzellen.

Herstellung des PET-Tracers [^{68}Ga]Ga-DOTA-TATE im Heißlabor, Qualitätskontrolle mittels High-Performance-Liquid-Chromatography (HPLC). Im Rahmen der PET-Messungen Durchführung der Inhalationsnarkose, Anlage eines venösen Gefäßzugangs und Verabreichung des Tracers. Nachprozessierung und Auswertung der Messdaten. Euthanasie der Versuchstiere sowie Konservierung und Einbettung der Organe.

Statistische Auswertung der erhobenen Daten. Erstellung graphischer Darstellungen. Interpretation der Daten. Verfassung des Manuskripts zur Veröffentlichung. Einreichen des Manuskripts bei einem wissenschaftlichen Journal und Beantwortung der Kommentare im Rahmen des Review-Prozesses zusammen mit meinem Betreuer.

5. Veröffentlichung I

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Toxicity of a combined therapy using the mTOR-inhibitor everolimus and PRRT with [^{177}Lu]Lu-DOTA-TATE in Lewis rats

Johannes Zellmer[1], Hsi-Yu Yen[2,3,4], Lena Kaiser[1], Erik Mille[1], Franz Josef Gildehaus[1], Guido Böning[1], Katja Steiger[2,4], Marcus Hacker[5], Peter Bartenstein[1], Andrei Todica[1], Alexander R. Haug[5] and Harun Ilhan[1*]

Abstract

Purpose: Peptide receptor radionuclide therapy (PRRT) with [^{177}Lu]Lu-DOTA^{0},TYR^{3}-octreotate ([^{177}Lu]Lu-DOTA-TATE) and the mechanistic target of rapamycin (mTOR) inhibitor everolimus are both approved for the treatment of neuroendocrine tumours (NET). However, tumour progression is still frequent, and treatment strategies need further improvement. One possible approach could be to combine different therapy options. In this study, we investigated the toxicity of a combined treatment with everolimus and [^{177}Lu]Lu-DOTA-TATE in female Lewis rats.

Methods: Animals received 200 MBq of [^{177}Lu]Lu-DOTA-TATE once and/or 5 mg/kg body weight everolimus or placebo weekly for 16 weeks and were divided into four groups (group 1, placebo; group 2, everolimus; group 3, placebo + [^{177}Lu]Lu-DOTA-TATE; group 4, everolimus + [^{177}Lu]Lu-DOTA-TATE). Blood levels of creatinine and blood urea nitrogen (BUN) were assessed weekly to monitor nephrotoxicity, and a full blood count was performed at the time of euthanasia to monitor myelotoxicity. Additionally, renal function was analysed by sequential [^{99m}Tc]Tc-mercaptoacetyltriglycine ([^{99m}Tc]Tc-MAG3) scintigraphies. Histopathological examination was performed in all the kidneys using a standardized renal damage score (RDS).

Results: Rats receiving everolimus showed a significantly lower increase in creatinine levels than those receiving placebo. Everolimus therapy reduced white blood count significantly, which was not observed for [^{177}Lu]Lu-DOTA-TATE. Functional renal scintigraphies using [^{99m}Tc]Tc-MAG3 showed a compromised initial tracer uptake after PRRT and slower but still preserved excretion after everolimus. Histology showed no significant RDS differences between groups.

Conclusion: Renal scintigraphy is a highly sensitive tool for the detection of renal function impairment after a combination of everolimus and PRRT. Additional treatment with everolimus does not increase renal and haematological toxicity of PRRT with [^{177}Lu]Lu-DOTA-TATE.

Keywords: [^{177}Lu]Lu-DOTA-TATE, PRRT, Everolimus, Scintigraphy, Renal clearance

Introduction

Neuroendocrine tumours (NET) are a relatively rare entity of malignancies with increasing incidence and prevalence during the last decades [1, 2]. Around 20% of patients present with metastatic disease at the time of diagnosis and up to 38% during follow-up [1]. As opposed to localized NET, where surgical resection represents a curative approach, the therapy of advanced, metastatic NET remains challenging, and the median survival is reported to be about 12 months [1].

The novel targeted drugs sunitinib, everolimus and telotristat etiprate, which were highly effective in randomized controlled trials, complement pharmacologic

* Correspondence: harun.ilhan@med.uni-muenchen.de
[1]Department of Nuclear Medicine, University Hospital, Ludwig-Maxilimians-University Munich, Munich, Germany
Full list of author information is available at the end of the article

therapeutic options such as chemotherapy and the use of somatostatin analogues [3–5].

Peptide receptor radionuclide therapy (PRRT) with [^{90}Y]Y-DOTA^{0},Tyr^{3}-octreotide [^{90}Y]Y-DOTA-TOC or [^{177}Lu]Lu-DOTA^{0},TYR^{3}-octreotate ([^{177}Lu]Lu-DOTA-TATE) has been successfully performed for almost 30 years. Recently, Strosberg et al. reported significantly longer progression-free survival for patients with advanced, metastatic midgut NETs treated with [^{177}Lu]Lu-DOTA-TATE in the randomized, multi-centric phase-III NETTER-1 trial [6], which led to the approval of [^{177}Lu]Lu-DOTA-TATE by the FDA and EMA. However, up to now combined therapy algorithms have not been evaluated in larger cohorts. A possible approach could be the administration of two or more different agents simultaneously. Since everolimus is known to increase the radiosensitivity in solid tumours treated with external radiation therapy [7, 8], the effects of PRRT and everolimus might be potentiated. These considerations gave rise to the phase-I NETTLE study exploring the maximum tolerated dose of everolimus in a combined therapy with [^{177}Lu]Lu-DOTA-TATE [9]. In a small cohort of patients who received a standard PRRT regime, no severe adverse effects where seen up to a daily administered dose of 7.5 mg everolimus. However, groups examining the effect of such combined therapies showed that the combination is less effective and can even promote metastasis in preclinical models using the tumour cell line CA20948 [10, 11]. Moreover, there are several adverse effects for both therapies such as haemato- and nephrotoxicity, which also have to be taken into consideration. Using PRRT with [^{177}Lu]Lu-DOTA-TATE, haematotoxicity is rare, and dose limiting nephrotoxicity can be reduced by co-administration of basic amino acids [6]. Nonetheless, so far the augmentation of these toxicities using [^{177}Lu]Lu-DOTA-TATE in combination with everolimus has not been analysed in detail, yet. The aim of this work is to evaluate the toxicity of this combined treatment in a rat model using [^{99m}Tc]Tc-mercaptoacetyltriglycine ([^{99m}Tc]Tc-MAG3) scintigraphies for the longitudinal evaluation of renal function, laboratory chemical analyses (blood count, creatinine, blood urea nitrogen) to further assess nephro- and haematotoxicity as well as a histopathologic preparation and microscopic analysis of the kidneys to assess morphological damages to this organ.

Methods

Animals and experimental design

All animal experiments were conducted in accordance with institutional guidelines and approved by the Administrative Panel on Laboratory Animal Care (Government of Upper Bavaria, Germany). We used non-tumour bearing female Lewis rats (Charles River Laboratories, Sulzfeld, Germany), aged 10 weeks, with a median weight of 207 g, which were fed a standard diet and given free access to water. The body weight of all animals was monitored weekly. Animals were divided into four groups. Group 1 (n = 15) received placebo, group 2 (n = 17) everolimus (5 mg/kg body weight once weekly), group 3 (n = 14) a combination of placebo (once weekly) and [^{177}Lu]Lu-DOTA-TATE (single injection at the start of the study, mean 200 MBq, range 191–207 MBq) and group 4 (n = 16) a combined treatment with everolimus (5 mg/kg weekly) and a single injection of [^{177}Lu]Lu-DOTA-TATE at the start ot the study (mean 200 MBq; range 195–212 MBq). Based on the experience of Pool et al., the administered activity of 200 MBq [^{177}Lu]Lu-DOTA-TATE represents a trade-off between low and high dose therapy and a potential curative dose after a single injection [11]. Renal function was monitored weekly (respectively every 14 days after week 8) by determination of creatinine and blood urea nitrogen (BUN) in the blood serum by drawing approximately 0.5 ml blood from a tail vein. At the end of the observation period, blood samples from the heart were collected to assess the full blood count. Furthermore, renal function of the rats was evaluated with serial [^{99m}Tc]Tc-MAG3-scintigraphies in the remaining half of rats. A baseline scan was performed in a group of 21 randomly chosen, otherwise untreated rats 1 week before the start of the actual treatment. Control MAG3 scans were performed in all animals in the four groups (group 1: n = 7, group 2: n = 7, group 3: n = 6, group 4: n = 8) 1, 6, 11 and 16 weeks after the start of the treatment. Laboratory studies (n = 8 in groups 1, 3 and 4; n = 10 in group 2) and renal scintigraphies were performed in different animals of the same group. All animals were euthanized 16 weeks after the start of the treatment, and the kidneys were prepared for the histopathological examination. No animal had to be euthanized due to severe toxicity prior to the endpoint of 16 weeks post treatment.

Laboratory chemical analysis

Creatinine and BUN levels in the serum were quantified to monitor kidney function. A total blood count was performed right before euthanasia of animals at the end of the study. All laboratory analyses were performed according to the manufacturer's protocols and standardized methods at the Institute of Laboratory Medicine of the Medical Centre of the University of Munich. Blood was not diluted. Serum creatinine and BUN concentrations were measured using an Olympus AU5400 analyser (Beckman-Coulter) using the creatinine reagent OSR6178 and the urea reagent ORS6578. Blood count analysis was performed using an XN-2000 analyser (Sysmex, Kobe, Japan). All analyses were performed according to the manufacturer's protocols.

Pharmaceuticals and radiopharmaceuticals

Everolimus (formerly known as RAD001) and placebo were kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). We applied a weekly dose of 5 mg/kg body weight chosen in accordance with previously published data for single agent treatment [12]. The pharmaceuticals were freshly prepared from the pre-concentrate once weekly right before oral gavage. In accordance with the manufacturer's manual, the everolimus pre-concentrate was diluted with 5% glucose solution to a concentration of 2 mg/ml corresponding to an administered volume of ~ 0.5 ml. Equivalent amounts of pre-concentrate and glucose solution were used for the preparation of the placebo solution. ^{99m}Tc-mercaptoacetyltriglycine was purchased from Covidien, Neustadt/Donau, Germany, and prepared according to the manufacturer's manual. No carrier added ^{177}Lu was obtained from Isotope Technologies Garching GmbH (Garching, Germany). DOTA^0,TYR^3-octreotate was obtained from ABX advanced biochemical compounds (Dresden, Germany). Radiolabeling was performed using 125 µg DOTA^0,TYR^3-octreotate according to a previously described protocol [13]. Quality control was performed using thin layer chromatography and HPLC. Radiochemical yield 99.9% and purity > 99.5% (molar activity GBq/mol). All radiopharmaceuticals were administered via the tail vein (with an administered volume of ~ 0.5 ml).

Renal scintigraphy

[^{99m}Tc]Tc-MAG3-scintigraphy was performed as described in previously published protocols [14–17]. Inhalational anaesthesia with 2.0% of isoflurane in pure oxygen was induced and maintained with a concentration of 1.5%. Rats received a standard dose of [^{99m}Tc]Tc-MAG3 (50 MBq) solved in 0.3 ml of sterile saline as a bolus via tail vein. Whole body scintigraphic recordings were initiated at the moment of tracer administration. One head of a triple-headed gamma camera (Philips–former Picker–Prism 3000 XP, Cleveland, USA) equipped with a LEHR collimator was on hand. The dynamic planar acquisitions consisted of 420 frames of 5 s each to a total of 35 min. For the baseline scans, 240 frames (20 min) were acquired to reduce the duration of anaesthesia.

In order to analyse generated data sets, the Hermes Dynamic Study display software V4.0 was used (Hermes Gold V2.10, Hermes Medical Solutions, Stockholm/London). Standardized regions of interest (ROI) was drawn for the whole body, both the kidneys, peri-renal background reference regions, the bladder, blood pool in the heart and the site of injection [15]. Further, dynamic data sets of the ROIs were used to create renograms using Microsoft Excel, which depicted the proportion of the kidney activity corrected for the background regions and the whole-body activity corrected for the injection site. The baseline renograms were extrapolated to 35 min using a monoexponential fit of the excretion phase. Eventually, the parameters 'time to peak', 'peak', 'IA10min' and 'Delta10min' were extracted from the renograms.

Furthermore, the fractional uptake rate (FUR) was calculated to assess renal clearance from the renograms [16]. FUR is defined as the fractional uptake of a tracer in the blood by an organ per time unit and can be calculated in the following way: $FUR = P(0) \times (k_l + k_r)/[IA]$. $P(0)$ was obtained by extrapolating backwards, using a mono-exponential fit of $P(t)$. The figures k_l and k_r are the slopes of the linear uptake (LU) segment of the Patlak-Rutland (PR) plots for the left and right kidneys [17].

Histopathological analysis

After the kidneys were fixed in 10% neutral-buffered formalin solution, they were dehydrated under standard conditions and embedded in paraffin. All blocks were cut into 2 µm slices. Selected slices were stained with Periodic acid–Schiff (PAS), adjacent ones with Haematoxylin-Eosin (HE) according to standard protocols. Subsequently, renal damage was classified according to Rolleman's grading scale using a renal damage score (RDS) ranging from grade 0 (no damage) to grade 4 (severe damage) [18, 19]. Briefly, evaluation criteria included the following:

Grade 1—inflammatory infiltrate in the glomeruli, little dilatation of tubules; no basal membrane thickening or protein cylinders

Grade 2—same criteria as for grade 1, however in addition rough protein staining, more pronounced dilation of tubules, basal membrane thickening and mitotic activity; very little protein cylinders in tubules

Grade 3—same criteria as for grade 2, however additional shrinkage in a small number of glomeruli, smaller vascular lumina flat or lost tubular epithelium, strong tubule dilatation and more pronounced basal membrane thickening; more protein cylinders

Grade 4—same criteria as for grade 3, however increased shrinkage of glomeruli leading to optical emptiness; strongly dilated tubules with massive protein cylinders and signs of peripheral fibrosis

The findings of the histopathological examination were recorded using the Excel sheet.

Statistical analysis

Data are expressed as the means of the treatment groups and the corresponding 95% confidence intervals. A p value of $p < 0.05$ was considered as statistically significant. Normality and homogeneity of variance were tested using the Shapiro-Wilk test and Levene's test. To

adjust for multiple testing, two-way analysis of variance (ANOVA) was carried out for parameters measured only once at the end of the observation period. When normality and/or homogeneity requirements were not met, the Scheirer-Ray-Hare (SRH) test was used, with the administration of everolimus or placebo as one and the treatment with or without [^{177}Lu]Lu-DOTA-TATE as the second factor in both cases. For repeatedly measured parameters from the blood sampling, the ANOVA or SRH test was applied for the individual differences between the first and last measurement. By covering all events of a certain parameter (in our case the values of all animals in all our groups rather than only the animals of two specific groups), the validity of the tests used is increased. Moreover, the added value of ANOVA and SRH lies in the evaluation of an over-additive or synergistic effect by analysing the impact of a combination of PRRT and everolimus. Statistical analysis of scintigraphy results was performed after obtaining and averaging baseline parameters. Means at baseline were considered as the population standard. *T*-tests were conducted versus the population standard for the average of each group in the follow-up scintigraphies. Pearson's chi-squared test was used to test for differences among the ordinally scaled values of the histological grading.

Results

Body weight

No animal had to be sacrificed due to weight loss. Groups receiving everolimus showed slower weight gain than the corresponding groups receiving placebo. Table 1 gives an overview of the mean bodyweight at baseline and week 16 and the corresponding differences. ANOVA showed that everolimus was significantly associated with slower weight gain (p = 0.009), whereas there was no significant impact for [^{177}Lu]Lu-DOTA-TATE (p = 0.133) or the combination of everolimus and [^{177}Lu]Lu-DOTA-TATE (p = 0.809).

BUN, creatinine and blood count during follow-up

Tables 2 and 3 show the mean values and confidence intervals at baseline and at week 16 at the end of the study and the means of their individual differences. For the differences in BUN levels, no significant influence of the factors everolimus (p = 0.166) and [^{177}Lu]Lu-DOTA-TATE (p = 0.894) or their interaction (p = 0.397) was found. In contrast, the increase in serum creatinine levels was significantly lower in the groups receiving everolimus (p = 0.023). No significant differences were found for the factor [^{177}Lu]Lu-DOTA-TATE (p = 0.185) or the interaction of both factors (p = 0.308).

The results of the total blood count at week 16 are shown in Table 4. The mean values of red blood cell (RBC) count, haemoglobin and haematocrit showed similar trends among the different groups. Everolimus treatment had a significant impact on all three parameters, whereas no significant impact was found for [^{177}Lu]Lu-DOTA-TATE. Animals treated with everolimus showed higher RBC counts than those treated with placebo (p < 0.001). [^{177}Lu]Lu-DOTA-TATE treatment resulted in a non-significant reduction of RBC (p = 0.063) compared to animals without PRRT. The increase of reticulocytes rate due to everolimus was not significant (p = 0.085), whereas platelet counts were reduced significantly by everolimus (p = 0.043) and non-significantly by [^{177}Lu]Lu-DOTA-TATE treatment (p = 0.577). Two-way ANOVA showed a significant reduction in the number of leucocytes (white blood cells, WBC) in the everolimus group compared to placebo (p = 0.029). There was no significant effect for [^{177}Lu]Lu-DOTA-TATE (p = 0.508). Correspondingly, the impact of everolimus on WBC was significant (p = 0.002) both in the single treatment and combination group, whereas therapy with [^{177}Lu]Lu-DOTA-TATE had no significant impact (p = 0.628). Regarding neutrophil counts, the impact of everolimus was significant (p = 0.028) whereas [^{177}Lu]Lu-DOTA-TATE had no significant impact (p = 0.764). This was also the case in the combination of everolimus and [^{177}Lu]Lu-DOTA-TATE (p=0.854). Both factors had no significant impact on monocyte counts. Using ANOVA and SRH, no statistically significant interactions were detected for any of the aforementioned parameters.

Scintigraphy

Figure 1 illustrates the renograms in the various groups 16 weeks after start of each treatment compared to baseline values. Results of the scans at week one, six and eleven are not shown. As described previously, preserved renal function is observed by a fast and steep increase of [^{99m}Tc]Tc-MAG3 in the kidneys with rapid excretion as well as preserved FUR values comparable to baseline

Table 1 Mean bodyweight at baseline, week 16 and corresponding differences with confidence intervals

Bodyweight (g)	Baseline	Week 16	Difference (%)
Placebo	204.0 ± 7.5	230.2 ± 10.5	12.8 ± 2.7
Everolimus	204.7 ± 5.2	222.9 ± 5.6	9.0 ± 2.9
Placebo + [^{177}Lu]Lu-DOTA-TATE	208.4 ± 3.8	230.5 ± 10.1	10.6 ± 4.5
Everolimus + [^{177}Lu]Lu-DOTA-TATE	208.4 ± 4.5	221.1 ± 10.8	6.0 ± 4.0

Table 2 Mean BUN at baseline, week 16 and corresponding differences with confidence intervals

BUN (mg/dl)	Baseline	Week 16	Difference (%)
Placebo	16.9 ± 2.1	19.4 ± 1.8	19 ± 27
Everolimus	15.7 ± 2.0	18.3 ± 1.2	15 ± 11
Placebo + [^{177}Lu]Lu-DOTA-TATE	17.9 ± 1.7	19.1 ± 2.2	7 ± 10
Everolimus + [^{177}Lu]Lu-DOTA-TATE	16.9 ± 1.3	19.9 ± 1.1	19 ± 12

[16, 17]. The renal curve in group 1 (placebo) is almost unchanged compared to baseline. Whereas the initial slope and late excretion in group 2 (everolimus) is also comparable to baseline, the peak is slightly higher (p = 0.063) and delayed (p = 0.621). The initial slope of both PRRT groups 3 (placebo + [^{177}Lu]Lu-DOTA-TATE) and 4 (everolimus + [^{177}Lu]Lu-DOTA-TATE) is less steep compared to baseline and to groups 1 and 2. This is reflected by significantly lower FUR values at day 112 (see Fig. 2; p = 0.003 for group 3 and p = 0.002 for group 4 vs. baseline). Compared to placebo, the administration of everolimus induces a later and higher peak, as already demonstrated between groups 1 and 2. The late excretion appears to be preserved.

Histopathology

Figure 3 shows microscopic images of the kidney sections, which are representative for each group (1–4). Each kidney was classified based on the RDS. For the glomeruli, a minimal to slight cell reduction and glomeruli shrinkage was observed in four animals of group 1 and a minimal to moderate in almost all animals of groups 2, 3 and 4. In the tubules, a minimal to marked cell damage, respectively loss of epithelium, was perceived in all animals in all groups. A minimal to marked tubules dilatation was detected in all animals in group 1 and slight to marked tubules dilatation in all animals in groups 2, 3 and 4. A minimal focal inflammation in one animal in group 2 and minimal to slight mononuclear inflammation was found in seven animals in group 3 and group 4. Slight BM thickening, a minimal focal to multifocal protein cylinder as well as a minimal to slight vacuolization was observed in most animals of all groups. Regeneration was solely found in six animals of group 4 and one animal of group 3. Additionally, spontaneous and background lesions, as for example pelvis dilatation, small cysts and minimal focal hemosiderosis, occurred occasionally. According to the renal damage score criteria of Rolleman et al., group 1 has the lowest average score (RDS 2.94), followed by group 3 (3.19), group 2 (3.25) and group 4 (highest score, 3.31). The obtained grading values were used to calculate means for all four groups and 95% confidence intervals, which are displayed in Table 5. The lowest average renal damage score was found in the group receiving placebo only, higher damage scores in groups 2 to 4. However, Pearson's chi-squared test showed no significant difference between groups (p = 0.395).

Discussion

The range of therapeutic options in advanced or metastatic NET is limited. If possible, metastasis resection or ablative techniques are used. For patients inappropriate for the aforementioned strategies, medical options can be somatostatin analogues, interferon-α, chemotherapy, Sunitinib or everolimus [20, 21]. In the RADIANT-3 trial, the median progression-free survival of patients treated with everolimus was 11.0 months compared to 4.6 months under placebo treatment. [^{177}Lu]Lu-DOTA-TATE plus standard dose octreotide LAR has shown to be effective in midgut NETs [6]. As objective response rates are low (5% for everolimus in p-NETs, 18% for [^{177}Lu]Lu-DOTA-TATE + octreotide LAR in midgut NETs) [6, 22], there is still need for optimizing therapeutic strategies, for example by combining established therapies. There has been some effort to combine other targeted agents with everolimus, but studies show either inacceptable toxicities [23] or only moderate clinical activity when using the maximum tolerated doses [24]. As already mentioned, the combination of everolimus and PRRT seems theoretically reasonable, however, due to the proposed synergistic effect and the dissatisfactory results of other combination studies, a combined therapy with everolimus and PRRT can only be used with particular caution. This is the first preclinical study to investigate the potentially aggravated toxicity of a

Table 3 Mean serum creatinine at baseline, week 16 and corresponding differences with confidence intervals

Creatinine (mg/dl)	Baseline	Week 16	Difference (%)
Placebo	0.44 ± 0.05	0.45 ± 0.05	4 ± 13
Everolimus	0.44 ± 0.04	0.42 ± 0.04	− 4 ± 10
Placebo + [^{177}Lu]Lu-DOTA-TATE	0.45 ± 0.05	0.58 ± 0.16	26 ± 25
Everolimus + [^{177}Lu]Lu-DOTA-TATE	0.48 ± 0.04	0.46 ± 0.05	− 1 ± 16

Table 4 Overview of the haematologic parameters measured in the first part of the study at week 16. The ranges mark the 95% confidence intervals

	Placebo	Everolimus	Placebo + [^{177}Lu]Lu-DOTA-TATE	Everolimus + [^{177}Lu]Lu-DOTA-TATE
RBC (10^{12}/l)	8.33 ± 0.20	9.44 ± 0.45	8.05 ± 0.20	8.84 ± 0.35
Haemoglobin (g/l)	144 ± 3	163 ± 7	138 ± 2	153 ± 4
Haematocrit	0.442 ± 0.007	0.500 ± 0.022	0.431 ± 0.011	0.470 ± 0.016
Reticulocytes (‰)	20.5 ± 3.3	23.6 ± 2.5	21.8 ± 2.2	23.1 ± 3.6
Platelets (10^9/l)	597 ± 103	544 ± 118	545 ± 111	506 ± 137
WBC (10^9/l)	4.98 ± 0.86	4.49 ± 0.48	4.94 ± 0.61	4.16 ± 0.63
Neutrophils	0.81 ± 0.35	1.00 ± 0.30	0.71 ± 0.15	0.89 ± 0.13
Monocytes	0.12 ± 0.06	0.13 ± 0.07	0.17 ± 0.09	0.15 ± 0.10
Lymphocytes	4.03 ± 0.65	3.34 ± 0.48	4.03 ± 0.59	3.09 ± 0.57

combined treatment with everolimus and [^{177}Lu]Lu-DOTA-TATE.

Rats receiving everolimus showed a slower weight gain than rats receiving placebo regardless whether it was combined with PRRT or not. This coincides with findings reported by Ramadan et al., who investigated the effects of everolimus on proteinuria in rats [25], which is consistent with the characteristics of everolimus as an inhibitor of cellular proliferation. Nevertheless, since weight gain based on growth processes plays a minor role in treatments with adult patients, this fact should be of minor importance in clinical practice. The altered levels of RBC, WBC and platelet count are not entirely unexpected, since everolimus is not only a cytoreductive but also an immunosuppressive agent and therefore partially modifies bone marrow activity. However, despite reaching statistical significance in our analysis, the changes are very moderate. These findings, as well as the rise in neutrophil counts and the equality of the monocyte counts after administration of everolimus, are in line with observations by Chen et al., who monitored haematological parameters in patients with metastatic breast cancer treated with everolimus [26]. The mechanism of everolimus causing these changes remains unclear. Rolleman et al. showed that PRRT with [^{177}Lu]Lu-DOTA-TATE compromises haemoglobin levels in rats in a dose-dependent manner [19]. In our study, we used a slightly lower dose of [^{177}Lu]Lu-DOTA-TATE, which resulted in a non-significant decline in serum haemoglobin. This indicates that our dose

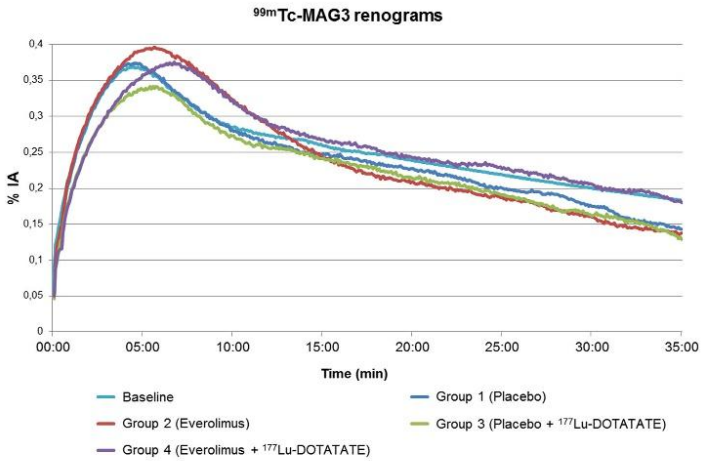

Fig. 1 Renograms at follow-up examination 16 weeks after the beginning of each treatment. Reduced steepness of the initial slope reflects impairment of renal function. For clarity, error bars are not shown. The baseline renogram is extrapolated to 35 min using a mono-exponential fit of the excretion phase

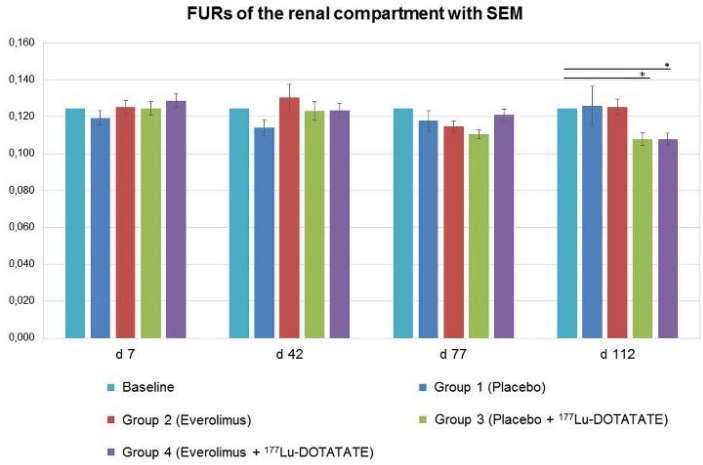

Fig. 2 FUR values of the renal compartment at weeks 1 (day 7), 6 (day 42), 11 (day 77) and 16 (day 112) after the start of each treatment compared to baseline values. A significantly decreased FUR was observed in groups 3 and 4 at day 112 compared to baseline values (*$p < 0.05$). Error bars represent the standard error of the mean

was selected reasonably, and haematotoxicity is increasing measurably when combining PRRT with everolimus. However, due to the effects of both therapies on these parameters, it is difficult to interpret the RBC, haemoglobin and haematocrit levels regarding the haematotoxicity in a combined regime. Both therapies reduce platelet and leucocyte counts. The group receiving the combined therapy showed the lowest group means for these parameters. In this context, the reduction of WBC was shown to be significant when everolimus was applied. These findings indicate that the impairment of both cellular immunity and platelet count might be a relevant issue for future studies on the combination of everolimus and [^{177}Lu]Lu-DOTA-TATE, particularly, as it is difficult to protect the bone marrow from radiation.

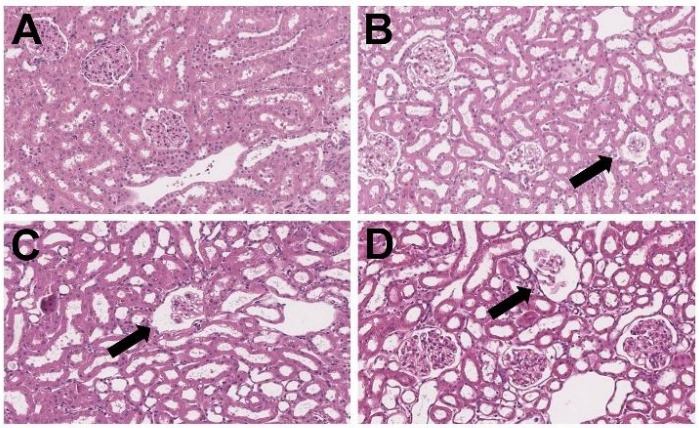

Fig. 3 Microscopic images of the kidneys of an animal in group 1 (**a**), group 2 (**b**), group 3 (**c**) and group 4 (**d**). Tubules appear dilated in all animals, and glomeruli shrinkage was found in most animals (black arrows), except for a small number of animals in group 1 (**a**). (HE, magnification × 15)

Table 5 Renal damage score (RDS) expressed as means and 95% confidence interval

Treatment	RDS
Placebo	2.94 ± 0.15
Everolimus	3.25 ± 0.39
Placebo + [^{177}Lu]Lu-DOTA-TATE	3.19 ± 0.32
Everolimus + [^{177}Lu]Lu-DOTA-TATE	3.31 ± 0.39

In terms of renal damage in the patient setting, kidneys are protected by administrating amino acids, which prevents severe adverse events as shown in the NETTER-1 trial [6]. However, in the present work, no amino acids or other nephroprotective agents were applied to protect the kidneys in order to be able to detect differences in the extent of renal function impairment. Laboratory analysis showed that creatinine levels increase particularly in the group receiving PRRT and placebo. The increase of creatinine levels is significantly lower in rats receiving everolimus, which is in line with better excretion revealed by the later and higher peak in the renograms. This fact may be an indication of nephroprotective characteristics of everolimus in animals treated with PRRT and might be explained by the fact that everolimus can inhibit the expression of the megalin receptor as reported by Gleixner et al. [27], which will reduce the re-uptake of [^{177}Lu]Lu-DOTA-TATE in the proximal tubules. Furthermore, Ramadan et al. confirmed that toxic effects of Adriamycin in rats can be mitigated significantly when everolimus is applied [25]. Additionally, renal scintigraphies showed that the FUR of [^{99m}Tc]Tc-MAG3 was significantly lower after 16 weeks (day 112) in groups 3 and 4, both receiving PRRT regardless whether everolimus was combined or not. Since [^{99m}Tc]Tc-MAG3 is mainly excreted by the proximal tubules, this finding is in accordance with a selective impairment of proximal tubular function after treatment with [^{177}Lu]Lu-DOTA-TATE. Considering the laboratory results for creatinine and BUN, the results of the scintigraphies and the histological analysis of the kidneys, a slight impairment of renal function is caused by [^{177}Lu]Lu-DOTA-TATE, which does not result in significant differences in renal damage scores. Interestingly, in a study on renal toxicity of [^{177}Lu]Lu-DOTA-TATE conducted by Rolleman et al., renal damage scores in untreated control animals were 0.5 on average, which is far below the average score of 2.94 found in rats treated with placebo in our study. Theoretically, a potential explanation of these findings could be a nephrotoxic effect of sequential renal scintigraphies. However, the fact that histological patterns of renal damage were also present animals that were used for laboratory analysis only contradicts this hypothesis. However, repeated application of inhalational anaesthesia with isoflurane, which was used for blood sampling and scintigraphies, could be nephrotoxic by inducing hypotension and, therefore, reducing renal blood flow. Measurements with an additional group of animals without any anaesthesia might be reasonable to verify this hypothesis; however, this was not performed due to restrictions by our institutional guidelines and the Administrative Panel on Laboratory Animal Care of Upper Bavaria. The average RDS, however, is not further compromised in rats treated with everolimus and/or [^{177}Lu]Lu-DOTA-TATE. This is in line with another finding by Rolleman et al. [18]. When analysing the long-term toxicity of the treatment with [^{177}Lu]Lu-DOTA-TATE in rats, no correlation of morphological renal damage and rise in creatinine levels was observed even after application of higher cumulative doses of PRRT. As hypothesized by Rolleman et al., the reason can be a potentially very inhomogeneous functional reserve in the severely damaged kidneys. This effect may also apply for this study, as all the kidneys seem to be strongly affected according to morphological criteria.

In summary, it is to be assumed that renal scintigraphies using [^{99m}Tc]Tc-MAG3 show high sensitivity for the detection of even slight changes of renal function. Nonetheless, our data do not indicate an increased renal or haematological toxicity by a combined treatment with everolimus and [^{177}Lu]Lu-DOTA-TATE compared to the mere treatment with [^{177}Lu]Lu-DOTA-TATE alone.

Conclusion

Our preclinical data on the combined toxicity of [^{177}Lu]Lu-DOTA-TATE and everolimus do not show increased toxicities compared to the monotherapies. Thus, further evaluation of the efficacy of a combined therapy using everolimus and [^{177}Lu]Lu-DOTA-TATE in tumour bearing animals is highly feasible. Potential synergistic anti-tumour effects on AR42J tumour bearing rodents are currently performed at our institution.

Abbreviations

ANOVA: Analysis of variance; BUN: Blood urea nitrogen; DOTA: 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; DOTA-TATE: DOTA^0,Tyr^3-octreotate; DOTA-TOC: DOTA^0,Tyr^3-octreotide; FUR: Fractional uptake rate; HE: Haematoxylin-eosin; LU: Linear uptake; MAG3: Mercaptoacetyltriglycine; mTOR: Mechanistic target of rapamycin; NET: Neuroendocrine tumour; PAS: Periodic acid-Schiff; PR: Patlak-Rutland; PRRT: Peptide receptor radionuclide therapy; RBC: Red blood cell; RDS: Renal damage score; ROI: Region of interest; SRH: Scheirer-Ray-Hare; WBC: White blood cell

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Authors' contributions

JZ performed and analysed the in vivo studies and contributed to the writing of the manuscript and preparation of the figures. HY and KS performed the histopathological analysis. LK, EM and GB contributed to the FUR calculation. FG was responsible for the production of [^{177}Lu]Lu-DOTA-TATE. MH, PB, AT and AR contributed to the study conception and critical

revision of the manuscript. HI designed the study, supervised the practical experiments and finalized the manuscript and figures. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
All experiments were carried out in compliance with the National Guidelines for Animal Protection, Germany, with the approval of the regional Animal Care Committee of the Government of Upper Bavaria (Regierung v. Oberbayern) and were overseen by a veterinarian.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department of Nuclear Medicine, University Hospital, Ludwig-Maxilimians-University Munich, Munich, Germany. [2]Department of Pathology, Technical University of Munich, Munich, Germany. [3]German Cancer Consortium (DKTK), German Cancer Research Centre (DKFZ), Heidelberg, Germany. [4]Comparative Experimental Pathology, Technical University of Munich, Munich, Germany. [5]Department of Biomedical Imaging and Image-guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, Vienna, Austria.

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Publisher's Note

6. Veröffentlichung II

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Evaluation of the Efficacy of a Combined Treatment Using the mTOR-Inhibitor Everolimus and [177Lu]Lu-DOTA-TATE in Nude CD1 Mice with SSTR-Expressing Pancreatic AR42J Xenograft Tumors

Johannes Zellmer [1], Hsi-Yu Yen [2,3,4], Lena Kaiser [1], Franz Josef Gildehaus [1], Guido Böning [1], Katja Steiger [2,4], Marcus Hacker [5], Peter Bartenstein [1], Andrei Todica [1,6], Alexander R. Haug [5] and Harun Ilhan [1,6,*]

1 Department of Nuclear Medicine, University of Munich, 81377 Munich, Germany
2 Department of Pathology, Technical University of Munich, 81377 Munich, Germany
3 German Cancer Consortium (DKTK), German Cancer Research Centre (DKFZ), 69120 Heidelberg, Germany
4 Comparative Experimental Pathology, Technical University of Munich, 81377 Munich, Germany
5 Department of Biomedical Imaging and Image-Guided Therapy, Division of Nuclear Medicine, Medical University of Vienna, 1090 Vienna, Austria
6 Die Radiologie, Private Practice for Radiology, Nuclear Medicine, and Radiation Oncology, 80331 Munich, Germany
* Correspondence: harun.ilhan@med.uni-muenchen.de; Tel.: +49-89-44007-7655

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Abstract: Therapy options for advanced pancreatic neuroendocrine tumors (pNETs) include the mTOR inhibitor everolimus and peptide receptor radionuclide therapy (PRRT) with [177Lu]Lu-DOTA-TATE, however further optimization in the therapeutic landscape is required as response rates are still low. In this study, we investigated the synergistic and potentially enhanced efficacy of a combined treatment with everolimus and [177Lu]Lu-DOTA-TATE in a mouse model. Baseline [68Ga]Ga-DOTA-TATE PET scans were obtained five days after athymic CD1 mice were inoculated with AR42J tumor cells, before separating the animals into four groups. Group 1 received a placebo, group 2 everolimus, group 3 a placebo and PRRT, and group 4 everolimus and PRRT. The treatment response was monitored by manually measuring the tumor volumes (manual tumor volume, MTV) and conducting sequential [68Ga]Ga-DOTA-TATE PET scans at one, two, and four weeks after treatment induction. The biological tumor volume (BTV) was derived from PET scans using threshold-based volume of interest (VOI) measurements. Tracer uptake was measured semi-quantitatively as a tumor to background ratio (TBR). Mice were euthanized due to excessive tumor growth according to the ethics protocol; blood samples were drawn for the preparation of full blood counts and kidneys were obtained for histological analysis. For the histological assessment, a standardized score (renal damage score, RDS) was used. Full blood counts showed significantly increased numbers of neutrophils and lymphocytes in the groups receiving PRRT. All other parameters did not differ relevantly. In the histological analysis, groups receiving PRRT had a significantly higher RDS, whereas everolimus only tended to cause an increase in the RDS. Mice in groups 1 and 2 had to be euthanized due to excessive tumor growth two weeks after the start of the therapy, whereas follow-up in groups 3 and 4 comprised four weeks. PRRT significantly inhibited tumor growth; the administration of everolimus did not induce an additional effect. A good correlation existed between MTV and BTV. PRRT significantly reduced the TBR. [68Ga]Ga-DOTA-TATE PET is suitable for monitoring tumor growth in the applied model. The high efficacy of [177Lu]Lu-DOTA-TATE is not enhanced by the combination with everolimus.

Keywords: Lu-177-DOTA-TATE; PRRT; everolimus; mTOR inhibitor; neuroendocrine tumors

1. Introduction

Neuroendocrine tumors (NETs) represent a rare entity of neoplasms with increasing incidence over the last years and high heterogeneity with respect to the primary tumor site, tumor grading, and pathophysiological properties such as hormonal activity. Despite a relatively high median overall survival of 9.3 years for all patients, there is a wide variety in survival times depending on the primary tumor site and stage. Patients with a primary tumor in the pancreas have an overall survival of 3.6 years, however in the presence of distant metastasis overall survival is only 12 months [1]. Novel treatment strategies established during the last years will hopefully improve these numbers. The RADIANT-3 trial, for instance, showed an improved progression-free survival (PFS) of 11 months for patients suffering from advanced, progressive pancreatic NETs (pNETs) treated with the mTOR inhibitor everolimus compared to 4.6 months for placebo [2]. Furthermore, the NETTER-1 trial significantly changed the therapeutic landscape of NETs. This study was the first phase 3 trial investigating the role of peptide receptor radiotherapy (PRRT) with [177Lu]Lu-DOTA-TATE in patients suffering from NETs, a therapy that has been used in clinical routines on a compassionate use basis for more than 20 years. It showed a significant improvement in PFS as well as quality of life for patients with NETs of midgut origin progressive on first line therapy treated with [177Lu]Lu-DOTA-TATE in combination with a standard dose of octreotide LAR compared to a high dose of octreotide LAR alone [3].

Despite the fact that everolimus and PRRT are used sequentially, a combination of these two therapy options seems reasonable from a theoretical point of view, as everolimus has been proven to enhance the efficacy of (external) radiotherapy in a broad range of solid cancer types in vitro [4–8]. Furthermore, preclinical data suggest that everolimus might even re-sensitize radioresistant tumor endothelial cells [9]. However, due to the proposed synergistic effect and the dissatisfactory results of other combination studies, severe safety concerns are raised. Claringbold et al. reported that the full recommended dose of everolimus was not tolerated in a phase I study combining everolimus and [177Lu]Lu-DOTA-TATE in humans [10].

Bison and Pool treated rats with CA20948 human pancreatic neuroendocrine tumor with everolimus and [177Lu]Lu-DOTA-TATE. They did not find the combined regime to be superior compared to PRRT alone. However, they observed the development of metastases in rats receiving the combined therapy when a complete remission was not achieved [11,12].

In a different rat model, we were recently able to show that a combined treatment with therapeutic doses of both everolimus and [177Lu]Lu-DOTA-TATE does not increase nephro- or hematotoxicity compared to mono-therapies [13]. However, in that previous work, we only evaluated therapy-related toxicity in animals without xenograft tumors.

The current study evaluates the potential synergistic therapeutic effect of everolimus and PRRT with [177Lu]Lu-DOTA-TATE in a mouse model using AR42J pancreatic tumors. Furthermore, an evaluation of the clinically most relevant toxicities, hemato- and nephrotoxicity is performed.

2. Materials and Methods

2.1. Animals, Tumor Cell Line, and Cultivation and Experimental Design

All animal experiments were performed following institutional guidelines and approved by the ethics committee and Administrative Panel on Laboratory Animal Care (Government of Upper Bavaria, Germany, reference 55.2-1-54-2532-201-12). Seven-week-old female nude CD1 mice weighing 21.5 to 30.6 g (Charles River Laboratories, Sulzfeld, Germany) were used. Mice were fed a standard diet and given free access to water. Body weight was monitored twice weekly.

AR42J cells were cultivated in bovine serum albumin nutritional medium at 37 °C and 5 % CO_2 atmosphere. These cells overexpress the somatostatin receptor type 2 (SSTR2) and are known to be suitable for [68Ga]Ga-DOTA-TATE PET-imaging [14]. Furthermore,

several studies have shown that this cell line seems to be feasible for preclinical PRRT and everolimus trials [15,16].

Mice were inoculated with 5×10^6 tumor cells in the right flank. Five days after the tumor injection, a pre-therapy/baseline [68Ga]Ga-DOTA-TATE PET scan was performed before the mice were randomly divided into four groups. Group 1 received a placebo (n = 7), group 2 everolimus (n = 8), group 3 a placebo in combination with PRRT (n = 7), and group 4 everolimus in combination with PRRT (n = 7). An everolimus + placebo group was omitted, as everolimus is already an established therapy for NET as shown in the RADIANT 3 and 4 trials [2,17]. The dose of everolimus was 5 mg/kg body weight every week and the dose of [177Lu]Lu-DOTA-TATE was 80 MBq once, on the day of the baseline scan. [68Ga]Ga-DOTA-TATE scans were repeated one, two, and four weeks after the baseline scan.

2.2. Laboratory Chemical Analysis

A total blood count was performed right before euthanizing the animals at the end of the study. The laboratory analyses were executed according to the manufacturer's protocols and standardized methods at the Institute of Laboratory Medicine of the Medical Centre of the University of Munich. Blood was not diluted. Blood count analysis was performed using an XN-2000 analyzer (Sysmex, Kobe, Japan).

2.3. Pharmaceuticals and Radiopharmaceuticals

Everolimus (formerly known as RAD001) and placebo were kindly provided by Novartis Pharma GmbH (Nuremberg, Germany). We applied a weekly dose of 5 mg/kg body weight as suggested by previously published studies [18]. The pharmaceuticals were freshly prepared from the pre-concentrate once a week right before the oral gavage. Following the manufacturer's manual, everolimus pre-concentrate was diluted with 5% glucose solution to a concentration of 0.25 mg/mL corresponding to an administered volume of ~0.5 mL. Equivalent amounts of pre-concentrate and glucose solution were used for the preparation of the placebo solution.

No-carrier added 177Lu was obtained from Isotope Technologies Garching GmbH (Garching, Germany). DOTA0, TYR3-octreotate was purchased from ABX advanced biochemical compounds (Dresden, Germany). Radiolabeling was performed according to a previously described protocol [19]. The amount of 80 MBq was chosen according to data by Svensson et al. as a trade-off between moderate toxicity and anti-tumor activity [20]. Radiolabeling of [68Ga]Ga-DOTA-TATE was performed by a radiochemist of the department of nuclear medicine according to protocols described elsewhere labeled with 68Ga obtained from a 68Ge/68Ga generator system (GalliaPharm, Eckert & Ziegler AG, Berlin, Germany) [21]. All radiopharmaceuticals were administered via a tail vein.

2.4. PET Imaging and Determination of Tumor Volume

[68Ga]Ga-DOTA-TATE PET imaging was performed with a dedicated small animal PET camera (Inveon Dedicated PET, Preclinical Solutions, Siemens Healthcare Molecular Imaging, Knoxville, TN, USA). After the induction of anesthesia with 1.5% of isoflurane in pure oxygen via a facial mask, 15 MBq of [68Ga]Ga-DOTA-TATE were administered through a tail vein. One static frame was obtained 45 min after the injection of the radiochemical for 30 min. The acquired image was reconstructed using an OSEM 3D algorithm (four iterations) and a MAP 3D algorithm (32 iterations).

In order to analyze the tracer uptake in the tumors, the semi-quantitative measure of the tumor-to-background ratio was calculated by the division of the count rates in standardized volumes of interest (VOIs) which were applied to the tumor and corresponding background regions (M. quadriceps femoris). To determine the tumor VOI, a region of high tracer uptake at the location of the tumor was drawn manually and the voxel of highest activity was selected. This voxel and all neighboring voxels down to a threshold activity of 30 % of the maximum activity were included in the tumor VOI. This method yielded the biological tumor

volume (BTV). Tumor volumes were also measured manually determining the maximum diameter of the tumor and two perpendicular diameters using a caliper. The measured tumor volume (MTV) was calculated using the ellipsoid formula $V = a \times b \times c \times \pi/6$.

2.5. Histopathological Analysis

The histopathological examination was performed on all kidneys of all mice euthanized due to excessive tumor growth. Left and right kidneys were fixed in 4% formaldehyde and stained with HE and PAS. The findings of the histopathological examination were recorded, evaluated, and presented using Excel sheet.

For the evaluation of the kidneys, criteria were used according to the renal damage score system (RDS) described by Rolleman et al. [22]. In case of divergent numbers for the two kidneys of one individual, the mean number was used for further analyses.

2.6. Statistical Analysis

Data are expressed as the means of the treatment groups and the corresponding 95%-confidence interval. A p-value of $p < 0.05$ was considered statistically significant. Normality and homogeneity of variance were tested using the Shapiro–Wilk test and Levene's test.

Two-way analysis of variance (ANOVA) was carried out for parameters measured only once in all groups. When normality and/or homogeneity requirements were not met, the Scheirer–Ray–Hare (SRH) test was used, with the administration of everolimus or placebo as one and the treatment with or without [177Lu]Lu-DOTA-TATE as the second factor in both cases. When parameters could only be obtained in two groups, a t-test was used.

The Kruskal–Wallis test was used to test for differences among the ordinally scaled values of the histological grading and Mann–Whitney tests were performed for post-hoc analyses between any two groups applying the Bonferroni correction.

All statistical tests were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, US) and SPSS Statistics (Version 26, IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Laboratory Chemical Analysis

The analysis of the total blood count at the end of the trial did not show any significant differences in the erythrocyte, leukocyte or platelet count, the hematocrit, hemoglobin, or the proportion of reticulocytes. Results are displayed in Table 1. A significant increase was only found in the number of neutrophils and lymphocytes due to the PRRT ($p = 0.003$ and $p = 0.002$, respectively). However, the increase in white blood cell count (WBC) due to PRRT was not significant ($p = 0.051$). All other hematologic parameters were also slightly elevated in the groups receiving PRRT. Everolimus increased RBC, hemoglobin, hematocrit, and platelet count and decreased the WBC and the number of neutrophils, monocytes, and lymphocytes, but for none of the parameters was the effect statistically significant.

Table 1. Results of the total blood count performed at euthanasia. PRRT causes a significant increase in lymphocyte and neutrophil counts. p-values result from a two-way ANOVA of the four groups.

	Placebo	Everolimus	Placebo + [177Lu]Lu-DOTA-TATE	Everolimus + [177Lu]Lu-DOTA-TATE	p-Values
RBC (10^{12}/L)	6.15 ± 1.32	7.96 ± 1.26	8.01 ± 3.57	7.82 ± 2.17	0.154
Hemoglobin (g/L)	99 ± 18	126 ± 18	114 ± 54	124 ± 31	0.181
Hematocrit	0.331 ± 0.058	0.419 ± 0.056	0.413 ± 0.103	0.414 ± 0.088	0.080
Reticulocytes (‰)	60.4 ± 30.5	39.6 ± 9.8	81.0 ± 88.9	71.7 ± 49.9	0.252
Platelets (10^9/L)	729 ± 459	965 ± 374	1028 ± 743	1079 ± 200	0.469
WBC (10^9/L)	3.29 ± 2.07	2.58 ± 1.03	5.06 ± 2.59	3.78 ± 1.80	0.158

Table 1. *Cont.*

	Placebo	Everolimus	Placebo + [177Lu]Lu-DOTA-TATE	Everolimus + [177Lu]Lu-DOTA-TATE	*p*-Values
Neutrophils (10^9/L)	0.83 ± 0.34	0.97 ± 0.49	1.91 ± 0.63	1.26 ± 0.58	0.013 *
Monocytes(10^9/L)	0.09 ± 0.06	0.05 ± 0.03	0.11 ± 0.07	0.10 ± 0.09	0.306
Lymphocytes (10^9/L)	0.94 ± 0.88	0.76 ± 0.34	2.36 ± 1.79	1.89 ± 1.06	0.014 *

Statistically significant differences are marked with an asterisk.

3.2. Histopathological Analysis of the Kidneys

Examples of the histological sections are presented in Figure 1.

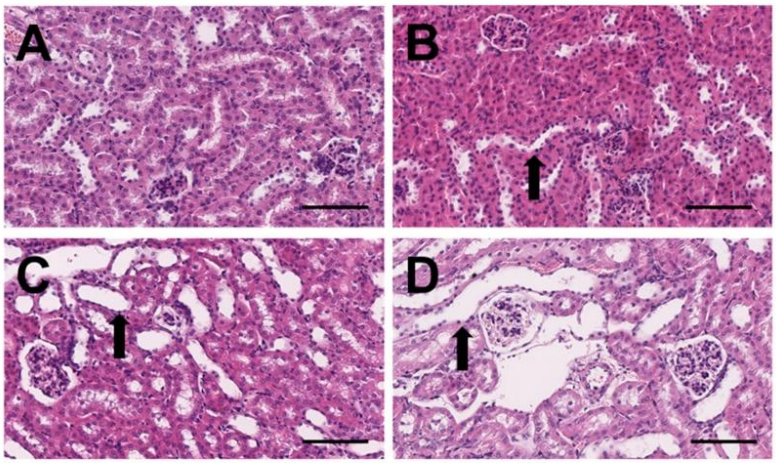

Figure 1. Microscopic images of the kidneys of individual mice in group 1 (**A**), group 2 (**B**), group 3 (**C**), and group 4 (**D**). Tubules are increasingly dilatated in animals of groups 2, 3, and 4 (black arrows). (HE, scale bars: 100 µm).

In the glomeruli, no finding was detected in the animals of groups 1 and 2. A minimal to slight multifocal cell reduction and apoptosis were observed in four animals in group 3, and a minimal to slight reduction in five animals in group 4.

In the tubuli, a minimal to slight cell damage or loss of epithelium was observed in five animals in group 2, and a minimal to moderate loss in all animals in groups 3 and 4. A minimal multifocal mononuclear cell infiltration was detected in two animals in group 3. Minimal protein cylinder formation was observed in two animals in group 1 and two animals in group 3, and minimal to slight protein cylinder formation was found in two animals in group 2. A minimal tubulus dilatation was detected in one animal in group 1, and a minimal to slight dilatation in five animals in group 2. In group 3, the tubulus dilatation was found to be minimal to moderate, and in group 4 it was slight to marked in all animals. A minimal multifocal vacuolization in the tubulus epithelium was found in one animal in group 3. A minimal focal regeneration was detected in three animals in group 2.

In summary, the median RDS for group 1 is 0 and for groups 2, 3, and 4 it amounts to 2, 2, and 3, respectively. The distribution of the scores is depicted in Figure 2.

The Kruskal–Wallis test showed significant differences in the RDS values ($p = 0.001$) and the post-hoc analyses revealed a significantly lower RDS in the placebo group compared to groups receiving PRRT ($p = 0.007$ for group 3 and $p = 0.008$ for group 4). No significant

difference was found between the everolimus and the PRRT group. Combined treatment induced a higher RDS compared to everolimus monotherapy without being statistically significant ($p = 0.22$).

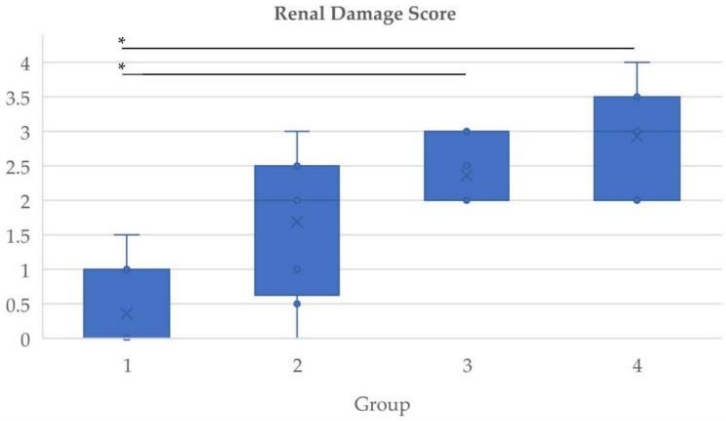

Figure 2. Renal damage scores in the different groups. Circles indicate individual values. Crosses represent means. Boxes cover the interquartile range and whiskers mark minimum and maximum values. Statistically significant differences are marked with an asterisk.

3.3. Tumor Growth

Mice were euthanized due to the penetration of the tumor through the skin or the tumor size, following animal welfare regulations as described in the ethics approval. Mice receiving placebo (group 1) had to be euthanized on day 15 (n = 3) or day 19 (n = 4). Mice receiving everolimus (group 2) also had to be euthanized on day 15 (n = 4) and day 19 (n = 4). Mice receiving [177Lu]Lu-DOTA-TATE and a placebo or everolimus (groups 3 and 4) were euthanized on day 33. One mouse in group 4 was lost on day 26 due to aspiration during the gavage of everolimus.

Tumor volumes were distributed homogeneously at the start of the treatment. Figure 3 shows the progression in tumor volume in the different groups.

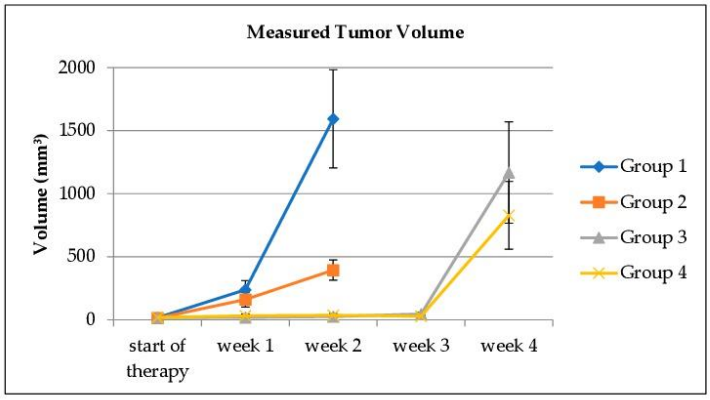

Figure 3. Time course of the manually measured tumor volumes averaged in the different groups. Error bars indicate the standard error of the mean.

At day 19, two weeks after the start of the treatment, MTVs were 1.6 ± 1.3 cm^3 in group 1, 0.39 ± 0.26 cm^3 in group 2, 0.026 ± 0.037 cm^3 in group 3, and 0.036 ± 0.034 cm^3 in group 4. Since the Shapiro–Wilk test revealed a significant deviation from normality, an SRH test was performed to analyze the differences between groups. Results showed significantly smaller MTVs only for [177Lu]Lu-DOTA-TATE ($p < 0.001$) but not for everolimus ($p = 0.55$).

MTVs did not differ significantly between groups 3 and 4 at day 33 ($p = 0.497$).

At euthanasia, the averaged masses of the xenografts were 1.2 ± 0.7 g in group 1, 0.8 ± 0.7 g in group 2 and did not differ significantly ($p = 0.363$). In group 3, the mean tumor mass was 1.1 ± 1.1 g, and in group 4 it was 0.7 ± 0.8 g. Again, no significant difference was found ($p = 0.481$).

3.4. Biological Tumor Volume

The BTVs obtained using the thresholding method (Figure 4) were plotted versus the respective MTVs (Figure 5). The result of the linear regression was BTV = 0.942 × MTV + 0.012 cm^3 (95%-CI for the correlation coefficient [0.9010, 0.983]) with the determination coefficient $R^2 = 0.8955$.

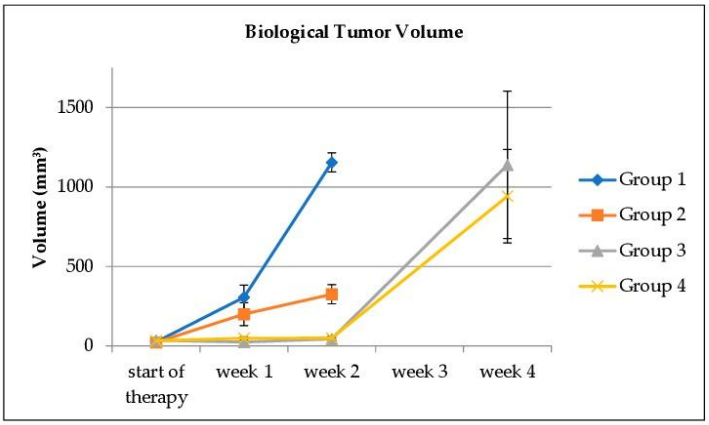

Figure 4. Time course of the tumor volumes determined from the PET scans averaged in the different groups. Error bars indicate the standard error of the mean.

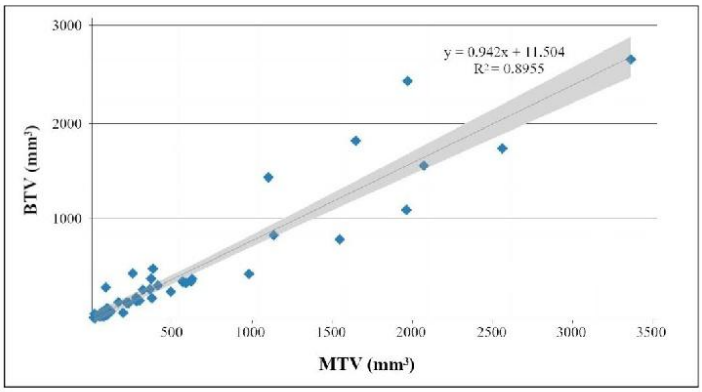

Figure 5. Correlation of manually measured tumor volumes (MTV) and the tumor volumes obtained from the PET scans (BTV). The gray area marks the confidence band of the linear regression.

3.5. Tumor to Background Ratio

TBR was determined separately for each scan and normalized to the individual TBRs in the baseline scans. TBRs are plotted in Figure 6 with the muscle as the background region. SRH test showed a significantly lower TBR for PRRT ($p < 0.001$) but not for everolimus ($p = 0.98$) as a factor.

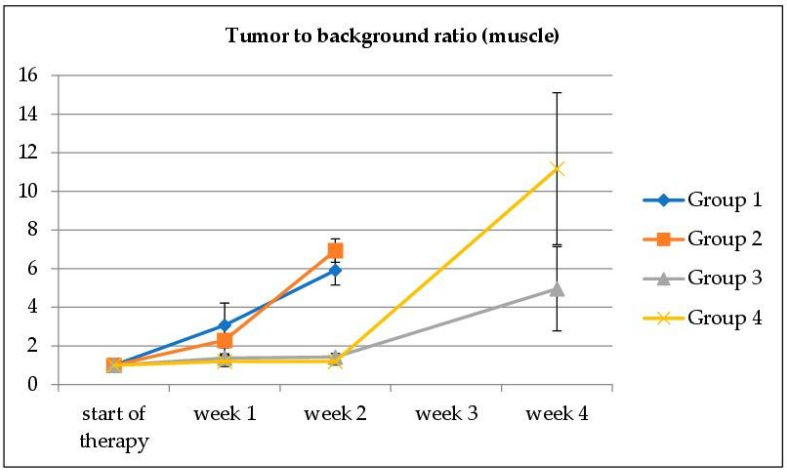

Figure 6. Tumor to background ratios with VOIs in the muscle (M. quadriceps femoris) as background. Error bars indicate the standard error of the mean.

4. Discussion

In the NCCN guidelines for the management of neuroendocrine and adrenal tumors, PRRT and everolimus represent first- or second-line therapy options in patients with metastatic NETs [23]. However, as objective response rates are relatively low, further optimization of therapy algorithms and sequences is needed. Our approach in the current preclinical trial was to combine both therapeutic options in AR42J tumor-bearing nude mice.

The results regarding hematotoxicity are in line with our findings in rats as previously reported [13]. Everolimus induced an increase in hemoglobin concentration and a decrease in white blood count. These findings did not reach statistical significance. However, the (statistically significant) increase in neutrophil, as well as the lymphocyte count due to PRRT, was not observed in the previous study. This divergence might be caused by the substantially shorter interval between the application of PRRT and the blood analysis and is considered a temporary, potentially reversible effect. There was no essential deterioration of the blood count in the group receiving the combined therapy compared to PRRT and placebo. In summary, with the given methods, no severe high-grade toxicity in terms of blood parameter elevation was observed with the treatment dose chosen for this study.

Histological evaluation revealed significant nephrotoxicity in mice receiving PRRT regardless of the addition of everolimus when compared to the group receiving only placebo. Considering the dose threshold of ~ 60 MBq [177Lu]Lu-DOTA-TATE for nephrotoxicity described by Svensson et al. [20], these findings are not surprising. The main consideration behind escalating PRRT doses was to maximize potential therapeutic synergistic effects by adding everolimus. In this regard, the combined treatment did not show increased nephrotoxicity compared to PRRT alone. Thus, we conclude that nephrotoxicity is also acceptable in the combined regime as implemented in this trial and is mainly dependent on the PRRT dose. As nephrotoxicity is rare when using [177Lu]Lu-DOTA-TATE at standard

doses of 7.4 GBq per administration, these results might not be transferable to human data anyway.

A significant deceleration in tumor growth was found for the treatment with [177Lu]Lu-DOTA-TATE. The observed delay in tumor growth due to PRRT is similar to the results reported by Cullinane et al., who evaluated the effect of [177Lu]Lu-DOTA-TATE in a combined regime with the PARP-inhibitor talazoparib using Balb/c nude mice with AR42J-tumors [16]. In this study, mice treated with [177Lu]Lu-DOTA-TATE showed tumor regression for two weeks after treatment and an overall survival of 37 days. This fits our observation of a two-week longer observation period after PRRT and the sacrifice due to tumor growth on day 28. The tendentially poorer performance of the mice used in our trial could be explained by the additional stress due to the PET scans and the necessary anesthesia.

Experimental data have demonstrated the role of the mTOR-signaling pathway in AR42J cells. mTOR-inhibition blocks signaling from mitogenic growth factors and mTOR-activation protects against inflammation [24,25]. Therefore, the treatment with everolimus should not only inhibit tumor growth but also aggravate the inflammatory response to DNA damage caused by PRRT. However, the current paper will not allow detailed insight into tumor biology and response pathways as it mainly evaluates the response assessment using SSTR-PET and tumor growth.

[68Ga]Ga-DOTA-TATE PET scans represent a suitable method for in vivo determination and monitoring of tumor burden with a good correlation of PET-derived BTVs and manually measured MTVs.

Despite being not significant, the addition of everolimus increased the TBR, which fits the clinical observation that everolimus can induce somatostatin receptor expression [26]. However, no significant differences in tumor size were observed.

The efficacy of everolimus as a radiosensitizer has been shown for external beam radiotherapy as mentioned earlier [4–8]. Unlike external beam radiotherapy, however, PRRT applies heterogeneous and prolonged irradiation and relatively low dose rates. Whether these differences urge the need for the development of distinctive radiosensitizers for PRRT is currently the subject of discussion as the underlying radiobiology is not yet fully understood [27]. Nonetheless, our preclinical data and a phase I trial in patients indicate that the combination of PRRT and everolimus might be associated with higher toxicity without higher anti-tumoral effects [10,13].

Unlike Bison, Pool, et al. who treated CD20948 bearing rats with a combination of everolimus and [177Lu]Lu-DOTA-TATE [11,12], we could not observe the development of metastasis. This divergence may be attributed to our rather short observation period, as mice had to be sacrificed according to the underlying study protocol as approved by the animal welfare committee that could be overcome in further experiments by resecting the primary tumor. Furthermore, our study represents localized disease, which is not the case in the patient scenario when PRRT and/or everolimus is applied as most patients suffer from metastatic disease.

In summary, the combination of PRRT and everolimus in the treatment of neuroendocrine tumors remains complex even in a preclinical setting. In an early Phase 1 clinical trial the combined regime could only be administered with reduced doses of everolimus due to unacceptable toxicity, further questioning this approach [10]. However, research into alternative options to optimize PRRT for neuroendocrine neoplasms like precise dosimetry for dose escalation seems promising.

5. Conclusions

[68Ga]Ga-DOTA-TATE PET scans are a suitable method for monitoring tumor size in SSTR2-positive AR42J tumors in mice. Combined treatment with everolimus and [177Lu]Lu-DOTA-TATE does not induce a significantly increased toxicity in this model. PRRT with [177Lu]Lu-DOTA-TATE shows good anti-tumor activity in this model inde-

pendent of a combination with everolimus without further synergistic effects for the combined treatment.

Author Contributions: J.Z. performed and analyzed the in vivo studies and contributed to the writing of the manuscript and preparation of figures. H.-Y.Y. and K.S. performed the histopathological analysis. L.K. and G.B. contributed to the analyses of the PET data. F.J.G. was responsible for the production of [177Lu]Lu-DOTA-TATE. M.H., P.B., A.T. and A.R.H. contributed to the study's conception and critical revision of the manuscript. H.I. designed the study, supervised the practical experiments, and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All experiments were carried out in compliance with the National Guidelines for Animal Protection, Germany, with the approval of the regional Animal Care Committee of the Government of Oberbayern (Regierung v. Oberbayern) and were overseen by a veterinarian.

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Erklärung zur Übereinstimmung der gebundenen Ausgabe der Dissertation mit der elektronischen Fassung

Ihr Name: Johannes Anton Josef Zellmer

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Hiermit erkläre ich, dass die elektronische Version der eingereichten Dissertation mit dem Titel

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