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Folate-based radiotracers for nuclear imaging and radionuclide therapy

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Abstract

Folate receptor α (FR α) is overexpressed on numerous tumorous cell types such as ovarian or endometrial cancer cells. Moreover, FR α is absent from most healthy tissues as it is normally expressed only on the surface of proximal tubules cells of kidneys and choroid plexus. Thus, folate-based radiopharmaceuticals have emerged this last two decades as FR α is a target of choice to diagnose and treat numerous cancers. Nuclear imaging is a performing diagnostic technology using highly sensitive detectors and specific radiopharmaceuticals used to detect tumors at an earlier stage. Herein, an overview of the development of folate-based radiopharmaceuticals to detect FR α -positive tumors by nuclear imaging using positron emission tomography (PET) and single photon emission computed tomography (SPECT) is exposed. Strategies developed to improve precursor synthesis, bioavailability, clearance, and affinity to FR α will be detailed. Advances made to decrease kidney uptake open the gate to targeted radionuclide therapy (TRT) using folate-based radiopharmaceuticals to treat FR α -positive tumors. Thus, radiofolates used in TRT and more precisely in a theranostic approach will be depicted in this review.

Keywords

Folic acid, Ovarian cancer, Radiopharmaceutical, Nuclear imaging, Targeted radionuclide therapy, Theranostic

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1. Introduction

1.1. Generalities

Folate receptor α (FR α) shows promising prospects in targeting ovarian, endometrial, colorectal, non-small cell lung and renal cancers and has a highly limited expression in normal tissues except for kidneys [1,2]. In fact, FR α is overexpressed in 72-100% of ovarian carcinomas [3–6]. Various studies have been initiated to develop folic acid (FA)-conjugated agents for diagnosis by molecular imaging or for the treatment of such diseases [7]. For example, the interest of FA as targeting agent for peritoneal metastasis treatment thanks to Photodynamic Therapy (PDT) has been largely proved by Frochot and coworkers [8–15]. FA can also be an ideal molecule for selective nuclear imaging using FR-targeted radiopharmaceuticals. In fact, radiofolates have been developed to diagnose rheumatoid arthritis [16,17], osteoarthritis [18,19] and atherosclerosis plaque [20,21] by detection of activated macrophages in concerned tissues which overexpressed FR β and tumorous cells overexpressing FR α . Moreover, radiopharmaceuticals using alternatives to FA as monoclonal antibodies like MOv-18 [22,23] and M9346A [24,25], and peptides like C7 [26] have been developed to target FR α . Since the emergence of nanoparticles as drug delivery system, a dozen of studies have been achieved on nanoparticles-based radiofolates to detect FR α -overexpressing tumors [27–29]. It is also worth noting that within the last decade, researchers have taken an interest in the use of small-molecule radiotherapeutics targeting receptors for a targeted radionuclide therapy (TRT) of cancers [30]. The development of folate-based radiopharmaceuticals for nuclear imaging or radionuclide therapy consists of similar strategies using α - or β -emitters radionuclides for therapy and β^+ or γ -emitters radionuclides for nuclear imaging (*i.e.*, β^+ for positron emission tomography (PET) and γ for single-photon emission computed tomography (SPECT)).

1.2. Folic acid receptors and carriers

Different high affinity receptors to folates are reported in the literature: reduced folate carrier (RFC) [31], proton-coupled folate transporter (PCFT) [32] and folate receptor (FR) are receptors having good affinity to folate and/or reduced folates. RFC is involved in the transport and internalization of reduced folates used in the *de novo* synthesis of DNA. PCFT is involved in carrier-mediated intestinal folate transport system. FR exists in three isomers: FR α , FR β and FR γ . These isomers differ from about 30% in sequence homology [33]. FR α and FR β are 38 kDa glycosylphosphatidylinositol anchored glycoproteins mediating the internalization of FA *via* endocytosis route [34]. FR α is absent from the majority of healthy tissues [35] as it is normally expressed only on the surface of proximal tubules cells of kidneys (for folate resorption) [36], lung and choroid plexus [37]. Salivary glands are also able to release a soluble form of FR α in the saliva [38]. FR α is a folate-binding protein overexpressed in 40% of solid tumors including epithelial cancerous cells (serous and endometrioid epithelial ovarian, endometrial, breast, cervical, colorectal, non-small cell lung and renal cancers). FR β is overexpressed on placenta [39,40] but also on activated (but not resting or quiescent) macrophages which are recognized either to cause or to contribute to inflammatory and auto-immune diseases like rheumatoid- and osteo-arthritis [41], lupus [42], Crohn's disease or atherosclerosis [43]. FR γ is a secreted protein as the signaling sequence for membrane anchoring is missing in this isomer form. FR γ is secreted by lymphoid cells in the thymus, spleen and bone marrow and is related to malignancies with hematopoietic origins [44].

1.3. Targeting agent of folic acid receptors

Folic acid (FA) is a water-soluble molecule (441 Da), also known as the synthetic form of folate (vitamin B9) which has a strong affinity to FR α (1 nM). Its structure is divided in three distinct parts: pterin, aminobenzoate and glutamate entities (Fig.1, left). Pterin and aminobenzoate entities form the pterate moiety. In 2013, crystal structure of FA bound to FR α was successfully determined and gave tangible information on the recognition area of FA which is the pterate moiety [45]. FA is crucial to ensure normal functions of the human body in various metabolic (amino acids, nucleic acids) and biochemical processes. The human body does not store FA, thus, a FA deficiency can lead to several defects, such as spina bifida or anencephaly [46]. FA deficiency has also been associated with certain types of congenital defects (heart and neural tube defects) or malformations (cleft lip) or pregnancy complications [47].

5-methyl-tetrahydrofolate (5-Me-THF) is the active form of folate. It differs from this latter by the presence of a methyl group on 5-N of the pterin entity and the presence of an asymmetrical carbon (6-C). Thus,

5-Me-THF is found under two forms: (6*S*)- and (6*R*)-diastereomers (Fig. 1, right). It is the cofactor of Thymidylate Synthase (TS) involved in the *de novo* synthesis of DNA building block 2'-deoxy-thymidylate (dTMP) [48].

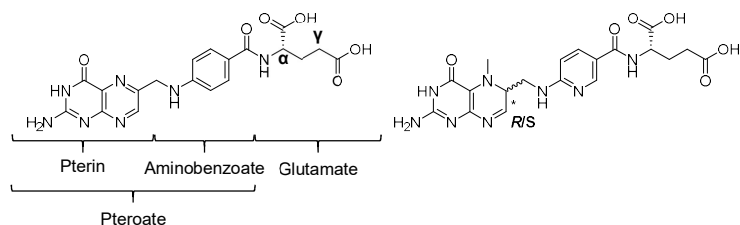


Figure 1: Chemical structure of folic acid (FA) (left) and 5-Me-THF (right)

1.4. Nuclear medicine

Nuclear medicine is a medical domain where i) diagnosis are determined thanks to nuclear molecular imaging exams (PET and SPECT) and ii) treatments are performed thanks to radiopharmaceuticals administered to patients, which can target tumor cells and damage their DNA (TRT).

Molecular imaging is defined as the *in vivo* targeted imaging of biological process and takes an important place to establish diagnosis of many diseases. PET and SPECT are two nuclear imaging techniques which had numerous impacts of access to health care [49–52]. These techniques consist in injecting molecular imaging agents named radiotracers or radiopharmaceuticals to detect interactions with specific cellular target.

Radionuclides [53] can be directly integrated in the targeting agent or be coupled with it thanks to a radiolabeled prosthetic group or inserted into a chelating agent *via* coordination chemistry for metal radionuclides. Most widespread radionuclides are radiometals labeled with chelating agent. Depending on their nature, they do not form the same number of coordination bonds with chelating agent and do not have the same oxidation level. There is a wide variety of chelating agents which can be cyclic (*i.e.* 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA), 1,4,7-triazacyclononane-1,4,7-trisacetic acid (NOTA), or linear (*i.e.* *N,N*-Bis(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED), deferoxamine (DFO), diethylene-triaminepentaacetic acid (DTPA), 6-hydrazinonicotinic acid (HYNIC)) [54]. Most representative chelating agents of folate-based radiopharmaceuticals are presented in Fig. 2.

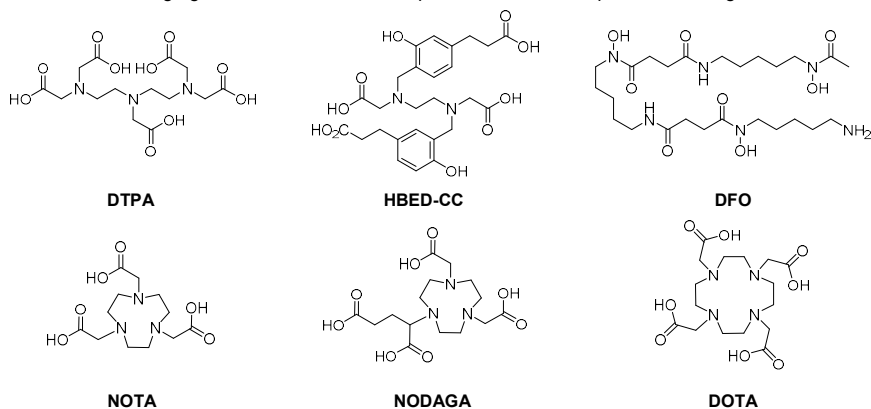


Figure 2: Chemical structures of linear and cyclic chelating agents

Radionuclides used for **PET imaging** [55–59] (Table 1) are positrons (β^+) emitting. The PET scanner detects the pair of 511 keV photons produced by annihilation of β^+ with an electron. An image is reconstructed to locate exactly the production site of the pair of photons. As this technique is very sensitive [60,61],

radiopharmaceuticals are injected in picomolar concentrations with activity range of 0.37 to 600 MBq for preclinical studies and from 300 to 600 MBq for clinical trials.

Fluorine-18 (^{18}F) is the most widespread radionuclide for PET imaging. Its radiochemistry is essentially performed by nucleophilic substitution. ^{18}F fixation on biomolecules is a big issue as fluorination reactions need harsh conditions. As biomolecules are quite sensitive, the development of conjugation approach has emerged. It consists in fixing ^{18}F to a small molecule (a prosthetic group) which can react with biomolecules in soft conditions *via* amino, thiol or click chemistry [62–65]. In 2009, a novel radiochemistry strategy as emerged in which ^{18}F is used as co-ligand in the coordination of aluminum [66,67].

PET metal radionuclides like gallium-68 (^{68}Ga) are incorporated into macrocyclic compounds (DOTA [68], NOTA [69,70], NODAGA [71] or acyclic chelators (HBED) [72] by coordination chemistry in aqueous phase. The use of ^{68}Ga is growing to radiolabeled biomolecules for clinical applications. New PET metal radionuclides like scandium-44 (^{44}Sc) [73], copper-64 (^{64}Cu) [74,75] and cobalt-55 (^{55}Co) [76] can be coordinated to the same chelating agents as ^{68}Ga and are currently under study to evaluate their capability to be good diagnostic radionuclides.

Table 1: Chemical properties of PET radionuclides used to radiolabel folate-based probes

| Isotope | Half-life | Decay mode | E _{max} (keV) | Production | Oxidation number | Number of coordination | References |
|-------------------|-----------|--|------------------------------------|--|------------------|------------------------|------------|
| ^{18}F | 109.8 min | β^+ (95%) EC (3%) | β^+ , 635 γ , 1655 | $^{18}\text{O}(p,n)^{18}\text{F}$ | 1- 3+ | NA 6 | [66,77] |
| ^{44}Sc | 4.04 h | β^+ (94%) EC (6%) | γ , 1157 β^+ , 632 | $^{44}\text{Ti}/^{44}\text{Sc}$ generator $^{44}\text{Ca}(p,xn)^{44}\text{Sc}$ $^{44}\text{Ca}(p,n)^{44}\text{Sc}$ | 3+ | 8-9 | [73,78] |
| ^{55}Co | 17.5 h | β^+ (76%) | β^+ , 570 | $^{55}\text{Ni}(p,\alpha)^{55}\text{Co}$ $^{56}\text{Fe}(p,n)^{55}\text{Co}$ $^{54}\text{Fe}(d,n)^{55}\text{Co}$ | 2+ | 6 | [79] |
| ^{64}Cu | 12.7 h | β^+ (19%) EC (41%) β^- (40%) | β^+ , 653 | $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ | 2+ | 6 | [74] |
| ^{68}Ga | 67.7 min | β^+ (89%) EC (11%) | β^+ , 830 | $^{68}\text{Ge}/^{68}\text{Ga}$ generator $^{68}\text{Zn}(p,n)^{68}\text{Ga}$ | 3+ | 6 | [73,80] |
| ^{152}Tb | 17.5 h | β^+ (17%) | β^+ , 1080 | Spallation of Tantalum target $^{155}\text{Gd}(p,n)^{155}\text{Tb}$ $^{159}\text{Tb}(p,5n)^{155}\text{Dy} \rightarrow ^{155}\text{Tb}$ | 3+ | 6-9 | [81] |

EC: Electron capture; n: neutron; p: proton; d: deuteron; α : alpha particle; xn: one or more neutron; NA: Not applicable

Radionuclides used for SPECT imaging [55,56,59,82] (Table 2) are γ -emitting with an energy range of 25 to 300 keV. γ radiation in a range of 25-250 keV can be detected by small animal SPECT devices whereas γ radiation in a range of 60 to 300 keV can be detected by clinical SPECT devices. However, since the development of whole-body SPECT apparatus [60,61], the development of SPECT imaging agents to have access to quantifiable information for therapy purpose became interesting [83,84].

The most used SPECT radionuclide is technetium-99m ($^{99\text{m}}\text{Tc}$). Its radiochemistry is well-known, and it is one of the cheapest radionuclides available for nuclear imaging. $^{99\text{m}}\text{Tc}$ can be used under different oxidation state with a large variety of chelators [85]. Gallium-67 (^{67}Ga) is incorporated into macrocyclic compound such as NOTA, NODAGA and TRAP (1,4,7-triazacyclononane-1,4,7-tris[methyl(2-carboxyethyl)phosphinic acid]) and acyclic chelating agents like deferoxamine, HBED or H_2dedpa (1,2-[[6-(carboxy)-pyridin-2-yl]methylamino]ethane). Indium-111 (^{111}In) is incorporated into macrocyclic compounds like DOTA and acyclic chelating agents like CHX-A''-DTPA [54].

Table 2: Chemical properties of SPECT radionuclides used to radiolabel folate-based probes

| Isotope | Half-life | Decay mode | E (keV) | Production | Oxidation number | Number of coordination | References |
|-------------------|-----------|------------|----------------------|---|------------------|------------------------|------------|
| ⁶⁷ Ga | 3.26 d | EC (100%) | γ, 93, 185, 300 | ⁶⁷ Zn(p,n) ⁶⁷ Ga | 3+ | 6 | [54] |
| ^{99m} Tc | 6.02 h | γ (88%) | γ, 141 | ⁹⁹ Mo/ ^{99m} Tc generator | 5+ | 6-8 | [86] |
| ¹¹¹ In | 2.80 d | EC (100%) | γ, 172, 245 | ¹¹¹ Cd(p,n) ¹¹¹ In | 3+ | 7-8 | [54] |
| ¹⁵⁵ Tb | 5.32 d | EC (100%) | γ, 86, 105, 180, 262 | Spallation of Tantalum target ¹⁵⁵ Gb(p,n) ¹⁵⁵ Tb | 3+ | 6-9 | [81] |

EC: Electron capture; n: neutron; p: proton

Radionuclides used for **therapeutic application** (Table 3) are α or β⁻ emitting and could directly treat tumors thanks to the TRT approach. Most widespread radionuclides are radiometals coordinated with chelating agents like DOTA [87] or DOTAGA [88].

The most used therapeutic radionuclide is lutetium-177 (¹⁷⁷Lu). Scandium-47 (⁴⁷Sc), yttrium-90 (⁹⁰Y), terbium-161 (¹⁶¹Tb), ¹⁷⁷Lu and rhenium-188 (¹⁸⁸Re) are radionuclides used in β⁻-therapy, but they also could be used to performed SPECT imaging due to their γ radiation to evaluate biodistribution and tumor uptake, and to give dosimetry information for example. Terbium-149 (¹⁴⁹Tb) is a radionuclide used in α-therapy and the choice of the therapeutic radionuclide will depend on the nature of tumors.

Table 3: Chemical properties of therapeutic radionuclides used to radiolabel folate-based probes

| Isotope | Half-life | Decay mode | E (keV) | Production | Oxidation number | Number of coordination | References |
|-------------------|-----------|---|---|---|------------------|------------------------|------------|
| ⁴⁷ Sc | 3.35 d | β ⁻ (100%) γ | β ⁻ , 441, 600 γ, 159 | ⁴⁷ Ti(n,p) ⁴⁷ Sc ⁴⁶ Ca(n,γ) ⁴⁷ Ca→ ⁴⁷ Sc | 3+ | 8-9 | [73,78] |
| ⁹⁰ Y | 2.67 d | β ⁻ (100%) | β ⁻ , 2280 | ⁹⁰ Zr(n,p) ⁹⁰ Y | 3+ | 8-9 | [54,89] |
| ¹⁴⁹ Tb | 4.12 h | α (17%) β ⁺ (7%) EC(76%) | α, 3967 β ⁺ , 730 | Spallation of Tantalum target ¹⁵² Gd(p,4n) ¹⁴⁹ Tb ¹⁴² Nb(¹² C,5n) ¹⁴⁹ Dy→ ¹⁴⁹ Tb | 3+ | 6-9 | [81,90] |
| ¹⁶¹ Tb | 6.89 d | β ⁻ (100%) | β ⁻ , 154 γ, 25, 49, 57, 74 | ¹⁶⁰ Gb(n,γ) ¹⁶¹ Gd → ¹⁶¹ Tb | 3+ | 6-9 | [73,81] |
| ¹⁷⁷ Lu | 6.65 d | β ⁻ (100%) | β ⁻ , 177,385, 498 γ, 208 | ¹⁷⁶ Lu(n,γ) ¹⁷⁷ Lu ¹⁷⁶ Yb(n,γ) ¹⁷⁷ Yb → ¹⁷⁷ Lu | 3+ | 6-9 | [73,89] |
| ¹⁸⁸ Re | 16.9 h | β ⁻ γ | β ⁻ , 2120 γ, 155 | ¹⁸⁸ W/ ¹⁸⁸ Re generator ¹⁸⁷ Re(n,γ) ¹⁸⁸ Re | 1+ (CO) 4+ | 6-8 | [86,91] |

EC: Electron capture; n: neutron; p: proton; γ: gamma particle

The research of **new radiopharmaceuticals** is mostly based on the development of targeting specific agents for tumor-associated receptor. Recent radiopharmaceuticals are now used in clinical routine diagnosis and for an approach of personalized medicine [92]. It is the case for two well-known ⁶⁸Ga-radiopharmaceuticals: [⁶⁸Ga]Ga-DOTATOC (DOTA-Tyr³-octreotide) and [⁶⁸Ga]Ga-PSMA-11. This type of biological targeting specific agent is often a biomolecule such as peptide, protein or antibody able to bind a membrane receptor overexpressed on cancer cells. They allow to establish diagnosis and staging of diseases, treatment selection, guidance or for PET-guided radiotherapy as theranostic probes [93,94].

Theranostic is composed of the term diagnostic and therapy. It aims at developing radiopharmaceuticals usable both in nuclear imaging to perform diagnostic and TRT. It is a novel approach associated to personalized medicine. This approach has the advantage to give the good treatment to the good patient. Radionuclides used in this approach must emit rays detected by diagnostic tools (β⁺ for PET and γ for SPECT) and rays with high energy (α and β⁻) for therapy. Thus, radionuclides like lutetium-177 and terbium-161 are a perfect choice for the development of theranostic agents [73,95–97]. In this theranostic approach radionuclide used could be different (⁶⁸Ga/¹⁷⁷Lu for example) [97] or used as matched pair (⁶⁴Cu/⁶⁷Cu, ⁴⁴Sc/⁴⁷Sc,

¹⁴⁹Tb-¹⁵²Tb-¹⁵⁵Tb/¹⁶¹Tb [81,98,99]. Four promising terbium radioisotopes (¹⁴⁹Tb, ¹⁵²Tb, ¹⁵⁵Tb and ¹⁶¹Tb) were identified for applications in theranostic [81]. Thanks to identical chemical characteristics and complementary physical decay characteristics, these terbium radioisotopes could be used to compare the effectiveness of PET (¹⁵²Tb or ¹⁴⁹Tb) vs. SPECT (¹⁵⁵Tb) imaging and α vs. β -therapy (¹⁴⁹Tb, ¹⁶¹Tb). Thus, it also has the advantage to combine preclinical study of imaging and therapy for the same radioconjugate.

1.4. Structure of folate-based radiopharmaceuticals

Folate-based radiopharmaceuticals are composed of FA that has been modified by nucleophilic substitution with ¹⁸F or functionalized with a chelating agent (metal radionuclide) or a prosthetic group possessing a ¹⁸F radionuclide. Hence, as the recognition part of FA is the pteroyl moiety, the main challenge is to fix selectively the chelating agent/prosthetic group on the glutamic acid unit. Some studies demonstrated the necessity to preserve the α -carboxylic acid of glutamic residue to keep a good FR α -affinity [100,101]. On the other side, other scientists like Müller and coworkers [102,103] demonstrated that both α - and γ -analogs present similar *in vivo* properties. Wedeking *et al.* [104] confirmed these results after description of good *in vivo* properties of α -analog. Hence, α -carboxylic acid of glutamic residue was included to this recognition part for most research teams. Consequently, Boss *et al.* decided to confirm previous results by studying 3 pairs of α - and γ -¹⁸F-radiofolates [105]. The necessity to fix chelating agent on γ -carboxylic acid of glutamic acid residue led to develop regioselective synthesis strategies. These syntheses need lots of steps and overall yields are consequently low. However, some teams decided to circumvent this problem by fixing chelating agent to γ -carboxylate of glutamic acid and then, fixed this compound onto pteroyl [106]. Others made the choice to develop folate-like radiopharmaceuticals by removing or replacing glutamic acid [107]. In this way, recognition part is maintained but cannot be considered as FA anymore.

1.5. Focus and content of the review

Targeting FR α in diagnosis and therapy has attracted a lot of attention from researchers and various studies have already shown the frequent high-level expression of FR α in ovarian cancer, in a broad cross-section of non-small cell lung cancer patients, as well as in other cancers [6,108–110]

Herein, this review will be focused on folate-based radiopharmaceuticals used to diagnose FR α -overexpressing tumors using nuclear imaging (PET and SPECT), and to treat them more effectively by TRT. FR β -targeted radiopharmaceuticals, non-folate-based radiopharmaceuticals targeted FR α and nanoparticles-based radiofolates will not be depicted in this review but are reviewed by other researchers [111–119]. The different FA(α)- and/or FA(γ)-conjugates (*i.e.*, functionalization of FA at the α - and/or γ -position of the glutamate moiety) that have been developed to be radiolabeled with SPECT isotopes (^{99m}Tc, ¹¹¹In, ⁶⁷Ga) and/or PET isotopes (⁶⁸Ga, ⁶⁴Cu, ⁴⁴Sc, ¹⁸F) or for therapeutic application (¹⁷⁷Lu, ¹⁶¹Tb) will be described and compared. Improvement of radiofolate synthesis and *in vivo* properties using folate-derivatives will be presented. Albumin-binding (AB) folate radiopharmaceutical, an innovative class of radiofolate allowing the improvement of diagnostic (*i.e.*, improvement of the tumor-to-kidney ratio by reducing kidney uptake) will be described. Finally, TRT studies inscribe or not in a theranostic approach will be exposed.

In vitro studies as well as *in vivo* tests (biodistribution and imaging) in tumor-bearing mice will be reported. Stability tests were performed for all described radiopharmaceuticals in PBS or serum and no significant degradation was observed within two half-life time. *In vitro* studies were performed using FR α -positive cells such as HeLa (human cervical carcinoma cell line), KB and KB-V1 (human cervical carcinoma cells from a subclone of HeLa), IGROV-1 (human ovarian carcinoma), SKOV-3ip (human ovarian adenocarcinoma), HT29 and LoVo (colorectal carcinoma), 24JK-FBP (mice sarcoma), CT-26 (mouse colon cancer cell), MA104 (embryonic monkey cell), M109 (murine lung carcinoma) and MT-1 (human breast carcinoma) and FR α -negative cells such as WI38 (human lung fibroblast primary cell), A549 (human lung carcinoma), HT1080 (human fibrosarcoma) and PC-3 (human prostate cancer).

2. Radiofolates developed for SPECT imaging application

2.1 Gallium-67 radiofolates

2.1.1. [⁶⁷Ga]Ga-deferoxamine-folate

The first radiofolate was described in 1996 by Wang *et al.* [100]. They designed the radioconjugate [⁶⁷Ga]Ga-1 ([⁶⁷Ga]Ga-DFO-(γ)-folate) (Fig. 3) composed of a DFO chelating agent linked to FA via an amine bond on the γ-carboxylate function of the glutamic acid entity. Cellular uptake study was performed on KB and WI38 cells. Results showed that [⁶⁷Ga]Ga-1 was uptake at 32.3 ± 2.4% in KB cells. Uptake of [⁶⁷Ga]Ga-1 in WI38 was only of 0.22 ± 0.04% confirming the FRα endocytosis-mediated radiofolate uptake. Biodistribution and imaging tests were performed in a second study [120]. For biodistribution tests, KB cells were subcutaneously inoculated in male athymic nude mice to induce tumor formation and [⁶⁷Ga]Ga-1 was i.v. injected. Significant tumor and kidney uptakes were respectively 5.2 ± 1.5 %IA/g and 2.02 ± 0.32 %IA/g. No significant uptake was observed for other organs leading to an excellent tumor-to-background contrast. Tumor-to-kidney ratio was of 2.6. KB cells were subcutaneously injected in abdomen of mice for imaging study. γ-Scintigraphy images were obtained 46 hours after i.v. injection of [⁶⁷Ga]Ga-1 (6.66 MBq). Only kidneys and tumor were clearly visible on images and the contrast was of a good quality. To conclude, [⁶⁷Ga]Ga-1 was suitable for further investigations as it was fast cleared from mice body via renal way. Moreover, uptake was observed only for tumor and kidneys with one of the best tumor-to-kidney ratios for FRα-positive tumor-bearing mice and images managed to clearly reveal tumor.

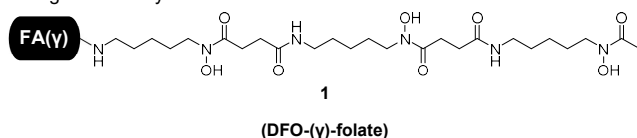


Figure 3: Chemical structure of 1 [100]

2.1.2. [⁶⁷Ga]Ga-DOTA-folates

Mindt *et al.* developed in 2009 another ⁶⁷Ga-radiofolate: [⁶⁷Ga]Ga-2 ([⁶⁷Ga]Ga-DOTA-click-(γ)-folate) [101]. DOTA chelator was regio-specifically coupled to γ-carboxylic acid of FA via Copper-catalyzed Azide-Alkyne Cycloaddition (CuAAC) click chemistry reaction to obtain compound 2 (Fig. 4A). IC₅₀ of [⁶⁷Ga]Ga-2 was determined on KB cells and revealed a high affinity of 1.6 nM for FRα. KB cells were subcutaneously inoculated in female BALB/c mice to prepare small animal SPECT/CT imaging. Images were recorded 18 hours after i.v. injection of 15 MBq of [⁶⁷Ga]Ga-2 in this mice model. Only tumors and kidney were visible and activity intensity was more important for tumors than kidneys (Fig. 4A). Imaging test results were quite promising, but further investigations, notably the achievement of biodistribution tests were required to confirm promising results and to hope the fulfilment of clinical trials.

Müller *et al.* worked on the DOTA radiofolate [⁶⁷Ga]Ga-3 ([⁶⁷Ga]Ga-DOTA-Bz-EDA-(γ)-folate also called [⁶⁷Ga]Ga-EC0800) [121]. DOTA chelator was fixed to γ-carboxylic acid of folate by the ethylenediamine (EDA) linker (Fig. 4B). *In vitro* uptake tests were performed in KB cells. Good results were obtained as at 4 hours, 30% of radioconjugate was internalized. [⁶⁷Ga]Ga-3 were injected in female athymic nude mice bearing KB tumor for biodistribution and SPECT/CT imaging (1 and 20 MBq respectively). Uptake in tumor and kidneys were of 6.08 ± 0.89 %IA/g and 84.53 ± 14.10 %IA/g, respectively. This led to a low tumor-to-kidney ratio of 0.07 ± 0.01. Significant uptake was also observed in salivary glands as the uptake was of 6.93 ± 1.67 %IA/g. Nevertheless, radioconjugate uptake in salivary glands significantly decreased in time whereas tumor and kidney uptake stayed stable. This indicated a fast-renal clearance, and that salivary gland uptake is not due to the presence of FRα. SPECT/CT images (Fig. 4B) showed a moderate activity intensity in tumor and kidneys. High uptake in bladder confirmed a fast renal clearance. To conclude, this radioconjugate was well internalized by tumors but also in non-targeting organ which can be problematic for ovarian cancer diagnostic.

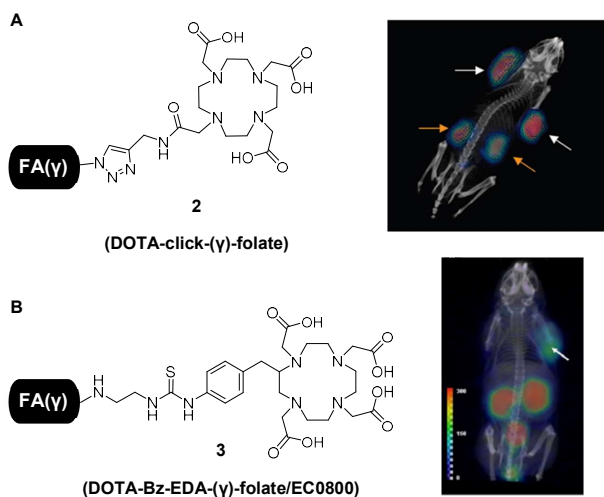


Figure 4: (A) Chemical structure of **2** and SPECT image of KB tumor-bearing mice at 18 hours p.i. of [^{67}Ga]Ga-**2** (15 MBq). Reprinted with permission from Mindt *et al.* *Bioconjug. Chem.* 20 (2009) 1940–1949. Copyright 2022 American Chemical Society. (B) Chemical structure of **3** and SPECT image of KB tumor-bearing mice at 4 hours p.i. of [^{67}Ga]Ga-**3** (20 MBq). Reprinted with permission from [121]. Copyright 2022 Elsevier

2.2. Indium-111 radiofolates

2.2.1. [^{111}In]In-DTPA-folate

The first folate-based radioconjugate using ^{111}In was described by Wang *et al.* in 1997 [122,123]. Compound **4** is composed of a DTPA chelating agent linked to γ -carboxylic acid of FA via an EDA linker (Fig. 5). Evaluation of the affinity of [^{111}In]In-**4** ([^{111}In]In-DTPA-(γ)-folate) using KB cells showed significant fixation of the derivative on FR α . Biodistribution study in KB tumor-bearing athymic nude mice was realized with [^{111}In]In-**4**. It was demonstrated that this radioconjugate was significantly uptake in kidneys and tumors. The tumor-to-kidney ratio was of 1.00 which appears to be an excellent value knowing the important presence of FR α in kidneys. Tumor uptake was 3.1 ± 0.6 %IA/g for [^{111}In]In-**4**. However, uptake in lungs, liver, spleen and kidneys was also important and resulted in a very poor tumor-to-background contrast. KB tumor-bearing mice were i.v. injected with [^{111}In]In-**4** 1 hour before imaging. γ -Scintigraphy of KB tumor-bearing mice at 5 and 24 hours p.i. of [^{111}In]In-**4** revealed a significant accumulation of the radioconjugate only in the tumor and kidneys. [^{111}In]In-**4** is also rapidly excreted primarily in the urine. These suggest that imaging tests of FR α -positive tumors on humans using [^{111}In]In-**4** could be explored to develop a non-invasive imaging method to diagnose FR α -positive tumors (section 2.4.1.).

In 2008, Müller *et al.* published further investigations on the suitability of [^{111}In]In-**4** for SPECT imaging [124]. Biodistribution experiments were performed on three types of mice model: IGROV-1 tumor-bearing mice, SKOV-3ip tumor-bearing mice and SKOV-3ip metastasis bearing mice. At 4 hours p.i. of [^{111}In]In-**4** in IGROV-1 or SKOV-3ip tumor-bearing mice, radioconjugate uptake in the IGROV-1 tumor was of 9.79 ± 3.21 %IA/g and 7.57 ± 0.61 %IA/g for SKOV-3ip tumor. Kidney uptake was also observed in both IGROV-1 and SKOV-3ip tumor-bearing mice with 85.61 ± 11.90 and 103.38 ± 18.79 %IA/g, respectively. Salivary gland uptake was also observed in both model with 12.18 ± 1.23 and 12.12 ± 1.62 %IA/g, respectively. Thus, a low tumor-to-kidney ratio was observed for both IGROV-1 or SKOV-3ip tumor-bearing mice, with 0.11 ± 0.04 and 0.08 ± 0.01 , respectively. These low ratios are unfavorable for imaging and diagnostic quality as an important kidney uptake could hide metastasis in the intraperitoneal region for example. SPECT/CT imaging was performed on the three mice models at 4 hours p.i. of [^{111}In]In-**4** (Fig. 5A). Images showed that tumor and kidneys fix [^{111}In]In-**4**. Tumor-to-background contrast was excellent, but metastases were not clearly visible on SPECT/CT images as kidney

uptake was too important. This article concluded that results were quite promising and showed that [^{111}In]In-4 is suitable to detect ovarian carcinoma or ovarian adenocarcinoma. Nevertheless, this radiofolate is not suitable to detect SKOV-3ip metastasis using PET imaging.

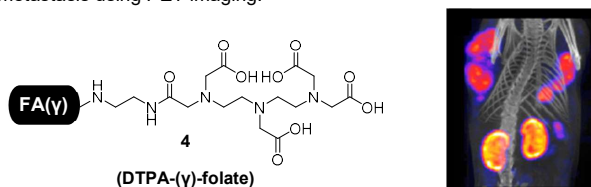


Figure 5: Chemical structure of 4 and SPECT image of IGROV-1 tumor-bearing mice at 4 hours p.i. of [^{111}In]In-4 (30-50 MBq). Image reprinted with permission from [124]. Copyright 2022 SNMMI

2.2.2. [^{111}In]In-DOTA-folate

Müller *et al.* developed new ^{111}In -radiofolate tracer by designing a radioconjugate called [^{111}In]In-5 ([^{111}In]In-DOTA-butanamide-click-(γ)-folate) (Fig. 6). FA was coupled to a DOTA chelating agent using CuAAC click chemistry [125]. The advantage of this chelating agent over DTPA is its capacity to coordinate with a wide range of diagnostic (e.g., ^{111}In , $^{67/68}\text{Ga}$, ^{64}Cu) and therapeutic (e.g., ^{177}Lu , ^{67}Cu , ^{90}Y) radionuclides. Tissue distribution of [^{111}In]In-5 was performed on KB tumor-bearing female athymic nude mice. Tumor uptake was of 5.80 ± 0.55 %IA/g whereas kidney uptake was ten times superior with 55.88 ± 3.91 %IA/g. In addition, liver and salivary glands uptake were also observed with 5.20 ± 2.00 and 6.13 ± 1.84 %IA/g, respectively. The tumor and kidney uptake values stayed stable 24 hours after injection whereas liver and salivary glands uptake significantly decreased after 4 hours. Tumor-to-kidney ratio was insufficient but stayed stable for 24 hours (0.11 ± 0.02). SPECT/CT imaging was performed on KB tumor-bearing mice at 24 hours p.i. of [^{111}In]In-5. Tumors and kidneys were clearly visible on images and no liver or salivary glands fixation were visible at 24 hours. Rapid renal clearance of the radioconjugate contributes to its safety and the high quality of images. To conclude, compared to [^{111}In]In-5, [^{111}In]In-4 is more suitable for clinical trials.

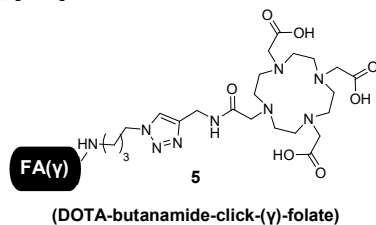


Figure 6: Chemical structure of 5 [125]

2.3. Technetium-99m radiolofates

2.3.1. [$^{99\text{m}}\text{Tc}$]Tc-HYNIC-folates

In 1999, Guo *et al.* developed the first $^{99\text{m}}\text{Tc}$ -radiofolate named [$^{99\text{m}}\text{Tc}$]Tc-6 ([$^{99\text{m}}\text{Tc}$]Tc-HYNIC-(γ)-folate)(tricine/TPPTS) [126]. FA was coupled to HYNIC chelator *via* an amide bond (Fig. 7). HYNIC conjugate was used because, unlike DTPA, its radiolabelling with $^{99\text{m}}\text{Tc}$ was effective even at low concentration. The authors decided to perform *in vivo* experiments with 24JK-FBP tumor-bearing C57BL/6 female mice because 24JK-FBP was more representative of tumors in humans than KB cells. The biodistribution results showed an important tracer uptake in the kidneys and tumors. Tumor-to-kidney ratio at 4 hours was quite low (0.26 ± 0.03). 24JK-FBP tumor-bearing mice expressed less $\text{FR}\alpha$ on tumor cell surfaces explaining a tumor-to-blood ratio seven times lower than in KB tumor-bearing mice. On γ -scintigraphy images, kidneys appeared with a significant brilliance. Tumors were quite visible even if their radiation intensities were lower than kidneys one.

Lu *et al.* synthesized HYNIC-NHHN-FA which was formed of a hexane-1-6-diamine (NHHN) linker between HYNIC chelator and FA [127]. HYNIC-NHHN-FA was radiolabeled with $^{99\text{m}}\text{Tc}$ in presence of tricine/diphenylphosphinobenzene-3-sulfonic acid sodium (TPPMS) or tricine/trisodium triphenylphosphine-

3,3',3''-trisulfonate (TPPTS) to form $[^{99m}\text{Tc}]\text{Tc-7}$ ($[^{99m}\text{Tc}]\text{Tc}-(\text{HYNIC-NHHN}-(\gamma)\text{-folate})(\text{tricine}/(\text{TPPMS}))$) and $[^{99m}\text{Tc}]\text{Tc-8}$ ($[^{99m}\text{Tc}]\text{Tc}-(\text{HYNIC-NHHN}-(\gamma)\text{-folate})(\text{tricine}/(\text{TPPTS}))$), respectively (Fig. 7). These co-ligands played an important role in improving pharmacokinetic properties and increasing the stability of folate-based radioconjugate. Biodistribution of the two radioconjugates in KB tumor-bearing athymic nude mice revealed tumor and kidney uptake of 0.19 ± 0.05 and 3.62 ± 1.43 %IA/g for $[^{99m}\text{Tc}]\text{Tc-7}$ and 9.79 ± 1.66 and 114.88 ± 8.12 %IA/g for $[^{99m}\text{Tc}]\text{Tc-8}$, respectively. Thus, no significant tumor uptake was observed for $[^{99m}\text{Tc}]\text{Tc-7}$ whereas TPPTS analog exhibited good FR α -positive cell uptake and retention and a good tumor-to-background contrast. These results highlighted the important of the choice of co-ligands to chelate ^{99m}Tc as they play an important role on biological properties. They concluded that the TPPMS analog was quickly excreted due to its high hydrophobicity ($\log P$ of -2.34 ± 0.01) which conferred it different pharmacokinetic properties. Tumor-to-kidney ratio was low with 0.085 for $[^{99m}\text{Tc}]\text{Tc-8}$. This study demonstrated that only $[^{99m}\text{Tc}]\text{Tc-8}$ presented favorable biological characteristics and could lead to promising results for small animal imaging experiments.

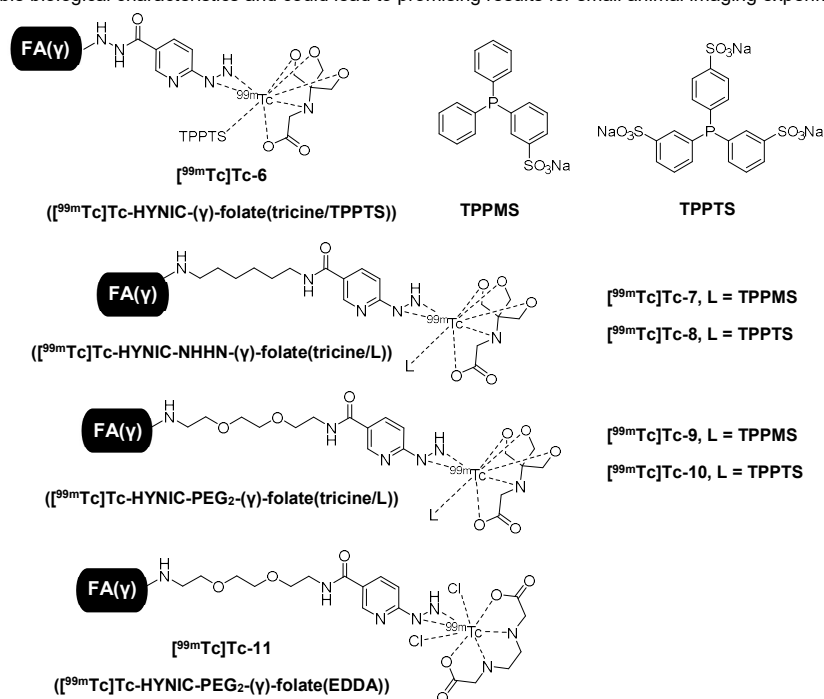


Figure 7: Chemical structures of $[^{99m}\text{Tc}]\text{Tc-6}$, co-ligands TPPMS and TPPTS, $[^{99m}\text{Tc}]\text{Tc-7}$, $[^{99m}\text{Tc}]\text{Tc-8}$, $[^{99m}\text{Tc}]\text{Tc-9}$, $[^{99m}\text{Tc}]\text{Tc-10}$ and $[^{99m}\text{Tc}]\text{Tc-11}$ [126,127]

To pursue their work, the same team synthesized HYNIC-PEG₂-FA which was composed of HYNIC chelator linked to FA thanks to 2,2'-(ethylenedioxy)-bis-ethylamine (PEG₂). Tricine/TPPTS, tricine/TPPMS and ethylenediamine-*N,N*-diacetic acid (EDDA) were co-ligands used to coordinate ^{99m}Tc [128]. The three complexes formed were respectively $[^{99m}\text{Tc}]\text{Tc-9}$ ($[^{99m}\text{Tc}]\text{Tc}-(\text{HYNIC-PEG}_2-(\gamma)\text{-folate})(\text{tricine}/\text{TPPTS}))$, $[^{99m}\text{Tc}]\text{Tc-10}$ ($[^{99m}\text{Tc}]\text{Tc}-(\text{HYNIC-PEG}_2-(\gamma)\text{-folate})(\text{tricine}/\text{TPPMS}))$ and $[^{99m}\text{Tc}]\text{Tc-11}$ ($[^{99m}\text{Tc}]\text{Tc}-(\text{HYNIC-PEG}_2-(\gamma)\text{-folate})(\text{EDDA}))$ (Fig. 7). Biodistribution was performed on KB tumor-bearing mice and a major accumulation in kidneys for $[^{99m}\text{Tc}]\text{Tc-9}$, $[^{99m}\text{Tc}]\text{Tc-10}$ and $[^{99m}\text{Tc}]\text{Tc-11}$ with 149.70 ± 19.57 , 24.38 ± 3.25 and 121.41 ± 11.5 %IA/g, respectively, was observed. Tumor uptake of $[^{99m}\text{Tc}]\text{Tc-9}$, $[^{99m}\text{Tc}]\text{Tc-10}$ and $[^{99m}\text{Tc}]\text{Tc-11}$ were respectively of 11.35 ± 0.67 , 0.67 , 1.24 ± 0.01 and 5.52 ± 0.58 %IA/g leading to tumor-to-kidney ratios of 0.075, 0.05, 0.045, respectively. The tumor-to-kidney ratios of $[^{99m}\text{Tc}]\text{Tc-10}$ and $[^{99m}\text{Tc}]\text{Tc-11}$ were the lowest never observed in radiofolate studies. These results clearly demonstrated that $[^{99m}\text{Tc}]\text{Tc-9}$ was the best of the three developed complexes. The linker used here was more hydrophilic than NHHN previously described ($\log P$ of -

3.40 ± 0.12 for [^{99m}Tc]Tc-9 vs. -3.26 ± 0.08 for [^{99m}Tc]Tc-8) and enhanced the hydrophilicity of the tracer and so its renal clearance. Through this, the team wanted to improve SPECT imaging quality. [^{99m}Tc]Tc-9 was selected for SPECT imaging studies on KB tumor-bearing mice. Only tumor and kidneys were visible on images which confers to [^{99m}Tc]Tc-9 a good tumor-to-background contrast ability. Moreover, high activity found in bladder confirmed the renal clearance way. To conclude, [^{99m}Tc]Tc-9 was the most suitable compound developed in this study for nuclear imaging.

Guo *et al.* studied [^{99m}Tc]Tc-12 ([^{99m}Tc]Tc-HYNIC-T-(γ)-folate(tricine/TPPTS)) [129]. HYNIC-T-(γ)-folate was composed of HYNIC chelator linked to the γ-carboxylate group of glutamate residue of FA using CuAAC click chemistry reaction (Fig. 8). Biodistribution study in KB tumor-bearing athymic nude mice revealed accumulation in tumor with a concentration in radiofolate of 8.14 ± 0.45 %IA/g at 4 hours and especially in kidneys with 57.72 ± 4.50 %IA/g. This induced a low but correct tumor-to-kidney ratio equal to 0.14. SPECT imaging was performed 4 hours after radioconjugate injection and brought forward tumor and kidneys but also bladder (Fig. 8). Guo *et al.* came to the conclusion that [^{99m}Tc]Tc-12 has a strong tumor uptake, a low uptake in non-specific organs such as in the liver and muscles and a fast renal clearance way. In short, this study shows that [^{99m}Tc]Tc-12 was a good candidate to track the evolution of therapies by SPECT imaging.

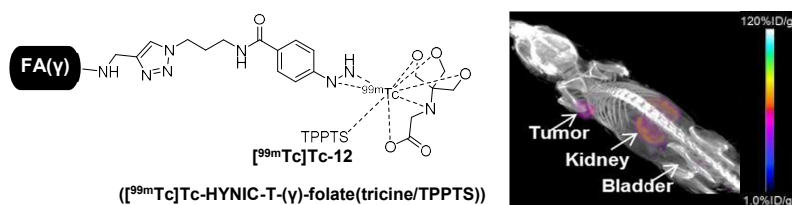


Figure 8: Chemical structure of [^{99m}Tc]Tc-12 and SPECT image of KB tumor-bearing mice at 4 hours p.i. of [^{99m}Tc]Tc-12 (18 MBq). Image reprinted with permission from [129]. Copyright 2022 Elsevier

Guo *et al.* coupled HYNIC to the ε-amine of lysine-pteroyl via an amide bond to give the HYNIC-Lys-Pteroyl compound. Tricine and TPPTS were used as co-ligands to coordinate ^{99m}Tc, affording [^{99m}Tc]Tc-13 ([^{99m}Tc]Tc-(HYNIC-lys-pteroyl)(tricine/TPPTS)) (Fig. 9) [130]. Biodistribution in KB tumor-bearing BALB/c nude male mice showed that the largest accumulation of [^{99m}Tc]Tc-13 was found in kidneys with 89.60 ± 5.06 %IA/g whereas tumor uptake was only of 9.60 ± 1.46 %IA/g at 2 hours. Tumor-to-kidneys ratio was equal to 0.11 and tumor-to-liver ratio was low (3.89) leading to a massive hepatobiliary excretion of the radiofolate which was not optimal for a good diagnostic as renal excretion way is more suitable. SPECT imaging was performed on KB tumor-bearing mice at 2 hours p.i. of [^{99m}Tc]Tc-13 (Fig. 9) and a major activity was found in tumor, kidney, liver and bladder. This confirmed hepatobiliary excretion of the radiofolate that is not an optimal way of clearance. Moreover, the intense activity found in the abdomen area could mask a potential tumor or metastasis in this area. Thus, this radiofolate was not suitable for FRα-overexpressing tumor diagnosis.

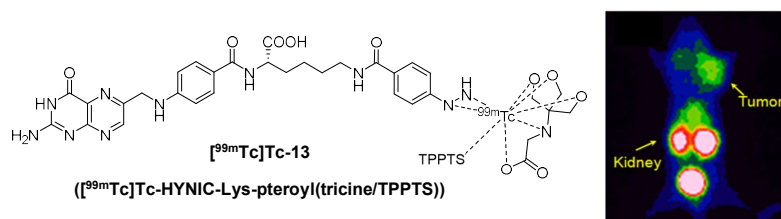


Figure 9: Chemical structure of [^{99m}Tc]Tc-13 and SPECT image of KB tumor-bearing mice at 2 hours p.i. of [^{99m}Tc]Tc-13 (7.4 MBq). Image reprinted with permission from [130]. Copyright 2022 Elsevier

2.3.2. [^{99m}Tc]Tc-peptide-folates

Kim *et al.* synthesized [^{99m}Tc]Tc-14 ([^{99m}Tc]Tc-ECG-EDA-(γ)-folate) by coupling the peptide H-Gly-Cys-Glu-OH, to FA thanks to an EDA linker [131] (Fig. 10). The peptide moiety played the role of the chelating agent.

Biodistribution in KB tumor-bearing BALB/c athymic nude mice showed significant accumulation of [^{99m}Tc]Tc-14 in the tumor, kidney, liver, intestines and gallbladder. These non-specific accumulations hepatobiliary clearance way might be due to the hydrophobic nature of [^{99m}Tc]Tc-14. Scintigraphy imaging was performed at 4 hours p.i. of radioconjugate and revealed KB tumor, kidneys, liver, intestines and bladder with a huge activity intensity. SPECT/CT imaging allowed to well visualize KB tumor, intestines and kidneys even 22 hours p.i. of radioconjugate. [^{99m}Tc]Tc-14 showed good tumor absorption but further studies have to be performed to overcome the problem of non-specific organ uptake which conduct to a poor tumor-to-background contrast.

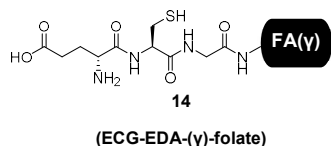


Figure 6. Chemical structure of 14 [131]

2.3.3. [^{99m}Tc]Tc-ethylenedicysteine-folate

Ligan *et al.* developed [^{99m}Tc]Tc-15 ([^{99m}Tc]Tc-ethylenedicysteine-(γ)-folate) which was formed of the ethylenedicysteine chelator linked to FA on its γ-carboxylate function via an EDA linker (Fig. 11) [132]. Biodistribution in female Fischer 344 rats inoculated with mammary tumor cells of the cell line 13762 revealed a significant uptake of [^{99m}Tc]Tc-15 in kidneys with 5.102 ± 0.276 %IA/g at 2 hours whereas tumor uptake was low and of the same order than other tested organs. This led to the lowest tumor-to-kidney ratio (0.04) observed for a ^{99m}Tc-radiofolate. These let to think that tumor cells selected for this study did not express enough FR α on cell surface. SPECT images were recorded at 4 hours p.i. of radioconjugate and exhibited intense activity in the tumor and kidneys. Furthermore, a renal excretion way as intense activity in bladder was also observed. These results suggested that [^{99m}Tc]Tc-15 might not be a good candidate for imaging of FR α -positive tumors.

2.3.4. [^{99m}Tc]Tc-TEPA-folate

A radiodiagnostic agent [^{99m}Tc]Tc-16 ([^{99m}Tc]Tc-TEPA-(γ)-folate) was developed by Panwar *et al.* [133] (Fig. 11), which was composed of the TEPA chelating agent coupled to FA on the γ-carboxylic acid of the glutamic entity via an amide bond. Affinity binding assay was performed on KB cells and revealed that [^{99m}Tc]Tc-16 had a K_d of 5 ± 0.06 μM meaning an affinity 5000 times lower for FR α than FA (1 nM). Biodistribution in KB tumor xenografted nude mice revealed a major uptake of [^{99m}Tc]Tc-16 at 4 hours in the kidneys following by liver, intestines and tumor (*i.e.*, 16.0 ± 1.2 , 16.1 ± 2.0 , 4.56 ± 0.3 and 4.26 ± 1.3 %IA/g, respectively). Important non-specific uptakes were not a good result for the suitability of this radioconjugate in diagnostic imaging. Scintigraphy was performed with the same mice model at 1 hour p.i. of radioconjugate and displayed intense activity in kidneys, liver, intestines and tumors leading to a real poor image quality. These non-specific targeting may be explained by the hydrophobicity of the molecule, the chelating agent having any electronegative groups expected for the 5 amine functions. This increased the hepatobiliary clearance and so accumulation in liver and intestines. In summary, modifications on the compound structure are required to increase the tumor specificity.

2.3.5. [^{99m}Tc]Tc-DO3A-EA-folate

[^{99m}Tc]Tc-17 ([^{99m}Tc]Tc-DO3A-EA-(γ)-folate) has been developed by Mishra *et al.* [134]. DO3A chelator and FA composed this tracer (Fig. 11). The affinity coefficient of 4.2 ± 0.8 μM indicated that [^{99m}Tc]Tc-17 was not as specific as FA and could lead to non-specific binding. Biodistribution on KB tumor-bearing nude mice showed a major accumulation of [^{99m}Tc]Tc-17 at 4 hours in liver and kidneys, less but significant uptake was also observed for tumors and intestines with respectively 12.2 ± 1.5 , 13.2 ± 1.1 , 6.26 ± 0.8 and 4.1 ± 0.8 %IA/g. SPECT scintigraphy images were recorded at 4 hours p.i. of radioconjugate and tumor was well visualized. Massive activity was detected in the whole abdomen of mice leading to poor quality images. High uptake in intestines presumes hepatobiliary clearance that is not recommended for good pharmacokinetic properties of radioconjugate and could prevent to see tumor in abdomen area. Too important non-specific uptake and poor image quality were problematic and jeopardize further investigations.

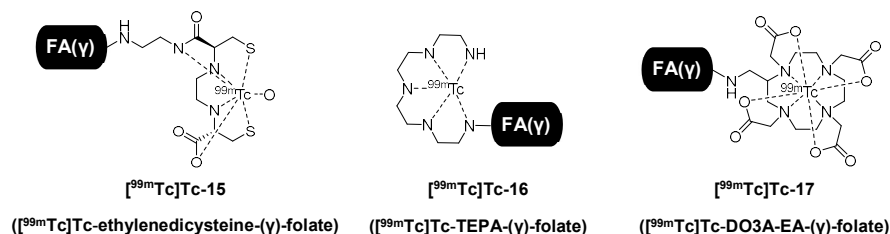


Figure 7: Chemical structures of $[^{99m}\text{Tc}]\text{Tc-15}$, $[^{99m}\text{Tc}]\text{Tc-16}$ and $[^{99m}\text{Tc}]\text{Tc-17}$ [132–134]

2.3.6. $[^{99m}\text{Tc}]\text{Tc}(\text{CO}_3)\text{-folates}$

Müller *et al.* also worked on $[^{99m}\text{Tc}]\text{Tc}(\text{CO}_3)\text{-radiofolates}$ by developing three radioconjugates: $[^{99m}\text{Tc}]\text{Tc-18}$ ($[^{99m}\text{Tc}]\text{Tc}(\text{CO}_3)\text{-PAMA-}(\alpha)\text{-folate}$), $[^{99m}\text{Tc}]\text{Tc-19}$ ($[^{99m}\text{Tc}]\text{Tc}(\text{CO}_3)\text{-PAMA-}(\gamma)\text{-folate}$) and $[^{99m}\text{Tc}]\text{Tc-20}$ ($[^{99m}\text{Tc}]\text{Tc}(\text{CO}_3)\text{-PAMA-pteroyl}$) [102,135]. The chelating agent picolylamine monoacetic acid (PAMA) was developed to chelate ^{99m}Tc using three carbon monoxide co-ligands. Compounds **18** and **19** were composed of PAMA entity linked to the α - and γ -carboxylic acids of FA, respectively. Compound **20** was composed of PAMA entity coupled *via* an amide bond to the pteroyl entity (Fig. 12). For these three radioconjugates, binding assay on KB cells showed excellent and similar cell binding (48% for $[^{99m}\text{Tc}]\text{Tc-20}$, 57% for $[^{99m}\text{Tc}]\text{Tc-19}$ and 60% for $[^{99m}\text{Tc}]\text{Tc-18}$). Excellent K_d constant was obtained compared to free FA (2.51, 2.09, 14.52 and 7.22 nM for $[^{99m}\text{Tc}]\text{Tc-18}$, $[^{99m}\text{Tc}]\text{Tc-19}$, $[^{99m}\text{Tc}]\text{Tc-20}$ and FA, respectively). Biodistribution in KB tumor-bearing athymic nude mice indicated no significant uptake of $[^{99m}\text{Tc}]\text{Tc-20}$ in tumor and kidneys at 4 hours p.i. and was rejected from the further studies. Significant uptake was observed only for $[^{99m}\text{Tc}]\text{Tc-19}$ and $[^{99m}\text{Tc}]\text{Tc-18}$ in tumor (2.33 ± 0.36 and 1.24 ± 0.19 %IA/g, respectively) and kidneys (18.48 ± 0.72 %IA/g and 12.36 ± 1.86 %IA/g, respectively) leading to tumor-to-kidney ratios of 0.13 and 0.10, respectively. Indeed, $[^{99m}\text{Tc}]\text{Tc-19}$ expressed slightly better biodistribution results than $[^{99m}\text{Tc}]\text{Tc-18}$ and was chosen for SPECT imaging study performed on KB tumor-bearing mice. At 24 hours, tumors, kidneys and intestines were highly visible on images. This could be explained by the high radioconjugate concentrations in the content of intestines due to a hepatobiliary excretion way. To conclude, $[^{99m}\text{Tc}]\text{Tc-19}$ successfully assessed *in vitro* and *in vivo* tests compared to $[^{99m}\text{Tc}]\text{Tc-20}$. $[^{99m}\text{Tc}]\text{Tc-19}$ and $[^{99m}\text{Tc}]\text{Tc-18}$ showed similar properties and are both suitable for further investigations notably to improve their bioavailability. Müller *et al.* pursued their investigations with $[^{99m}\text{Tc}]\text{Tc-19}$ by exploring its targeting behavior for eight FR α -positive cancer cell lines (*i.e.*, KB, IGROV-1, SKOV-3ip, HT29, LoVo, 24JK-FBP, KB-V1 and HeLa cells) [136]. *In vitro* cell uptake study and *in vivo* biodistribution of $[^{99m}\text{Tc}]\text{Tc-19}$ on the 8 different FR α -positive tumor-bearing mice models using revealed that $[^{99m}\text{Tc}]\text{Tc-19}$ is suitable to detect all tested FR α -positive cell lines and so different cancerous cell types.

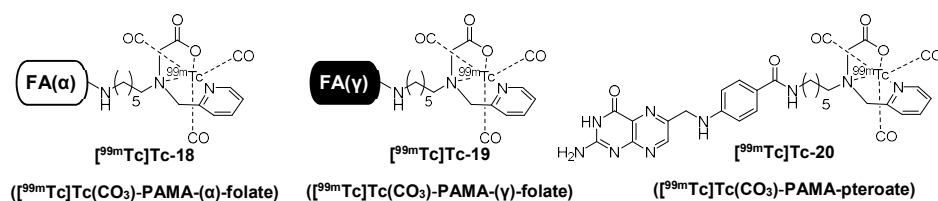


Figure 12: Chemical structures of $[^{99m}\text{Tc}]\text{Tc-18}$, $[^{99m}\text{Tc}]\text{Tc-19}$ and $[^{99m}\text{Tc}]\text{Tc-20}$ [102,135]

Müller and coworkers designed $[^{99m}\text{Tc}]\text{Tc-21}$ ($[^{99m}\text{Tc}]\text{Tc}(\text{CO}_3)\text{-triazole-}(\gamma)\text{-folate}$) through an innovative strategy called click-to-chelate [137,138]. This strategy consisted in conjugating a small molecule to FA by CuAAC click chemistry reaction and using the small molecule and the formed triazole ring as chelator moieties (Fig. 13). The team also synthesized an analog of $[^{99m}\text{Tc}]\text{Tc-21}$ by replacing the triazole entity by a histidine to give $[^{99m}\text{Tc}]\text{Tc-22}$ ($[^{99m}\text{Tc}]\text{Tc}(\text{CO}_3)\text{-N}(\text{H})\text{-}(\gamma)\text{-folate}$). *In vitro* cell uptake assay of $[^{99m}\text{Tc}]\text{Tc-21}$ and $[^{99m}\text{Tc}]\text{Tc-22}$ was performed on KB cells and showed a similar uptake of 50%. Biodistribution on KB tumor-bearing mice revealed similar profile for both radiofolates as the only significant different was a high liver uptake for $[^{99m}\text{Tc}]\text{Tc-22}$ than for $[^{99m}\text{Tc}]\text{Tc-21}$ (3.83 ± 1.49 vs. 0.89 ± 0.42 %IA/g at 4 hours p.i., respectively). Significant uptake was

observed in kidneys and tumors in the same level. A second biodistribution study was performed on KB tumor-bearing mice using $[^{99m}\text{Tc}]\text{Tc-22}$ and similar results were obtained, excepted for salivary glands showing a significant non-specific uptake of $5.72 \pm 0.63\% \text{I/A/g}$ [138]. *Ex vivo* autoradiography confirmed biodistribution results as uptake in tumor, kidneys and salivary glands was significant. SPECT/CT images were recorded at 24 hours p.i. of $[^{99m}\text{Tc}]\text{Tc-22}$. High activity was measured in KB tumors, kidneys and salivary glands. These results confirm the suitability of $[^{99m}\text{Tc}]\text{Tc-22}$ for SPECT/CT diagnostic as no non-specific uptake in the abdominal area was observed (Fig. 13).

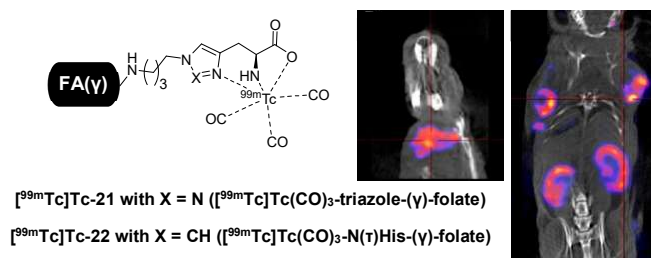


Figure 13: Chemical structures of $[^{99m}\text{Tc}]\text{Tc-21}$ and $[^{99m}\text{Tc}]\text{Tc-22}$, and SPECT images of KB tumor-bearing mice at 24 hours p.i. of $[^{99m}\text{Tc}]\text{Tc-22}$ (500 MBq). Image reprinted with permission from [138]. Copyright 2022 SNMMI

2.3.7. $[^{99m}\text{Tc}]\text{Tc-EC20}$

Leamon *et al.* used the innovative folate-based radioconjugate for SPECT imaging $[^{99m}\text{Tc}]\text{Tc-23}$ ($[^{99m}\text{Tc}]\text{Tc-EC20}$ also called $[^{99m}\text{Tc}]\text{Tc-etarfolatide}$) [139] (Fig. 14). ^{99m}Tc formed 4 coordination bonds with the chelating agent and 2 with an oxygen atom as co-ligand. The IC_{50} of 128 nM obtained was closed to those of FA and $[^{111}\text{In}]\text{In-4}$ meaning that $[^{99m}\text{Tc}]\text{Tc-23}$ is as specific for $\text{FR}\alpha$ as a successful clinical trial radioconjugate. Biodistribution profile was achieved on M109 tumor-bearing BALB/c mice. Significant uptake was observed 4 hours p.i. in tumor and kidneys (17.2 ± 1.02 and $138 \pm 12.4\% \text{I/A/g}$, respectively) and no significant uptake was observed in non-specific organs. Tumor-to-kidney ratio was of 0.12 for $[^{99m}\text{Tc}]\text{Tc-23}$ consolidating the promising of the observed results. Metabolism study revealed that $[^{99m}\text{Tc}]\text{Tc-23}$ is predominantly eliminated from the body via the renal way. SPECT imaging was performed on the same mice model at 4 hours p.i. of radioconjugate. Tumor and kidneys were clearly visible on images and other organs did not express activity intensity. To conclude, these preliminary results are very encouraging to push further tests with clinical trials.

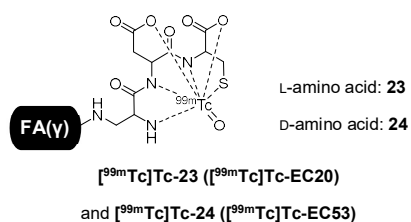


Figure 14: Chemical structures of $[^{99m}\text{Tc}]\text{Tc-23}$ and $[^{99m}\text{Tc}]\text{Tc-24}$ [139]

The study of $[^{99m}\text{Tc}]\text{Tc-23}$ was continued, and comparative experiments were made with its D-amino acid analog $[^{99m}\text{Tc}]\text{Tc-24}$ ($[^{99m}\text{Tc}]\text{Tc-EC53}$) (Fig. 14) [140]. Affinity tests were performed on KB cells. $[^{99m}\text{Tc}]\text{Tc-23}$ and $[^{99m}\text{Tc}]\text{Tc-24}$ afforded K_d of 0.92 and 0.23 nM, respectively, meaning that $[^{99m}\text{Tc}]\text{Tc-24}$ had a highest specificity to $\text{FR}\alpha$. Biodistribution study was performed in M109 and 4T1 tumor-bearing BALB/c mice, and revealed a significant uptake in KB tumor and kidneys for both radioconjugates. The uptake in almost all organs was lower of 33% for $[^{99m}\text{Tc}]\text{Tc-24}$ compared to $[^{99m}\text{Tc}]\text{Tc-23}$, leading to the same tumor-to-kidney ratio of 0.15. Additional biodistribution tests were done in KB tumors-bearing mice after i.v. and i.p. injections of $[^{99m}\text{Tc}]\text{Tc-23}$, respectively. Biodistribution profiles were quite similar and led to conclusion that uptake is independent of tumor and injection localization. With the objective of enhancing the tumor-to-kidney ratio, KB

tumor-bearing mice were pre- or co-injected with free FA without success. Despite a general decrease of organs uptake in a dose-dependently manner that led to the same tumor-to-kidney ratio, injection of 1 equivalent of free FA improved the tumor-to-background contrast. As [^{99m}Tc]Tc-24 gave no better results than [^{99m}Tc]Tc-23, they focused their research on the first developed radioconjugate.

2.4. Clinical evaluation of SPECT radiofolate

Two radiofolates were evaluated in clinical trials for SPECT imaging diagnosis of FRα-overexpressing tumors: [¹¹¹In]In-4 and [^{99m}Tc]Tc-23. The first-in-human application was performed with [¹¹¹In]In-4. [^{99m}Tc]Tc-23 is the only SPECT folate radioconjugate that was used clinically and in official studies. It is currently used in clinical routine. This following section resumed both clinical trials of [¹¹¹In]In-4 and [^{99m}Tc]Tc-23.

2.4.1. [¹¹¹In]In-DTPA-folate

Regarding promising preclinical results, Phase I and II clinical trials were pursued using [¹¹¹In]In-4 as a diagnostic imaging agent for ovarian cancer [141]. These trials were performed to study its safety, biodistribution and efficacy. 33 women from 31 to 82-years-old participated in this study, out of which 14 with malignant tumors (7 ovarian tumors, 2 recurrent endometrial tumors and 5 recurrent ovarian tumors), 18 with benign pelvic masses and 1 with a borderline malignancy. After SPECT imaging at 4 hours p.i. (Fig. 15A), a biopsy was performed on patients to clearly define the malignant nature or not of masses. Only 5 patients reported minor adverse reactions like rashes or kidney discomfort that disappeared causing no sequelae. Uptake was observed in kidneys for all patients. Few patients also presented liver, spleen or gallbladder uptake. Two nuclear medicine physicians were charged with reading images based on two approaches: masked vs. unmasked. The images were read without (*i.e.*, masked) or with (*i.e.*, masked) complementary data of anatomic imaging studies (CT, sonography, MRI). For the masked study, 78% and 75% of the patient's diseases were correctly diagnosed, compared to 82% and 81% for the unmasked one. All malignant masses were correctly diagnosed. However, two false positive diagnoses of malignant tumors were made for two patients (*i.e.*, one suffering from endometriosis and one from adenomatoid tumor/hilar Leydig cell hyperplasia). For the recurrent malignancies only, the correct diagnostic ratio was only of 35% for the masked study and increased to 85% in the unmasked one. This indicated the importance of the correlation between the complementary data obtained with previous anatomical imaging studies and scintigraphy images. [¹¹¹In]In-4 radiopharmaceutical was safe and effective for ovarian malignancies imaging because injection was safe and rapid uptake in the tumor and quick clearance from non-targeting tissues were observed. Images were similar and malignant tumors were well diagnosed by both nuclear medicine physicians.

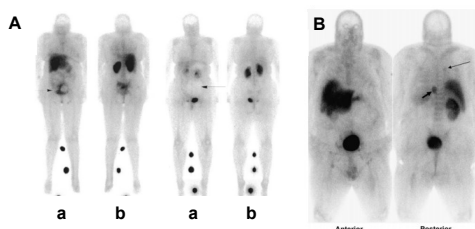


Figure 15: (A) Planar scintigrams of patients at 4 hours p.i. of [¹¹¹In]In-4 (183 MBq) (a : anterior, b: posterior). Image reprinted with permission from [141]. Copyright 2022 SNMMI. (B) SPECT images of 57-year-old man with pulmonary metastases from renal cell carcinoma at 1-2 hours p.i. of [^{99m}Tc]Tc-23 (555 MBq). Image reprinted with permission from [142]. Copyright 2022 SNMMI

2.4.2. [^{99m}Tc]Tc-EC20

Phase I clinical study was performed on 6 healthy untreated males (aged 20-65 years old) with no renal and hepatic dysfunction [143–145]. Subjects were *i.v.* pre-treated with FA before *i.v.* injection of [^{99m}Tc]Tc-23. Health constants was then monitored up to 7 days, highlighting dizziness, diarrhea and increased white blood cells percentage in 50% of subjects. These events were related to radioconjugate injection but not considered as severe events to stop the study. Pharmacokinetic was evaluated during 24 hours by measuring activity in blood and activity decreased rapidly as the half-life resident time in blood was of 12 min. Urine was also collected

for 24 hours to evaluate activity absorbed dose in organs. It was showed that urinary bladder and kidneys were the two organs absorbing the most radioconjugate. Whole-body planar imaging was performed at 5 min to 24 hours p.i.. Activity was clearly visualized in liver, kidneys and urinary bladder up to 24 hours p.i. but also moderately in the bone marrow at 1 and 4 hours p.i.. Imaging confirmed results of pharmacokinetics. Other phase I clinical trials were pursued in 2014 leading to the same results. [^{99m}Tc]Tc-23 was well tolerated by subjects leading to the succeed of phase I.

Fisher *et al.* published phase II clinical trials results of [^{99m}Tc]Tc-23 imaging to identify patients with FR α -positive tumors [142]. 154 patients with proven (CT, MRI) or suspected renal, ovarian, endometrial, breast or pituitary cancer participated in this study. Chosen patients should have lesions > 1.5 cm, no renal dysfunction and a tissue sample from surgery or biopsy. This latter was necessary to perform immunohistochemistry assay for analyzing FR α expression in tumors. Pre-treatment with FA before i.v. injection of [^{99m}Tc]Tc-23 was necessary to obtain images with good tumor-to-background contrast. SPECT imaging was performed at 1 to 2 hours p.i. (Fig. 15B) and, for most patients, specific uptake was observed in tumors and kidneys, which was not the case in bladder, liver and spleen. The monitoring of patient's health for 7 days p.i. revealed abdominal pain, nausea and vomiting for 8% of patients and were considered relative to radioconjugate injection. These non-severe events allowed to conclude that one injection of [^{99m}Tc]Tc-23 was well tolerated by patients. 66.7% of patients were diagnosed with a cancer after nuclear imaging against 68% regarding immunohistochemistry assay results. Comparison of imaging and immunohistochemistry results showed an agreement in diagnostic of only 61%. This poor value is explained by the time spending between cancerous tissues sampling and imaging (from few days to few years). To conclude, clinical trial results were promising, and the team conducted further clinical tests to prove the suitability and efficiency of [^{99m}Tc]Tc-23 to diagnose FR α -positive tumors.

2.5. Conclusion

First folate-based radioconjugates were developed in the late 90s for SPECT devices using ⁶⁷Ga and ¹¹¹In. These searches conducted to the first application of a folate-based radiopharmaceuticals for clinical diagnostic of ovarian and endometrial tumors. The first radiofolate [⁶⁷Ga]Ga-1 described in the literature presented one of the best tumor-to-kidney ratio observed for radiofolate injection in FR α -positive tumor-bearing mice. Nevertheless, ⁶⁷Ga-folate-based radioconjugates also showing non-significant advantages in image and diagnostic quality compared to [¹¹¹In]In-4, which may be one of the reasons why no new folate-based tracers using ⁶⁷Ga were developed since 2011. Moreover, studies on ⁶⁷Ga-radioconjugates helped the development of ⁶⁸Ga-radiochemistry for PET applications. [¹¹¹In]In-4 was used for first-in-human application and its clinical use led to the development of new folate-based radioconjugates using ¹¹¹In, but without significant improvement in diagnostic quality. The disinterest to develop new folate-based tracers using ⁶⁷Ga and ¹¹¹In was also probably caused by the keen interest for ^{99m}Tc which was the most affordable radionuclide for SPECT imaging. Huge numbers of ^{99m}Tc-folate-based-radioconjugates were developed within the last 20 years. Different chelating strategies were implemented such as the use of chelating agents with or without co-ligand like (CO)₃ or TPPTS. These experimentations led to the discovery of [^{99m}Tc]Tc-23 which is now used in clinical routine to diagnose ovarian tumors and other tumors overexpressing FR α . Most representative radiofolates developed for SPECT imaging have been depicted in this section and main results are described in Table 4. Following, classical folate-based tracers labelled with β^+ -emitted isotopes to diagnose FR α -positive tumors by PET imaging will be described.

Table 4: Summary of radiochemistry, *in vitro* and *in vivo* results of radiofolates depicted in this section

| Radiofolate | RCY (%) | Log P | K _d for FR α (nM) | Tumor uptake (%IA/g) (4h) | Tumor-to-kidney ratio (4h) | References |
|--------------------------|---------|-------|-------------------------------------|---------------------------|----------------------------|------------|
| [⁶⁷ Ga]Ga-1 | NC | NC | 2 | 5.2 \pm 1.5 | 2.6 | [100,120] |
| [⁶⁷ Ga]Ga-2 | >90 | NC | 1.6 ^d | NC | NC | [101] |
| [⁶⁷ Ga]Ga-3 | NC | NC | NC | 6.08 \pm 0.89 | 0.07 \pm 0.01 | [121] |
| [¹¹¹ In]In-4 | >98 | NC | NC | 3.1 \pm 0.6 | 1.00 \pm 0.34 | [122,123] |
| [^{99m} Tc]Tc-4 | >97 | NC | NC | 3.65 \pm 0.23 | 0.14 \pm 0.01 | [146] |

| | | | | | | |
|---------------------------|--------|---------------------------|--------------------|----------------------------|--------------------|---------------|
| [¹¹¹ In]In-5 | >90 | -4.21 ± 0.11 ^c | NC | 5.80 ± 0.55 | 0.10 ± 0.01 | [125] |
| [¹⁷⁷ Lu]Lu-5 | >90 | NC | NC | 7.51 ± 1.25 | 0.13 ± 0.02 | [125] |
| [^{99m} Tc]Tc-6 | NC | NC | NC | 17.84 ± 11.23 | 0.26 ± 0.03 | [126] |
| [^{99m} Tc]Tc-7 | >90 | -2.34 ± 0.01 | NC | 0.19 ± 0.05 | 0.05 | [127] |
| [^{99m} Tc]Tc-8 | >90 | -3.23 ± 0.08 | NC | 9.79 ± 1.66 | 0.09 | [127] |
| [^{99m} Tc]Tc-9 | >90 | -3.40 ± 0.12 | NC | 11.35 ± 0.67 ^b | 0.08 ^b | [128] |
| [^{99m} Tc]Tc-10 | >90 | -2.46 ± 0.04 | NC | 1.24 ± 0.01 ^b | 0.05 ^b | [128] |
| [^{99m} Tc]Tc-11 | >90 | -3.05 ± 0.35 | NC | 5.52 ± 0.58 ^b | 0.05 ^b | [128] |
| [^{99m} Tc]Tc-12 | >95 | -2.40 ± 0.17 | NC | 8.14 ± 0.45 | 0.14 | [129] |
| [^{99m} Tc]Tc-13 | NC | -2.89 ± 0.06 | NC | 7.85 ± 1.37 | 0.09 | [130] |
| [^{99m} Tc]Tc-14 | >94 dc | NC | NC | 3.12 ± 0.06 | NC | [131] |
| [^{99m} Tc]Tc-15 | NC | NC | NC | 0.147 ± 0.026 ^a | 0.036 ^a | [132] |
| [^{99m} Tc]Tc-16 | >95 | NC | 5000 ± 06 | 4.26 ± 1.3 | 0.27 ± 0.10 | [133] |
| [^{99m} Tc]Tc-17 | >97 | NC | 4.2 ± 0.8 | 6.26 ± 0.4 | 0.47 | [134] |
| [^{99m} Tc]Tc-18 | 92 dc | NC | 2.51 ^d | 1.24 ± 0.19 | 0.10 ± 0.00 | [102,135,136] |
| [^{99m} Tc]Tc-19 | 92 dc | NC | 2.09 ^d | 2.33 ± 0.36 | 0.13 ± 0.02 | [102,135,136] |
| [^{99m} Tc]Tc-20 | 92 dc | NC | 14.52 ^d | 0.43 ± 0.17 | 0.13 ± 0.05 | [102,135,136] |
| [^{99m} Tc]Tc-21 | 95 dc | -3.41 ± 0.06 | NC | 4.84 ± 0.10 | 0.18 | [137] |
| [^{99m} Tc]Tc-22 | 95 dc | -3.47 ± 0.05 | NC | 4.29 ± 0.67 | 0.17 | [137] |
| [^{99m} Tc]Tc-23 | 95 dc | NC | 0.92 | 17.68 ± 1.95 | 0.20 ± 0.01 | [139,140] |
| [^{99m} Tc]Tc-24 | 95 dc | NC | 0.23 | 11.77 ± 4.26 | 0.2 ± 0.1 | [140] |

RCY: Radiochemical yield; Log P: Logarithm of the Partition coefficient; NC: Not communicated; dc: Decay corrected; ^a: 1h; ^b: 2h; ^c: Log D_{7.4} (Logarithm of the Distribution coefficient at pH 7.4); ^d: IC₅₀ (nM)

3. Radiofolates developed for PET imaging application

3.1. Fluorine-18 radiofolates

3.1.1. ¹⁸F-folates synthesized using the conjugation approach

Bettio *et al.* presented the first radiofolate labeled with ¹⁸F for visualization of FRα-positive cells using PET [147]. 4-fluorobenzylamine was fixed to α- or γ-carboxylate function of glutamate residue of FA to obtain the [¹⁸F]25/26 ([¹⁸F]FBA-(α/γ)-folates) regioisomer mixture (Fig. 16). Postmortem biodistribution and *in vivo* PET imaging were achieved using KB tumor-bearing athymic nude mice. Postmortem biodistribution results at 125 min p.i. of [¹⁸F]25/26 presented a major uptake in kidneys (40.65 ± 12.81 %IA/g) following by tumors (6.56 ± 1.80 %IA/g) and duodenum (5.01 ± 2.01 %IA/g). High biodistribution of [¹⁸F]25/26 found in bile, urine and feces let conclude on both hepatobiliary and renal clearance ways. PET imaging highlighted high activity in gallbladder, bladder and part of intestines that confirmed also hepatobiliary and renal elimination pathways. The dorsal image had a good contrast and showed that KB tumors were quite visible using [¹⁸F]25/26 (Fig. 16). To conclude, even if [¹⁸F]25/26 was not the ideal PET radiofolate, these first results demonstrated that ¹⁸F radiotracers were promising to diagnose FRα-positive tumors.

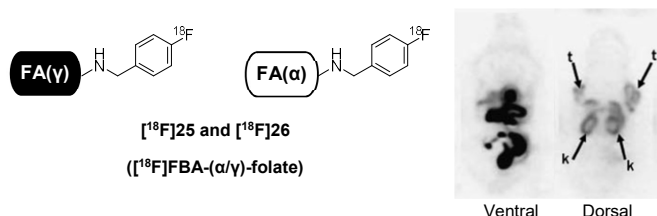


Figure 16: Chemical structures of $[^{18}\text{F}]\text{25}$ and $[^{18}\text{F}]\text{26}$, and PET image of mice bearing KB tumor with $[^{18}\text{F}]\text{25/26}$ (12.5 MBq). Image reprinted with permission from [147]. Copyright 2022 SNMMI

Ross *et al.* synthesized $[^{18}\text{F}]\text{27}$ ($[^{18}\text{F}]\text{F}$ -click-fluorobutyl-(γ)-folate) [148] with CuAAC click reaction between azide-FA and $[^{18}\text{F}]\text{fluoro-1-hexyne-propargyl}$ (Fig. 17). *In vitro* experiment in KB cells allowed to determine a low radiotracer affinity coefficient of 9.76 ± 3.13 nM, which boded well for the good radiotracer specificity. *In vivo* experiments were performed in KB tumor-bearing male NMRI nude mice. Tissue distribution at 45 min p.i. of $[^{18}\text{F}]\text{27}$ revealed a massive uptake in kidney (16.53 ± 2.22 %IA/g) contrary to tumor uptake (3.13 ± 0.93 %IA/g). High uptake was also observed in the intestines (19.59 ± 59 %IA/g) concluding on the hepatobiliary clearance of $[^{18}\text{F}]\text{27}$, which could reduce diagnostic accuracy. Moreover, a renal clearance was by far preferred. Higher doses of $[^{18}\text{F}]\text{27}$ was injected to perform PET imaging at 15-45 min. Images demonstrated high radioactivity concentrations in gallbladder, intestines, urinary bladder and kidneys, but moderate level in the tumor. The imaging study revealed too important non-specific accumulations impeding the clear visualization of potential ovarian tumors. Unsatisfactory *in vivo* results depleted the possibility to push further preclinical study using this radiotracer.

Fisher *et al.* developed $[^{18}\text{F}]\text{28}$ ($[^{18}\text{F}]\text{F}$ -click-FDG-(γ)-folate), based on the original combination of 2-deoxy-2- $[^{18}\text{F}]\text{fluoroglucopyranosyl}$ azide with propargyl- γ -FA (Fig. 17) [149]. Binding affinity assay demonstrated an affinity of 0.63 ± 0.05 nM meaning that $[^{18}\text{F}]\text{28}$ was more affine to $\text{FR}\alpha$ than FA. Biodistribution study was performed in KB tumor-bearing nude mice. Major uptake was found in kidney and gallbladder (27.08 ± 1.53 and 27.42 ± 7.57 %IA/g, respectively) followed by tumors and liver (9.05 ± 2.12 and 8.37 ± 1.19 %IA/g, respectively) at 90 min p.i. of $[^{18}\text{F}]\text{28}$, thus reflecting a moderate non-specific uptake. PET imaging was performed at 75-105 min p.i. of $[^{18}\text{F}]\text{28}$. Images revealed a high accumulation of $[^{18}\text{F}]\text{28}$ in kidneys and urinary bladder and, to a lesser extent, in KB tumors and liver. These results confirmed also that a huge $[^{18}\text{F}]\text{28}$ accumulation in liver, kidneys and gallbladder could complicate the diagnostic *via* imaging tools. In conclusion, the innovative $[^{18}\text{F}]\text{28}$ is a hopeful radiotracer which has to be improved with future research to become an even more specific radiotracer.

Boss *et al.* studied 3 pairs of α - and γ - ^{18}F -radiofolate regioisomers synthesized by CuAAC click chemistry [105]: $[^{18}\text{F}]\text{F}$ -click-fluorobutyl-(α/γ)-folates ($[^{18}\text{F}]\text{27}$ and $[^{18}\text{F}]\text{29}$), $[^{18}\text{F}]\text{F}$ -click-FDG-(α/γ)-folates ($[^{18}\text{F}]\text{28}$ and $[^{18}\text{F}]\text{30}$), and $[^{18}\text{F}]\text{F}$ -click-fluoroethyl-(α/γ)-folates ($[^{18}\text{F}]\text{31}$ and $[^{18}\text{F}]\text{32}$) (Fig. 17). *In vitro* binding assay on KB cells confirmed the high affinity to $\text{FR}\alpha$ for each radiotracer (1.4-2.2 nM). Biodistribution and PET imaging studies were performed in KB tumor-bearing athymic nude mice. All radiofolates showed highest uptake at 90 min p.i. (16.9 - 52.9 %IA/g). Tumor and liver exhibited a slightly and significantly higher uptake, respectively, for α -analogs compared to γ -analogs, thereby inducing a lower clearance of α -analogs from the body by hepatobiliary way. PET images recorded at 75-105 min p.i. confirmed that $[^{18}\text{F}]\text{27}$ and $[^{18}\text{F}]\text{29}$ were not suitable for nuclear imaging as no activity was detected in tumor region. The most important activity in tumor areas was detected with $[^{18}\text{F}]\text{31}$ and $[^{18}\text{F}]\text{32}$ which both conducted to the best tumor-to-background contrast images. Using $[^{18}\text{F}]\text{28}$, $[^{18}\text{F}]\text{31}$ and $[^{18}\text{F}]\text{32}$, major activity intensities were detected in kidneys and urinary bladder. The highest activity intensity was observed in intestines and bladder for $[^{18}\text{F}]\text{30}$ and intestines were also quite visible on PET images using $[^{18}\text{F}]\text{32}$. Nevertheless, images contrast was sufficient for tumor detection. PET/CT scans revealed that $[^{18}\text{F}]\text{28}$, $[^{18}\text{F}]\text{30}$, $[^{18}\text{F}]\text{31}$ and $[^{18}\text{F}]\text{32}$ were suitable for $\text{FR}\alpha$ -positive tumor detection. To conclude, site conjugation has no impact on the *in vitro* characteristics of these six ^{18}F -radiofolates but has a real impact on *in vivo* distribution patterns. This work highlighted the important of the prosthetic group choice to improve biodistribution ability of the radiotracer.

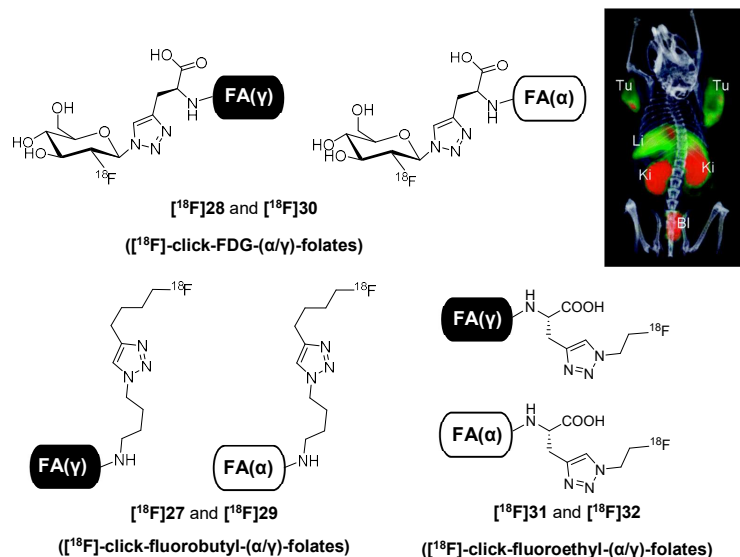


Figure 17: Chemical structures of [¹⁸F]27, [¹⁸F]F-28, [¹⁸F]F-29, [¹⁸F]F-30, [¹⁸F]F-31 and [¹⁸F]32, and PET/CT image of KB tumor-bearing mice at 75-105 min p.i. of [¹⁸F]28 (10-14 MBq). Image reprinted with permission from [149]. Copyright 2022 SNMMI

Based on a CuAAC click chemistry strategy, Schieferstein *et al.* designed [¹⁸F]33 ([¹⁸F]fluoro-OEG-(γ)-folate) which had an oligoethylene glycol (OEG) spacer between ¹⁸F and FA to improve its pharmacokinetics properties (Fig. 18) [150]. [¹⁸F]33 exhibited a reduced lipophilicity compared [¹⁸F]27 (capacity factor k' of 1.12 and 2.28, respectively) and showed an excellent FR α affinity (K_i of 1.6 nM). Biodistribution and PET imaging were performed in KB tumor-bearing nude mice. Biodistribution results at 90 min p.i. of [¹⁸F]33 showed a tumor uptake of 3.54 ± 0.68 %IA/g, and the higher uptake was observed in gallbladder and kidneys (55.13 and 41.04 ± 7.04 %IA/g, respectively). As expected, [¹⁸F]33 exhibited a better tumor-to-background contrast than [¹⁸F]27. PET images at 90 min p.i. of [¹⁸F]33 revealed major accumulation in kidneys, liver, urinary bladder and intestines and, to a lesser extent, in tumor. This research proved the benefits of incorporating the OEG spacer in ¹⁸F-radiofolate, such as improved tumor-to-background contrast. Nevertheless, results obtained with [¹⁸F]40 and [¹⁸F]41 presented section 3.1.2. are better.

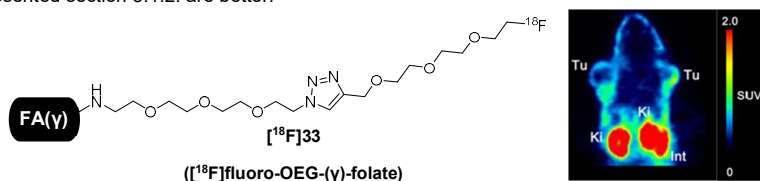


Figure 18: Chemical structure of [¹⁸F]33 and PET image of KB tumor-bearing mice at 60-90 min p.i. of [¹⁸F]33 (13 MBq). Image in open access from [150].

After experimentation using [¹⁸F]33, the team concluded that a too lipophilic profile was unfavorable as it favored hepatobiliary excretion. Kettenbach *et al.* pursued their investigations around lipophilicity impact on *in vivo* distribution patterns using two ¹⁸F-radiofolates: [¹⁸F]34 ([¹⁸F]fluoro-dibenzocyclooctyne-(γ)-folate) and [¹⁸F]35 ([¹⁸F]fluoro-Alakyne-(γ)-folate) (Fig. 19) [151,152]. [¹⁸F]34 is more lipophilic (Log $D_{7.4}$ of 0.6) than [¹⁸F]35 (log $D_{7.4}$ of -1.4) as it contains a four cycles formation. Binding affinity assay was performed on KB cells and high FR α -affinity was achieved with [¹⁸F]34 and [¹⁸F]35 (IC_{50} of 11.2 ± 3.7 and 6.4 ± 0.5 nM, respectively). *Ex vivo* biodistribution was carried out in KB tumor-bearing BALB/c nude mice and a 3-times higher tumor uptake was observed for [¹⁸F]35 compared to [¹⁸F]34. The main uptake was found in kidneys and a low tumor-to-kidney

ratio was observed for [^{18}F]34 (0.05) compared to that generally found in literature (0.12), which was the case for [^{18}F]35. Significant non-specific uptake in intestines and liver was also observed for both radiotracers. Background contrast was bad for [^{18}F]34 as it was excreted by hepatobiliary way due to its lipophilic character. Finally, *in vivo* PET imaging showed a massive activity in intestines for [^{18}F]34, followed by bladder, kidneys and liver. No activity was found in KB tumor. On the contrary, important activity was found in KB tumor using [^{18}F]35 but main activity was visualized in bladder, intestines, and kidneys. To sum up, [^{18}F]34 was not suitable for PET imaging. On its stands, [^{18}F]35 was more appropriate for future observations and experiments for scanning FR α -positive tumors, but the tracer design should be optimized to improve its hydrophilicity character and to reduce intestines uptake and hepatobiliary clearance.

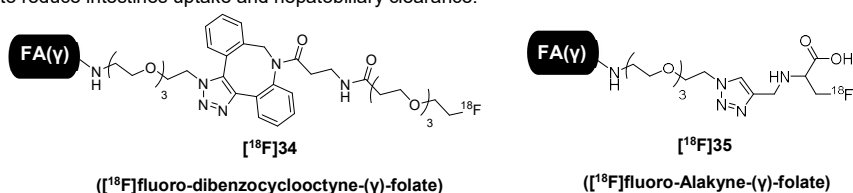


Figure 19: Chemical structures of [^{18}F]36 and [^{18}F]37 [152]

Boss *et al.* wished to know whether the 5-Me-THF could replace FA to target FR α -positive cells. The team synthesized α - and γ -regioisomers (both in enantiomeric pure forms) of a ^{18}F -5-Me-THF-radiotracer using CuAAC click reaction: [^{18}F]36 ((6S)- α -[^{18}F]fluoro-5-Me-THF), [^{18}F]37 ((6S)- γ -[^{18}F]fluoro-5-Me-THF), [^{18}F]38 ((6R)- α -[^{18}F]fluoro-5-Me-THF) and [^{18}F]39 ((6R)- γ -[^{18}F]fluoro-5-Me-THF) (Fig. 20) [153]. *In vitro* binding assay with KB cells revealed similar affinity to FR α in the range of 17.7 to 25.8 nM with close affinity to 5-Me-THF (20.6 to 25.8 nM). 5-Me-THF and its four derivatives had less affinity to FR α than FA and [^{18}F]41 (Fig. 21) but stayed sufficiently affine to be highly specific radiofolates with a cellular uptake of about 50-60%. Tissue distribution was performed in nude mice bearing KB tumors. Similar tumor uptake was found at 1 hour p.i. for all compounds ([^{18}F]36: 8.28 ± 1.2 , [^{18}F]37: 8.20 ± 2.4 , [^{18}F]38: 10.6 ± 2.8 and [^{18}F]39: 8.69 ± 1.5 %I/g). The tumor-to-kidney ratio was 0.5 for (6S)-compounds ([^{18}F]36 and [^{18}F]37) and 2-fold higher than for (6R)-analogs. α - And γ -compounds presented similar biodistribution patterns which was not the case for (6R)- and (6S)-compounds ([^{18}F]38 and [^{18}F]39). Kidney uptake was 2-fold higher for (6R)- compared to (6S)-compounds ([^{18}F]38: 35.5 ± 2.0 and [^{18}F]36: 15.1 ± 1.8 %I/g)) whereas liver uptake was 5-fold higher for (6S)- compared to (6R)-compound ([^{18}F]37: 14.1 ± 2.2 and [^{18}F]39: 2.48 ± 0.4 %I/g). PET/CT data was recorded at 1 hour p.i.. α -Compounds had bad tumor-to-background contrast as liver, kidneys, intestines, gallbladder, and urinary bladder expressed higher activity level than tumors. Contrast was better for γ -compounds. Tumor expressed the highest activity level for [^{18}F]39. This compound gave the better images quality as liver and intestines were not visible. To conclude, (6R)-analogs showed better *in vivo* properties than (6S)-analogs. No significant differences could be observed between α - and γ -compounds. These four compounds and more specifically [^{18}F]39 are suitable for clinical trials.

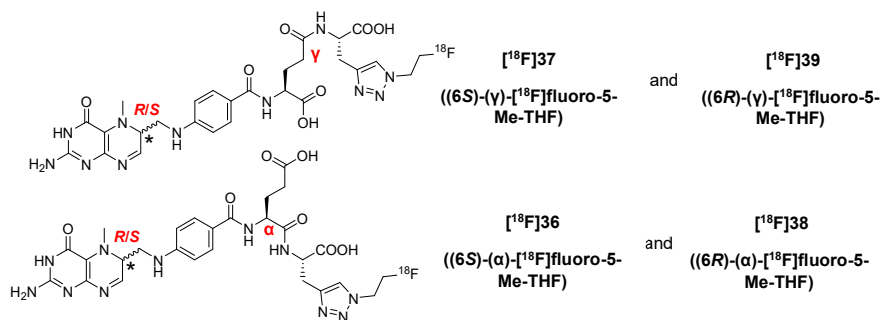


Figure 20: Chemical structures of [^{18}F]36, [^{18}F]37, [^{18}F]38 and [^{18}F]39 [153]

3.1.2. ^{18}F -folates synthesized by pendent approach

Ross *et al.* and Betzel *et al.* [154,155] were the first to implement an integrated approach using a direct labelling strategy by nucleophilic aromatic substitution. Ross *et al.* developed [^{18}F]40 (2'-[^{18}F]-fluorofolic acid or 2'-[^{18}F]FFA) (Fig. 21). *In vitro* binding assay using KB cells demonstrated the high affinity of [^{18}F]40 to FR α . *In* and *ex vivo* studies were performed in KB tumor-bearing NMRI nude mice at 75 min p.i. of [^{18}F]40. The major uptake was measured in kidneys followed by gallbladder, tumor and liver (46.06 ± 13.39 , 17.69 ± 10.35 , 9.37 ± 1.76 and 7.79 ± 1.23 %IA/g, respectively). High activity in urine concluded on the renal excretion way of [^{18}F]40. PET study was performed at 10 min p.i. of [^{18}F]40 and helped to determine non-specific organ uptake. Tumor and kidneys were well visualized as they presented the highest activity. Minor activity was also measured in abdominal region due to uptake in liver and intestines. Until now, PET images presented one of the best tumor-to-background contrasts and gave promising results to pursue experimentation on fluorinated-radiofolate.

The radiofolate [^{18}F]41 (3'-Aza-2'-[^{18}F]fluorofolic acid ([^{18}F]-AzaFol)) developed by Betzel *et al.* is a derivative of [^{18}F]40 where the ^{18}F is attached by nucleophilic aromatic substitution onto a pyridine ring for 41 instead of a phenyl ring for 40 (Fig. 21). *In vitro* stability studies on KB cells demonstrated that affinity to FR α of [^{18}F]41 was not affected by backbone modification as IC_{50} was of 1.4 ± 0.5 nM. Another promising finding was obtained with biodistribution studies showing a high KB tumor uptake (12.59 ± 1.77 %IA/g) at 90 min p.i. of [^{18}F]41 in KB tumor-bearing athymic nude mice. Organs such as kidneys, liver, salivary glands and gallbladder also taken up significant amount of radiofolate (57.33 ± 8.40 , 10.31 ± 2.37 , 14.09 ± 0.93 and 9.26 ± 0.59 %IA/g, respectively) which could be due to the short time between injection and collecting data. Indeed, clearance was still ongoing and folate derivative accumulation in gallbladder and feces demonstrated a partial hepatobiliary clearance. Compared to previous results, no significant uptake in intestines was observed which is a good point for a well visualization of tumors in the abdominal area. Furthermore, PET images recorded at 120-150 min p.i. of [^{18}F]41 presented good quality as major activity was detected in tumor and kidneys followed by moderate activity in liver and bladder (Fig. 21). The results were the more convincing ones until now and a new synthesis strategy was implemented to considerably decrease total synthesis time. Moreover, less non-specific uptake was observed, and the higher tumor uptake was registered. High contrasts images were the most encouraging results. Regarding all these promising results, clinical study using [^{18}F]41 was allowed and presented in section 3.3.

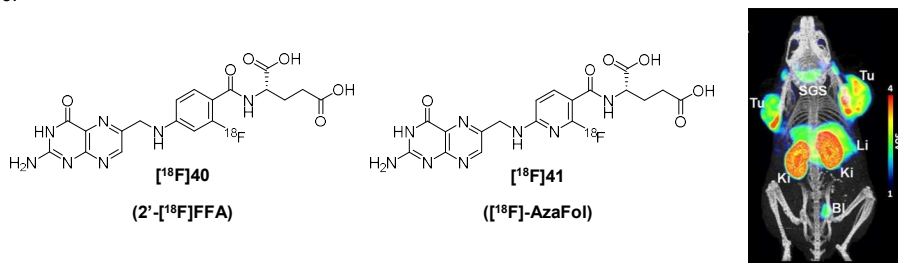


Figure 21: Chemical structures of [^{18}F]40 [154] and [^{18}F]41 and PET/CT image of KB tumor-bearing mice at 120-150 min p.i. of [^{18}F]41 (13 MBq). Image reprinted with permission from [155]. Reprinted with permission from Betzel *et al.* *Bioconjug. Chem.* 24 (2013) 205–214. Copyright 2022 American Chemical Society

Boss *et al.* modified 5-Me-THF to create a new FA derivative: 3'-Aza-5-Me-THF by changing the 3-C of the phenyl ring by a nitrogen. This new FA derivative exists in two diastereomeric forms: (6S)- (42) and (6R)- (43) derivatives (Fig. 22A). [^{18}F]42 ((6S)-3'-Aza-2'-[^{18}F]fluoro-5-Me-THF) and [^{18}F]43 ((6R)-3'-aza-2'-[^{18}F]fluoro-5-Me-THF) were synthesized using substitution approach [156]. FR α affinity of (6R)- and (6S)-analogs (27.1 and 23.8 nM, respectively) were close to 5-Me-THF one. *In vivo* properties were described and compared to results obtained in a previous study involving [^{18}F]41 [155]. The biodistribution study started with the injection of [^{18}F]41, [^{18}F]43 and [^{18}F]42 in KB tumor-bearing nude mice. Final data at 3 hours p.i. showed the highest tumor uptake ever observed for a radiofolate with a value of 32.3 ± 6.1 and 34.8 ± 6.0 %IA/g for [^{18}F]42 and [^{18}F]42, respectively. Tissue distribution profile using [^{18}F]41 was the same as previous results and showed tumor internalization 2-times lower than for [^{18}F]42 and [^{18}F]43 (15.0 ± 2.3 %IA/g). [^{18}F]43 showed a high tumor-to-kidney ratio of 1.63 ± 0.32 and this is the best ratio ever observed. PET/CT imaging recorded at 3 hours p.i. allowed well visualization of tumors and kidneys with a huge tumor-to-background contrast (Fig. 22B,C,D).

Biodistribution results were confirmed by imaging study ones as the major activity intensity in tumors was found for [¹⁸F]43 followed by [¹⁸F]42 and the lowest kidney uptake was found with [¹⁸F]43. Moreover, liver was not visible with [¹⁸F]42 compared to [¹⁸F]43 which is an advantage for tumor detection. Therefore, [¹⁸F]42 and [¹⁸F]43 are suitable for clinical trials as they provide images of a rare quality and contrast. Their use could significantly improve medical experiments for the FRα-positive tumors detection and could also be used for therapy monitoring and patient surveillance.

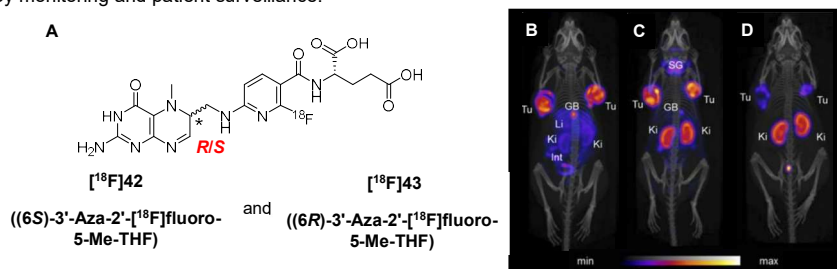


Figure 22: (A) Chemical structures of [¹⁸F]42 and [¹⁸F]43. PET/CT images of KB tumor-bearing mice at 3 hours p.i. of (B) [¹⁸F]43, (C) [¹⁸F]42 and (D) [¹⁸F]41 (5 MBq). Images reprinted with permission from [156]. Copyright 2022 SNMMI

3.1.3. [¹⁸F]F-AI-NOTA-folates

Conjugation approach has the drawback to require long synthesis time that is not in adequation with the short half-life time of ¹⁸F. Low and coworkers has perceived the need to explore another radiofluorination approach based on aluminum-¹⁸F coordination. Consequently, this team synthesized and explored *in vitro* and *in vivo* characteristics of [¹⁸F]F-AI-44 ([¹⁸F]F-AI-NOTA-(γ)-folate) to assess the relevance of using this as an alternative radiofluorination approach [157]. 44 was composed of a PEG₂ spacer inserted between NOTA chelator and FA (Fig. 23). The binding affinity coefficient determined using KB cells illustrated the good binding ability of the design radiofolate (IC₅₀ of 18.7 nM). *In vivo* studies were performed in KB tumor-bearing nude mice. PET data collected at 90 min p.i. of [¹⁸F]F-AI-44 showed that KB tumor and kidneys were the only two tissues visible on imaging with an important background contrast. Biodistribution data revealed a significant uptake only in tumor and kidneys (10.9 ± 2.7 and 78.6 ± 5.1 %IA/g, respectively) and a moderate one in liver (5.3 ± 0.5 %IA/g). Those preclinical results with [¹⁸F]F-AI-44 are the best observed for a radiofluorinated folate tracer by far. These conclusions open new investigation perspectives since [¹⁸F]F-AI-44 development is at its early stage.

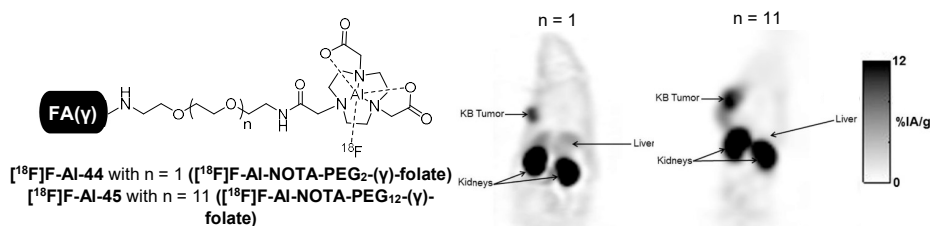


Figure 23: Chemical structures of [¹⁸F]F-AI-44 and [¹⁸F]F-AI-45, and PET/CT images of KB tumor-bearing mice at 90 min p.i. of [¹⁸F]F-AI-44 or [¹⁸F]F-AI-45 (4-11 MBq). Reprinted with permission from Chen et al. Mol. Pharm. 14 (2017) 4353–4361. Copyright 2022 American Chemical Society

Regarding promising results obtained with [¹⁸F]F-AI-44, the team decided to replace PEG₂ by a PEG₁₂ spacer to improve its *in vivo* behavior and thus synthesized [¹⁸F]F-AI-45 ([¹⁸F]F-AI-NOTA-PEG₁₂-(γ)-folate) (Fig. 23) [158]. The IC₅₀ of [¹⁸F]F-AI-45 determined with KB cells was 33.8 nM and KB or A549 cells were inoculated into right shoulder of female nu/nu mice for *in vivo* studies. PET data was collected at 90 min p.i. of [¹⁸F]F-AI-45 or [¹⁸F]F-AI-44. For both radiopharmaceuticals, only KB tumors and kidneys expressed high activity intensity. Background contrast was better with PEG₁₂-analog as liver was no more visible (Fig. 23). *Ex vivo* distribution was performed at 120 min p.i. of [¹⁸F]F-AI-45 or [¹⁸F]F-AI-44 and, as generally observed, the highest uptake

was found in kidneys (55.25 ± 7.38 and 87.86 ± 4.42 %IA/g, respectively). Tumor uptake was similar for both radiofolates. Liver uptake was significantly weaker for [^{18}F]F-AI-45 than [^{18}F]F-AI-44 (1.64 ± 0.3 vs. 5.40 ± 0.75 %IA/g) due to the most important hydrophilicity of PEG₁₂ compared to PEG₂. To conclude, the team successfully improved the *in vivo* properties of [^{18}F]F-AI-44 by increasing the spacer hydrophilicity. In conclusion, [^{18}F]F-AI-45 possesses good characteristics and constitutes an improved diagnostic agent to detect FR α -positive tumors. This novel radiopharmaceutical possesses all the required qualities to participate in clinical trials.

3.2. Gallium-68 radiofolates

3.2.1. [^{68}Ga]Ga-DOTA-folates

Fani *et al.* developed the two first ^{68}Ga -radiofolates consisting in γ -conjugation of DOTA chelator to FA through an EDA linker ([^{68}Ga]Ga-46, [^{68}Ga]Ga-P3026) or a 3-{2-[2-(3-amino-propoxy)-ethoxy]-ethoxy}-propylamine linker ([^{68}Ga]Ga-47, [^{68}Ga]Ga-P1254) (Fig. 24) [159]. *In vitro* studies were performed in KB tumor cells and both radiofolates showed a rapid and high cell uptake and retention. [^{68}Ga]Ga-46 revealed highest cell surface binding compared to [^{68}Ga]Ga-47. The internalization rate followed the order [^{68}Ga]Ga-46 > [^{68}Ga]Ga-47. [^{68}Ga]Ga-46 and [^{68}Ga]Ga-47 showed high FR α affinity with a K_d value of 4.65 ± 0.82 and 4.27 ± 0.42 mM, respectively. *In vivo* experiments were performed in nude mice bearing a dual KB and HT1080 tumor model. Biodistribution profiles for each radioconjugates were similar and characterized by efficient clearance from the blood. The major uptake at 2 hours p.i. was found in kidneys resulting in a low tumor-to-kidney ratio of 0.12 ± 0.02 for [^{68}Ga]Ga-46 and 0.11 for [^{68}Ga]Ga-47. PET/CT images were performed with [^{68}Ga]Ga-46 on mice at 1 hour p.i. with surgically kidney removal. PET images confirmed biodistribution results with a good visualization of FR α -positive tumor and negligible uptake in FR α -negative tumor. High uptake in salivary gland was also observed. To sum up, these two radiofolates give promising results but further investigations are necessary to develop a suitable ^{68}Ga -tracer for clinical trials.

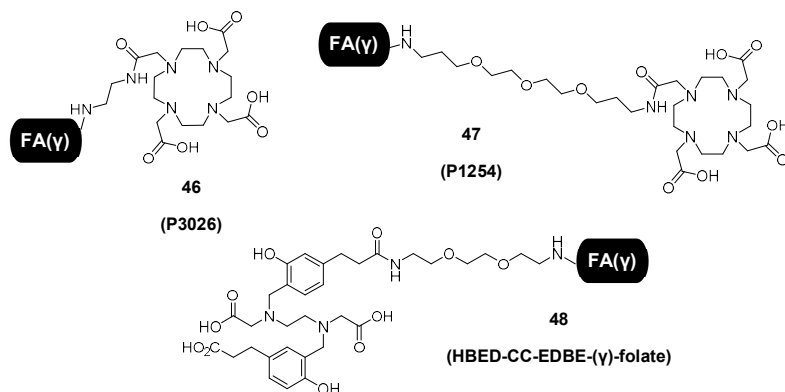


Figure 24: Chemical structures of 46, 47 and 48 [72,159]

3.2.2. [^{68}Ga]Ga-HBED-CC-EDBE-folate

Choi *et al.* described the synthesis of the new ^{68}Ga -folate-radioconjugate [^{68}Ga]Ga-48 ([^{68}Ga]Ga-HBED-CC-EDBE-(γ)-folate) consisting in γ -conjugation of HBED-CC chelator to FA through an 2,2'-ethylenedioxy-bis-ethylamine linker (Fig. 24) [72]. *In vitro* cellular uptake of [^{68}Ga]Ga-48 was evaluated in CT-26, KB cells and A549 cells. Binding percentage was determined over 2 hours and showed a higher cellular uptake of [^{68}Ga]Ga-48 in CT-26 and KB cells which expressed FR α than in FR α -negative A549 cells. PET imaging performed on

CT-26 tumor-bearing BALB/c mice at 2 hours p.i. of [^{68}Ga]Ga-48 indicated significant uptake in tumor, but also in other organs leading to a poor image quality. Thus, this radiofolate is not suitable for further investigations.

3.2.3. [^{68}Ga]Ga-NODAGA-Folate

Fani *et al.* pursued their studies on ^{68}Ga -radiofolates with the synthesis of [^{68}Ga]Ga-49 ([^{68}Ga]Ga-P3246) using this time a NODAGA chelator (Fig. 25) [160] compared to [^{68}Ga]Ga-41 (see section 3.1.2.). A saturation binding study was conducted on KB cells using different concentrations of [^{68}Ga]Ga-49 and revealed its high affinity binding to FR α ($K_d = 5.61 \pm 0.96$ nM). *In vivo* biodistribution and PET/CT studies was performed in KB tumor-bearing athymic female nude mice. The biodistribution profile was characterized by high tumor uptake (16.56 ± 3.67 %IA/g) and high activity accumulation in kidneys (91.52 ± 21.05 %IA/g) at 1 hour p.i., resulting in tumor-to-kidney ratio of 0.18. The blood clearance was fast while the concentration of activity was low in non-targeted organs, such as lungs, spleen, stomach, and muscles. Concerning PET tests (Fig. 25), time activity curves obtained on the dynamic acquisition with regions of interest (ROIs) analyses showed an increase of activity in tissues which express FR α like tumor, salivary glands and kidneys.

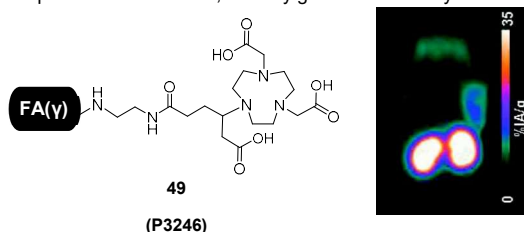


Figure 25: Chemical structure of **49** and PET image of KB tumor-bearing mice at 1 hour p.i. of [^{68}Ga]Ga-49 (10–12 MBq). Reprinted with permission from Fani *et al.* *Mol. Pharm.* 9 (2012) 1136–1145. Copyright 2022 American Chemical Society

3.2.4. [^{68}Ga]Ga-DOTA-Pteroyl

Zhang *et al.* described the synthesis of two novel radioconjugates named [^{68}Ga]Ga-50 ([^{68}Ga]Ga-DOTA-Lys-Pteroyl) and [^{68}Ga]Ga-51 ([^{68}Ga]Ga-DOTA-DAV-Lys-Pteroyl) (Fig. 26) in which glutamic acid of FA was replaced by a lysine (*i.e.*, Lys-Pteroyl ligand) and linked to a DOTA chelator [161]. In **51**, a δ -aminovaleric acid spacer was added between Lys-Pteroyl and DOTA. *In vitro* cellular uptake was performed in KB cells and showed a high cellular uptake of [^{68}Ga]Ga-50 and [^{68}Ga]Ga-51 with a cell binding of $52.52 \pm 1.64\%$ and $33.11 \pm 0.85\%$, respectively, and also a rapid cellular internalization. Biodistribution and PET imaging were carried out on Kunming mice bearing KB tumor. Both ^{68}Ga -complexes displayed a fast clearance and low activity accumulation in most organs except kidneys. Indeed tumor-to-kidney ratio was 0.11 for both ^{68}Ga -radioconjugates at 2 hours p.i.. [^{68}Ga]Ga-51 showed a better biodistribution and *in vivo* results: higher cellular uptake, faster clearance, higher tumor-to-muscle (6.76 vs. 4.34) and tumor-to-blood ratio (28.65 vs. 17.93) at 2 hours p.i.. This could be explained by a higher distance between the chelating agent and the targeting moiety allowing it to better interact with FR α . So PET images using [^{68}Ga]Ga-51 at 2 hours p.i. showed clear visualization of tumor and kidneys, and poor activity in non-specific organs leading to a good tumor-to-background contrast. To conclude, [^{68}Ga]Ga-51 is suitable for further investigations contrary to [^{68}Ga]Ga-50.

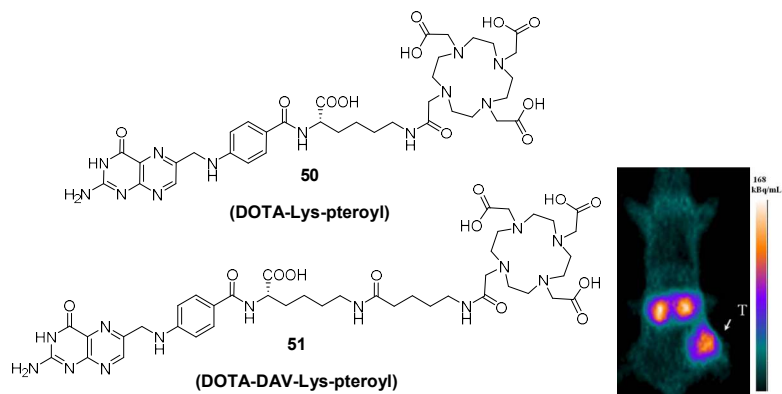


Figure 26: Chemical structures of **50** and **51** and PET/CT image of KB tumor-bearing mice at 2 hours p.i. of $[^{68}\text{Ga}]\text{Ga-51}$ (7.4 MBq). Image reprinted with permission from [161]. Copyright 2022 John Wiley and Sons

3.2.5. $[^{68}\text{Ga}]\text{Ga-NODAGA-dideaza-folate}$

$[^{68}\text{Ga}]\text{Ga-52}$ described by Fani *et al.* ($[^{68}\text{Ga}]\text{Ga-NODAGA-EDA-(}\gamma\text{)-5,8-dideaza-folate}$) consisted of 5,8-dideaza-folate linked on its γ -carboxylic acid to the spacer EDA functionalized by NODAGA (Fig. 27) [160]. *In vitro* and *in vivo* results of $[^{68}\text{Ga}]\text{Ga-52}$ were compared to those of $[^{68}\text{Ga}]\text{Ga-49}$ (analogue possessing a folate entity instead of dideaza-folate, see section 3.2.3.). Evaluation in KB cell culture showed an higher cell-associated uptake *in vitro* for $[^{68}\text{Ga}]\text{Ga-49}$ compared to $[^{68}\text{Ga}]\text{Ga-52}$ (60-72% vs. 50-60%). Internalized and binding fraction were higher for $[^{68}\text{Ga}]\text{Ga-49}$ ($14.8 \pm 0.2\%$) compared to $[^{68}\text{Ga}]\text{Ga-52}$ ($11.5 \pm 1.0\%$) at 4 hours. The cellular retention was slightly higher for $[^{68}\text{Ga}]\text{Ga-49}$ ($76.3 \pm 10.2\%$) compared to $[^{68}\text{Ga}]\text{Ga-52}$ ($70.9 \pm 3.9\%$). Soft modification of the FA entity resulted in the decrease in affinity to FR α ($K_d = 7.21 \pm 2.46$ nM). Biodistribution and PET/CT imaging were carried out on KB tumor-bearing athymic female nude mice. Biodistribution profile was similar to that of $[^{68}\text{Ga}]\text{Ga-49}$ (tumor and kidneys uptake values of 10.95 ± 2.12 and 62.26 ± 14.32 %IA/g at 1 hour p.i., respectively), resulting in tumor-to-kidney ratio of 0.18. The clearance from the blood and the specificity of $[^{68}\text{Ga}]\text{Ga-52}$ were also similar to those of $[^{68}\text{Ga}]\text{Ga-49}$. To sum up, modification of the FA entity does not improve properties of the radioconjugate.

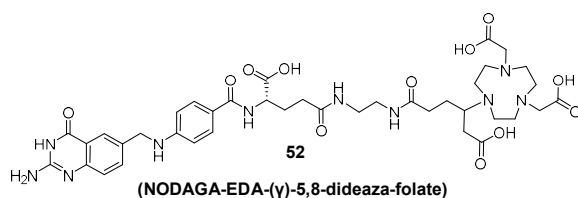


Figure 27: Chemical structure of **52** [160]

3.3. Clinical evaluation of PET radiofolate

Gnesin *et al.* exposed the first clinical trial of a PET radiotracer for FR α -overexpressing tumor imaging [162,163]. 6 patients with lung adenocarcinoma were involved in this study. Lung cancers were previously histologically confirmed for each patient. FA (1 mg) was i.v. injected before injection of 299-399 MBq of $[^{18}\text{F}]\text{41}$ and PET/CT was recorded during 1 hour after tracer injection (Fig. 28). Kidneys were quite visible on imaging only at 3 min p.i. and became moderately visible such as liver and tumor. Tumor-to-lung ratio slightly increased during image acquisition before reaching a plateau between 4.8 and 5 at 40 min. This high tumor-to-lung ratio allowed high tumor-to-background contrast and a good visualization of tumors in the thoracic area. Dosimetric study highlighted that the most irradiated organs were liver, kidney, bladder wall and spleen. Extrapolation from preclinical data differed from clinical results as in mice model, the highest exposure was in kidneys followed by

liver. The effective dose taken by patients were comparable to the one taken during a ^{18}F FDG PET imaging. To conclude, ^{18}F 41 is suitable to diagnose lung adenocarcinoma by PET imaging as it is safe and an effective radiotracer with good dosimetric properties. Nevertheless, regarding the low number of patients, this study has to be pursued to reinforce these promising results. ^{18}F 41 could also be employed in future clinical trials to diagnose ovarian and endometrial cancer or peritoneal carcinosis.

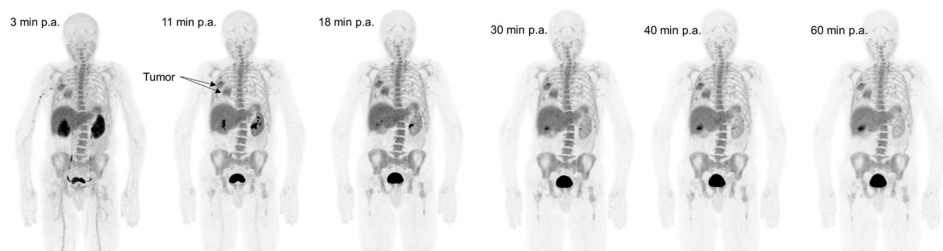


Figure 28: PET images of patient with lung adenocarcinoma at 3, 11, 18, 30, 40 and 60 min p.i. of ^{18}F 41 (299-399 MBq). Image in open access from [162].

3.4. Conclusion

As PET imaging for tumor diagnostic was implemented in clinical routine more recently than SPECT imaging, the development of folate-based radiopharmaceuticals has started later. Thus, the first radiofolate using β^+ -emitting isotope ^{18}F 27/26 was developed in 2006 whereas the first SPECT radiofolate ^{67}Ga Ga-1 was introduced ten years earlier, in 1996. ^{18}F -folate-tracers developed using substitution or chelating approach give better *in vivo* results than those developed using conjugation approach. In fact, less uptake in non-specific organs was observed and those radiopharmaceuticals are more excreted from body using renal clearance way. They also allow to record images with better tumor-to-background contrast and thus with better qualities. Until now, only ^{18}F -AzaFol (^{18}F 41) radiofolate using β^+ -emitting isotope is under clinical trials to detect FR α -positive tumors by PET/CT[163]. Results of this clinical trial were very promising to diagnose lung adenocarcinoma and should be used in another clinical study in a few years. Moreover, regarding promising results already obtained concerning ^{68}Ga -radiofolates, the ease to produce ^{68}Ga thanks to $^{68}\text{Ga}/^{68}\text{Ge}$ generator and the generalization of the use of PET compared to SPECT, future clinical trials could also be conducted soon. Main results for each radiofolate are described in Table 5.

Table 5: Summary of radiochemistry, *in vitro* and *in vivo* results of radiofolates depicted in this section

| Radiofolate | RCY (%) | Log P | K_d for FR α (nM) | Tumor uptake (%IA/g) | Tumor-to-kidney ratio | References |
|-----------------------|----------------|--------------------|------------------------------|---------------------------|-----------------------|------------|
| ^{18}F 25/26 | 15-45 | NC | 71 ± 8^b 62 ± 6^b | 6.56 ± 1.80 (125 min) | 0.16 | [147] |
| ^{18}F 27 | 19-23 dc | -2.7 ± 0.1^a | 2.1 ± 0.2^b | 3.22 ± 1.05 (90 min) | 0.10 | [105, 148] |
| ^{18}F 38 | 19-23 dc | -2.7 ± 0.1^a | 2.2 ± 0.1^b | 3.73 ± 0.62 (90 min) | 0.08 | [105] |
| ^{18}F 29 | 3-10 dc | -4.2 ± 0.1^a | 1.5 ± 0.3^b | 9.05 ± 2.12 (90 min) | 0.34 | [105, 149] |
| ^{18}F 30 | 3-10 dc | -4.2 ± 0.1^a | 1.6 ± 0.2^b | 10.9 ± 0.52 (90 min) | 0.2 ± 0.07 | [105] |
| ^{18}F 31 | 2-4 dc | -3.0 ± 0.1^a | 1.4 ± 0.2^b | 7.24 ± 0.99 (90 min) | 0.43 | [105] |
| ^{18}F 32 | 2-4 dc | -3.0 ± 0.1^a | 1.6 ± 0.2^b | 12.5 ± 1.04 (90 min) | 0.38 | [105] |
| ^{18}F 33 | 8.7 dc | NC | 1.6 | 3.54 ± 0.68 (90 min) | 0.09 ± 0.01 | [150] |
| ^{18}F 34 | 3.2 ± 1.8 | 0.6 ± 0.07^a | 6.3 ± 1.4 | 1.68 ± 0.13 (1h) | 0.05 | [151] |
| ^{18}F 35 | 19.3 ± 2.8 | -1.43 ± 0.08^a | 5.5 ± 0.4 | 0.56 ± 0.13 (1h) | 0.12 | [151] |

| | | | | | | |
|---------------------------|---------------|-------------------------|-------------------------|-----------------------|-------------|-------|
| [¹⁸ F]36 | 1-4 dc | -2.2 ± 0.1 ^a | 24.0 ± 4.6 ^b | 8.28 ± 1.2 (1h) | 0.55 ± 0.08 | [153] |
| [¹⁸ F]37 | 5-7 dc | -2.2 ± 0.1 ^a | 17.7 ± 7.2 ^b | 8.20 ± 2.4 (1h) | 0.44 ± 0.12 | [153] |
| [¹⁸ F]38 | 2-3 dc | -2.6 ± 0.2 ^a | 22.3 ± 3.0 ^b | 10.6 ± 2.8 (1h) | 0.30 ± 0.08 | [153] |
| [¹⁸ F]39 | 2-4 dc | -2.5 ± 0.2 ^a | 22.2 ± 6.9 ^b | 8.69 ± 1.5 (1h) | 0.22 ± 0.04 | [153] |
| [¹⁸ F]40 | 8 dc | 0.53 ± 0.2 | 1.8 ± 0.1 | 9.37 ± 1.76 (75 min) | 0.20 | [154] |
| [¹⁸ F]41 | 9 dc | -4.2 ± 0.1 | 1.4 ± 0.5 | 12.59 ± 1.77 (90 min) | 1.4 ± 0.5 | [155] |
| [¹⁸ F]42 | 1-5 dc | -4.2 ± 0.1 ^a | 23.8 ± 4.0 ^b | 34.8 ± 6.0 (3h) | 0.84 ± 0.12 | [156] |
| [¹⁸ F]43 | 1-5 dc | -4.8 ± 0.1 ^a | 27.1 ± 3.7 ^b | 32.3 ± 6.1 (3h) | 1.63 ± 0.32 | [156] |
| [¹⁸ F]F-AI-44 | 18.6 ± 4.5 dc | 1.0 | 18.7 ^b | 10.9 ± 2.7 (90 min) | 0.10 ± 0.03 | [157] |
| [¹⁸ F]F-AI-45 | 8.4 ± 1.3 dc | 0.4 | 3.38 ^b | 9.20 ± 0.62 (90 min) | 0.18 ± 0.02 | [158] |
| [⁶⁸ Ga]Ga-46 | >95 | NC | 4.65 ± 0.82 | 14.29 ± 4.14 (4h) | 0.12 ± 0.01 | [159] |
| [⁶⁸ Ga]Ga-47 | >95 | NC | 4.27 ± 0.82 | 13.10 ± 0.65 (4h) | 0.13 ± 0.03 | [159] |
| [⁶⁸ Ga]Ga-48 | >98 dc | 1.61 | NC | NC | NC | [72] |
| [⁶⁸ Ga]Ga-49 | >95 dc | NC | 5.61 ± 0.96 | 16.56 ± 3.67 (1h) | 0.18 | [160] |
| [⁶⁸ Ga]Ga-50 | 84 ± 3 | -3.22 ± 0.11 | NC | 11.05 ± 0.59 (2h) | 0.11 | [161] |
| [⁶⁸ Ga]Ga-51 | 93 ± 2 | -3.64 ± 0.19 | NC | 0.06 ± 0.60 (2h) | 0.10 | [161] |
| [⁶⁸ Ga]Ga-52 | >95 dc | NC | 7.21 ± 2.46 | 10.95 ± 2.12 (1h) | 0.18 | [160] |

RCY: Radiochemical yield; Log P: Logarithm of the Partition coefficient; NC: Not communicated; dc: Decay corrected; ^a: Log D_{7.4} (Logarithm of the Distribution coefficient at pH 7.4); ^b: IC₅₀ (nM)

4. Albumin-binding radiofolates for nuclear imaging

As FR α is present on proximal tubule cells of kidneys, all developed radiofolates are well internalized into them. This uptake makes kidneys quite visible on nuclear images and deplete the tumor-to-background contrast. Radiopharmaceuticals with the best tumor-to-kidney ratio are presented in this section which increase diagnostic's quality. High kidney uptake is also an issue for TRT and theranostic approach. Radionuclides used in therapy emit α or β^- radiations which are destructive for DNA. Thus, with a massive uptake in kidneys, these radiofolates can be nephrotoxic. Mathias *et al.* were the first to discover that moderate injection of FA before the injection of radiofolate improve the tumor-to-background ratio by decreasing the uptake of non-specific organs and kidneys [123]. Müller and coworkers were the main ones to work on solutions to decrease kidney uptake [164] by pre-injection of FA derivatives like antifolates [165–168] or by conjugation of an albumin-binding entity to radiofolates for preparing albumin binder entity (AB)-radiofolates [169]. So, this section is dedicated to these radiofolates, and the addition of an AB entity is aimed at improving the pharmacokinetic properties. Thanks to this addition, the affinity of the AB-radiofolates to plasma proteins increases and provides an enhancement of the circulation time in the blood. Enhancement of the blood circulating time allows radiofolates to fix more tumor and less kidneys. It exists different types of AB entities [170] like peptides [171] and portable binders like 4-(*p*-iodophenyl)butyric acid derivatives [172] which can be easily conjugated to drugs or tracers.

4.1. Albumin-binding ¹⁸F-folate

In 2013, Fischer *et al.* were the first to design and evaluate an AB-radiofolate called [¹⁸F]53 ([¹⁸F]FDG-AB-(γ)-folate) [173]. 53 was synthesized through CuAAC click chemistry approach from propargyl-AB-(γ)-folate and [¹⁸F]FDG-azide (Fig. 29). *In vitro* and *in vivo* experiments were carried out on KB tumor cells and KB tumor-bearing mice, respectively. A FR α -specific binding of [¹⁸F]53 to KB tumor cells was observed. The role of AB

entity was tested by comparison between [^{18}F]53 and [^{18}F]28 (studied in section 3.1.1.) [149]. The addition of the AB entity showed an increase in the partition coefficient ($\text{Log } D_{7.4}$ of -3.2 ± 0.4 for [^{18}F]53 vs. -4.2 ± 0.1 for [^{18}F]28), which led to slightly increase the hydrophilicity of compound 53. As expected, biodistribution tests revealed a high blood circulation time for [^{18}F]53 as blood uptake was still of 2.21 ± 0.15 %IA/g at 4 hours p.i. whereas it was only of 0.44 ± 0.09 %IA/g at 1 hour p.i.. This confirmed that AB entity had a real impact on biodistribution. *In vivo* tumor uptake of [^{18}F]53 increased over time to reach a maximum at 4 hours p.i. (15.2 ± 0.53 %IA/g). Kidneys uptake was reduced by four using AB entity (13.4 ± 3.62 %IA/g compared to 42.9 ± 1.13 %IA/g without). The tumor-to-kidney ratio was 0.88 at 1 hour p.i., whereas a much lower ratio of 0.23 was seen at 1 hour p.i. for [^{18}F]28. Nonetheless, non-specific uptake was observed in gallbladder, salivary glands and liver. SPECT/CT scan images of mouse taken at 4 hours p.i. of [^{18}F]53 clearly showed tumors. The addition of the AB entity improved hepatobiliary elimination leading to a good visualization of gallbladder and abdominal region that could decrease the reliability of imaging diagnostics.

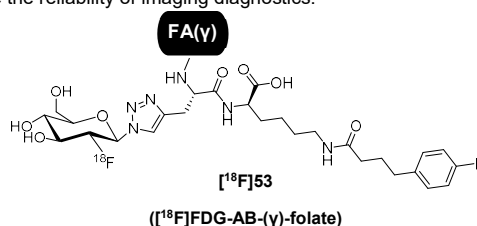


Figure 29: Chemical structure of [^{18}F]53 [173]

4.2. Albumin-binding ^{152}Tb -, ^{155}Tb - and ^{161}Tb -folates

Müller and coworkers developed compound 54 (cm09) composed of a DOTA chelator, FA and AB entities that were assembled around a modified lysine thanks to amide bond and CuAAC click reaction. Their objective was to investigate for the first time, in parallel, the respective contributions of each terbium radioisotopes on PET and SPECT imaging in KB tumor-bearing nude mice [81]. *In vivo* biodistribution studies for [^{161}Tb]Tb-54 revealed a high tumor uptake at 4 hours p.i. (23.8 ± 2.5 %IA/g) and persisting at 7 days p.i. (5.7 ± 1.9 %IA/g). No uptake was found in non-FR α -expressing organs and good tumor-to-tissue ratios were measured in blood, liver and kidneys (15, 6 and 0.8, respectively, at 24 hours p.i.), demonstrating the potential interest of [^{161}Tb]Tb-54 as contrast agent for SPECT imaging. Excellent PET and SPECT images with [^{152}Tb]Tb-54, [^{155}Tb]Tb-54 and [^{161}Tb]Tb-54 were obtained at 24 hours p.i., allowing clear visualization of the tumor (Fig. 30A,B,C). Interestingly, the relatively long half-life of ^{155}Tb allowed the acquisition with high longitudinal SPECT imaging resolution, leading to a clear visualization of the activity accumulation in the tumor and kidneys at 4 days p.i.. Müller *et al.* completed the evaluation of [^{155}Tb]Tb-54 properties using SPECT images on IGROV-1 tumor-bearing mice [174]. SPECT/CT images at 2 days p.i. of [^{155}Tb]Tb-54 revealed excellent activity accumulation in IGROV-1 tumors and kidneys. To sum up, the three terbium radiofolates are suitable for their respective applications.

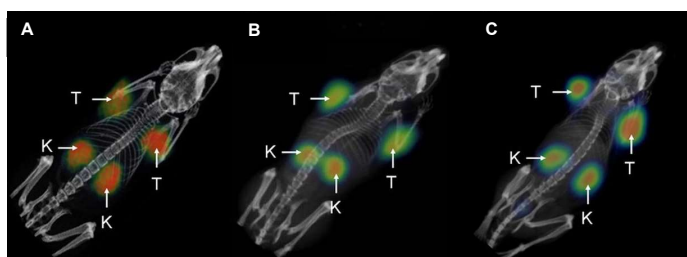
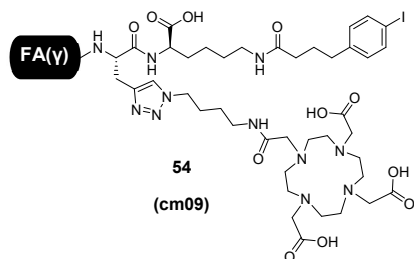


Figure 308: Chemical structure of **54**, (A) PET/CT image of KB tumor-bearing mice at 24 hours p.i. of [^{152}Tb]Tb-**54** (9 MBq), and SPECT/CT images of KB tumor-bearing mice at 24 hours p.i. of [^{155}Tb]Tb-**54** (B) 4 MBq and (C) 30 MBq. Images reprinted with permission from [81]. Copyright 2022 SNMMI

4.3. Albumin-binding ^{68}Ga -, ^{64}Cu -, and ^{55}Co -folates

Ocak *et al.* used [^{68}Ga]Ga-**54** to evaluate its diagnostic potential for a FR α -positive ovarian tumor cell line (MKP-L) [175]. Biodistribution analysis was performed at 4 hours p.i. of [^{68}Ga]Ga-**54** and showed a high fixation of the radioconjugate in tumors (4.2 ± 1.5 %IA/g) and kidneys (16.7 ± 4.7 %IA/g), conducted to a tumor-to-kidney ratio of 0.25. PET/CT images acquisition on mice C57BL/6 bearing MKP-L tumors at different stages of disease was performed with injection of [^{68}Ga]Ga-**54** and compared to control mice. Imaging of peritoneal tumors at 3-3.5 h p.i. showed specificity of [^{68}Ga]Ga-**54** and high tumor uptake. [^{68}Ga]Ga-**54** showed its suitability to monitor over time (between 2 and 7 weeks p.i.) the evolution of tumor size on the same mice model using PET imaging.

Müller and coworkers developed compound **55** (rf42) composed of a lysine residue functionalized with FA at its γ -position, AB entity and NODAGA chelator (Fig. 31) [176]. *In vivo* and *in vitro* results were compared to those obtained with its DOTA analogue **56** (cm10, Fig. 34). Compounds **55** and **56** were radiolabeled with ^{68}Ga and ^{64}Cu . Biodistribution data in KB tumor-bearing mice were acquired over a period of 4 hours for [^{68}Ga]Ga-**55** and 72 hours for [^{64}Cu]Cu-**55**. For [^{64}Cu]Cu-**55**, tumor, kidneys, salivary glands and liver showed significantly uptake at 4 hours p.i. with values of 14.52 ± 0.99 , 26.71 ± 2.34 , 7.08 ± 4.56 and 6.24 ± 0.56 %IA/g, respectively, leading to a tumor-to-kidney ratio of 0.55. Similar results were obtained for [^{68}Ga]Ga-**55**. [^{64}Cu]Cu-**55** showed accumulation in liver over the time (*i.e.*, 6.02 ± 0.41 %IA/g at 72 hours p.i.), thereby having a negative impact on the tumor-to-background contrast that could contribute to incorrect diagnoses. Chelating agent effect on the imaging efficiency was investigated by taking PET/CT images at 2 hours p.i. of [^{68}Ga]Ga-**55**, [^{68}Ga]Ga-**56**, [^{64}Cu]Cu-**55** and [^{64}Cu]Cu-**56**. PET/CT imaging results with [^{68}Ga]Ga-**55** and [^{64}Cu]Cu-**55** exhibited distinct tumor and kidneys visualization (Fig. 31A,B). [^{68}Ga]Ga-**55** and [^{68}Ga]Ga-**56** gave similar PET/CT images. Conversely, image comparison with [^{64}Cu]Cu-**55** and [^{64}Cu]Cu-**56** clearly showed a massive accumulation of activity of [^{64}Cu]Cu-**56** in liver due to a loss of ^{64}Cu from DOTA and its transfer to Cu-binding proteins induced by a limited *in vivo* stability of the ^{64}Cu -DOTA complex. Clear visualization of the tumor and kidneys on PET images accompanied by a low accumulation in the liver was obtained with [^{64}Cu]Cu-**55** even at 72 hours p.i.. [^{64}Cu]Cu-**55** was identified as the most promising ^{64}Cu -radiofolate for PET imaging of FR α -positive tumors.

In 2019, the same team developed and pre-clinically evaluated [^{55}Co]Co-**55** and [^{55}Co]Co-**56** [177]. Their biodistribution profiles were evaluated in KB tumor-bearing mice and compared to those of [^{64}Cu]Cu-**55** and [^{68}Ga]Ga-**55** [76,79,176,178–182]. The results showed that both ^{55}Co -radiofolates displayed higher tumor

uptake at 4 hours p.i. (17 %IA/g) compared to ^{68}Ga and ^{64}Cu -analogues. In addition, the biodistribution data at 4 hours p.i. revealed a higher renal uptake and a lower distribution in blood for ^{55}Co -**55** compared to ^{55}Co -**56** (53 ± 12 vs. 36 ± 7 %IA/g for kidney and 8 ± 3 vs. 14 ± 1 %IA/g for blood), which could be explained by the slightly higher lipophilicity of ^{55}Co -**56** compared to ^{55}Co -**55**. This data underlined the versatility importance of the chelating agent used for ^{55}Co labelling. The high affinity to FR α -expressing tissues was also evidenced by PET/CT imaging of KB tumor-bearing mice at 4 hours p.i..

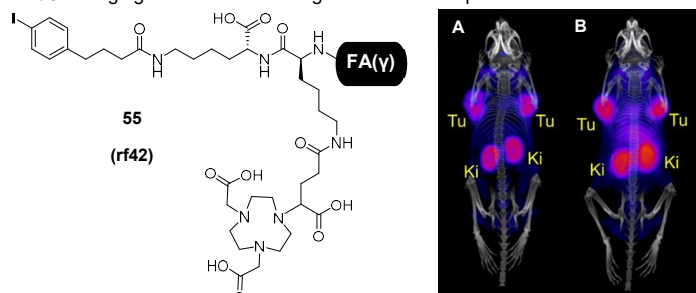


Figure 31: Chemical structure of **55** and PET/CT images of KB tumor-bearing mice at 2 hours p.i. of ^{64}Cu -**55** (3.5-7 MBq) (A) and ^{68}Ga -**55** (5 MBq) (B). Reprinted with permission from Farkas et al. *Mol. Pharm.* 13 (2016) 1979–1987. Copyright 2022 American Chemical Society

4.4. Albumin-binding ^{44}Sc -folate

In a proof-of-concept study published in 2013, Müller and co-workers focused on the use of ^{44}Sc for PET imaging and dosimetry using compound **54** [183,184]. The resulted ^{44}Sc -**54** was *in vitro* and *in vivo* compared to ^{177}Lu -**54** which showed a high KB tumor uptake (section 5.2.). High KB cellular uptake and internalization in KB cells (30-50% of total FR α -bound fraction) was observed. Biodistribution studies in KB tumor-bearing mice using ^{44}Sc -**54** revealed a high tumor accumulation at 2 hours p.i. (8.37 ± 0.41 %IA/g) that reached a maximum at 20 hours p.i. (14.05 ± 2.29 %IA/g). In addition, specific salivary and kidney accumulations of ^{44}Sc -**54** were observed (3-7 and 19-23 %IA/g from 2 to 20 hours p.i., respectively), indicating specificity of ^{44}Sc -**54** for organs expressing FR α . The biodistribution study showed a good tumor-to-kidney ratio between 0.44 and 0.65. All these tissue distribution data were broadly comparable, or even similar at 1 day p.i., to those of ^{177}Lu -**54**. PET/CT images with ^{44}Sc -**54** at 4 hours p.i. showed clear visualization of KB tumor and kidneys (Fig. 32A). The activity distribution profile of ^{44}Sc -**54** was similar to that observed on SPECT/CT images with ^{177}Lu -**54** at 4 hours p.i. (Fig. 32B).

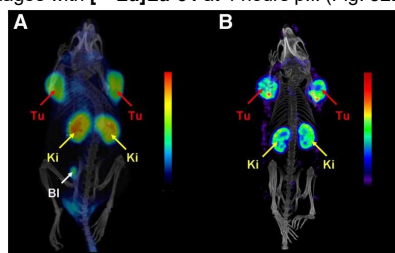


Figure 32: (A) PET/CT images of KB tumor-bearing mice at 4 hours p.i. of ^{44}Sc -**54** (25 MBq) and (B) SPECT/CT images of KB tumor-bearing mice at 4 hours p.i. of ^{177}Lu -**54** (35 MBq). Images reprinted with permission from [183]. Copyright 2022 SNMMI

4.5. Conclusion

Addition of an AB entity to radiofolate successfully decrease kidney uptake and lead to a significant improvement of tumor-to-kidney ratio of AB-radiofolate reaching 0.44 to 1.03 (Table 6). These results are similar with those obtained with the other strategy involving antifolate pre-injection [124,125]. In addition, regarding images, both strategies lead to a better tumor-to-background contrast resulting in more accurate diagnostic. It is interesting to note that the combination strategy showed a synergistic effect on tumor-to-kidney ratio (*i.e.*,

reaching 1.79 and on image contrast [164]. However, as the addition of AB entity to radiofolate is not toxic compared to antifolate pre-injection, this strategy is more suitable and AB-radiofolates could be validated by authorities to perform clinical trials. Moreover, by decreasing kidney uptake, TRT experimentations to treat FR α -positive tumors become possible as nephrotoxicity could be limited.

Table 6: Summary of radiochemistry, *in vitro* and *in vivo* results of radiofolates depicted in this section

| Radiofolate | RCY (%) | Log P | Tumor uptake (%IA/g) (4h) | Tumor-to-kidney ratio (4h) | References |
|---------------------------|---------|---------------------------|---------------------------|----------------------------|------------|
| [¹⁸ F]53 | 15-22 | -3.2 ± 0.4 | 15.2 ± 0.53 | 0.88 ± 0.04 | [173] |
| [⁶⁸ Ga]Ga-54 | >98 dc | NC | 4.2 ± 1.5 | 0.25 | [175] |
| [⁴⁴ Sc]Sc-54 | 96 | -4.49 ± 0.12 | 12.51 ± 1.74 | 0.59 ± 0.06 | [183] |
| [¹⁵² Tb]Tb-54 | >96 | NC | NC | NC | [81,185] |
| [¹⁵⁵ Tb]Tb-54 | >96 | NC | NC | NC | [81,185] |
| [¹⁶¹ Tb]Tb-54 | >98 | NC | 23.81 ± 2.53 | 0.87 ± 0.12 | [81,185] |
| [⁵⁵ Co]Co-55 | >95 | NC | 17.0 ± 4.0 | 0.32 | [177] |
| [⁵⁵ Co]Co-56 | >95 | NC | 17.0 ± 2.0 | 0.47 | [177] |
| [⁶⁸ Ga]Ga-55 | 95 | -3.85 ± 0.09 ^a | 11.92 ± 1.68 | 0.47 ± 0.06 | [176] |
| [⁶⁴ Cu]Cu-55 | >95 | -4.04 ± 0.48 ^a | 14.52 ± 0.99 | 0.55 ± 0.08 | [176] |

RCY: Radiochemical yield; Log P: Logarithm of the Partition coefficient; NC: Not communicated; dc: Decay corrected; ^a: Log D_{7.4} (Logarithm of the Distribution coefficient at pH 7.4)

5. Evaluation of Radiofolates used in theranostic approach

This section describes radiofolates design for diagnostic imaging (SPECT or PET) and/or for TRT in the viewpoint of developing a theranostic approach. Regarding its energy properties, lutetium-177 is a radionuclide of choice for TRT and treatment monitoring or dosimetry using SPECT/CT imaging. Moreover, lutetium can be easily complexed by the versatile DOTA chelator. Thus, it is favored for mismatched pair theranostic agents development using gallium-68 as diagnostic radionuclide [73,95–97]. Concerning FR α -positive tumors, [¹⁷⁷Lu]Lu-MOV18 [186] and ¹⁷⁷Lu-folates have been developed for therapy. For now, ¹⁷⁷Lu-folates have been developed only by Schibli and coworkers. The matched pairs (¹⁴⁹Tb/¹⁵²Tb/¹⁵⁵Tb/¹⁶¹Tb), (⁴⁴Sc/⁴⁷Sc) and (^{99m}Tc/¹⁸⁸Re) have also been tested in a theranostic approach using one radionuclide for diagnostic and the corresponding α or β -emitting-radionuclide for TRT. Finally, the ⁹⁰Y-radiofolate depicted here for TRT can be used in a mismatched pair with ⁶⁴Cu or ⁶⁸Ga-radiofolate using the same precursor for theranostic use [176].

5.1. Lutetium-177 radiofolate evaluation

Müller *et al.* completed the study of **5** by replacing indium-111 by lutetium-177 to observe the impact of the radionuclide choice on *in vivo* characteristics [125]. Radiolabelling and *in vitro* results were similar to those obtained with [¹¹¹In]In-5. *In vivo* study was performed with the same experimental conditions previously described in section 2.2.2. At 4 hours p.i. of [¹⁷⁷Lu]Lu-5, biodistribution data revealed massive uptake in kidneys (57.22 ± 11.05 %IA/g) and moderate uptake in KB tumors, liver and salivary glands (7.51 ± 1.25, 4.63 ± 1.12, 5.02 ± 1.07 %IA/g, respectively). Kidneys and non-specific uptakes in liver and salivary glands were similar to those observed with [¹¹¹In]In-5 (55.88 ± 3.91, 5.20 ± 2.00 and 6.13 ± 1.84 %IA/g, respectively) while tumor uptake was significantly higher (5.80 ± 0.55 %IA/g). This led to a higher tumor-to-kidney ratio of 0.15. Thus, the radionuclide type has a slightly but significant impact on the tracer biodistribution. SPECT/CT imaging showed moderate activity in KB tumors whereas high activity was detected in kidneys for both tracers. Small differences observed in biodistribution study did not have a significant impact on the image quality. Thus, [¹⁷⁷Lu]Lu-5 is as suitable as [¹¹¹In]In-5 for clinical trials.

5.2. Albumin-binding ^{177}Lu -folates evaluation

Müller and coworkers published three articles concerning [^{177}Lu]Lu-54 [184,185,187] and compared it with the conventional radiofolate [^{177}Lu]Lu-3 without AB entity. The aims of this study were to clarify whether the [^{177}Lu]Lu-54 could be compared to [^{177}Lu]Lu-3 and to highlight the effect of the presence of an AB entity on the tumor uptake and retention in kidneys. *In vitro* tests were performed on KB cells. High FR α affinities obtained with [^{177}Lu]Lu-54 and [^{177}Lu]Lu-3 were comparable to those of other radiofolates evaluated by the same team [188]. An increase in lipophilicity was observed with the addition of the AB entity (-4.25 ± 0.41 for [^{177}Lu]Lu-54 vs. -4.81 ± 0.36 for [^{177}Lu]Lu-3). The biodistribution studies with KB tumor-bearing mice showed a high tumor uptake of [^{177}Lu]Lu-54 (10.8 ± 1.32 %IA/g at 1 hour p.i.). At 24 hours p.i., tumor uptake of [^{177}Lu]Lu-3 was 7.00 ± 1.22 %IA/g concluding to a significant tumor uptake increase by adding an AB entity. For [^{177}Lu]Lu-54, kidney uptake was 30.09 ± 4.04 at 24 hours p.i. Comparatively, the kidney uptake of [^{177}Lu]Lu-3 at 24 hours p.i. was 57.95 ± 3.75 %IA/g confirming that a longer blood circulation time decreased the kidney uptake. Consequently, the tumor-to-kidney ratio was found to be higher for [^{177}Lu]Lu-54 compared to [^{177}Lu]Lu-3 (0.65 vs. 0.12). No significant uptake was observed in other tested organs for both radiofolates. SPECT/CT images showed a significant increase of the tumor-to-kidney ratio (~ 1.0 vs. ~ 0.2) in mice that received [^{177}Lu]Lu-54 (Fig. 33), compared with [^{177}Lu]Lu-3.

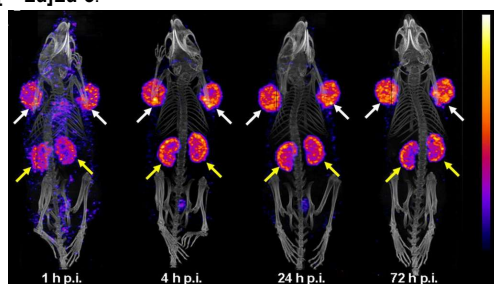


Figure 3310: SPECT/CT images of KB tumor-bearing mice at 1, 4, 24 and 72 hours p.i. of [^{177}Lu]Lu-54 (35 MBq). Image reprinted with permission from [184]. Copyright 2022 SNMMI

Müller's team developed four [^{177}Lu]Lu-DOTA-(γ)-folate conjugates deriving from [^{177}Lu]Lu-54 to increase pharmacokinetic properties of [^{177}Lu]Lu-54 and its interaction with albumin by modification of the linker between DOTA chelator and FA. They developed **56** (cm10: lead compound designed with a lysine residue functionalized with DOTA, FA and the AB entity), **57** (cm12: with a PEG₁₁ spacer between AB entity and lysine), **58** (cm13: with a short alkyl chain instead of PEG₁₁ spacer) and **59** (cm14: **56** without the AB entity) (Fig. 34) [188]. *In vitro* experiments were performed in KB and IGROV-1 tumor cells. Cell uptake was in the range of 13 to 22% at 30 min p.i. All four radiofolates showed high FR α affinities in a range of 4.0-7.2 nM. *In vivo* experiments were conducted in KB and IGROV-1 tumor-bearing mice. At 24 hours p.i., the kidneys uptake in KB tumor-bearing mice was between 19.5 and 46.6 %IA/g while tumor uptake was between 4.80 and 17.6 %IA/g. These led to tumor-to-kidney ratios between 0.43 and 0.63 for AB radiofolates and 0.10 for [^{177}Lu]Lu-59. Comparatively slightly high tumor-to-kidney ratio was found in IGROV-1 tumor-bearing mice. These results clearly indicated an increase in tumor uptake and a decrease in kidney uptake when an AB entity was employed. Results also revealed that the presence or not of a spacer and its length played a role in the biodistribution profile. The biodistribution study concluded that a short alkyl chain spacer gave better results than a long PEG₁₁ spacer for this type of radiofolate. SPECT/CT imaging was performed at 24 hours p.i. of the four radiofolates (Fig. 34) and an excellent visualization of tumors and kidneys was observed. Tumor-to-kidney ratios were calculated and confirmed results of biodistribution as tumor-to-kidney ratios of [^{177}Lu]Lu-56 (1.0) and [^{177}Lu]Lu-57 (1.1) were superior to those of [^{177}Lu]Lu-58 (0.7) and [^{177}Lu]Lu-59 (0.7).

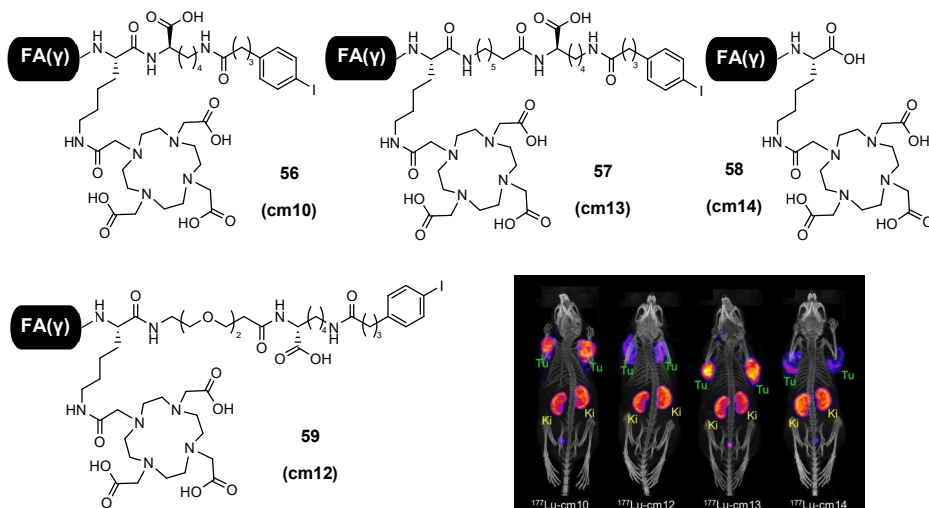


Figure 3411: Chemical structures of 56, 57, 58 and 59 and SPECT/CT images of KB tumor-bearing mice at 24 hours p.i. of [^{177}Lu]Lu-56, [^{177}Lu]Lu-57, [^{177}Lu]Lu-58 and [^{177}Lu]Lu-59 (50 MBq). Reprinted with permission from Siwowska et al. *Mol. Pharm.* 14 (2017) 523–532. Copyright 2022 American Chemical Society.

5.3. $^{99\text{m}}\text{Tc}/^{188}\text{Re}$ approach for theranostic folate application

Kim *et al.* worked on the matched pair ($^{99\text{m}}\text{Tc}/^{188}\text{Re}$) to develop a theranostic approach with folate tracer. ^{188}Re is a γ - and β -emitter which confer to him SPECT imaging and therapy properties. Technetium and rhenium have similar chemical properties and can be chelated on same molecules. Thus, authors synthesized two radiofolates [$^{99\text{m}}\text{Tc}$]Tc-60 and [^{188}Re]Re-60 [189]. Compound 60 ((γ)-folate-Gly-Gly-Cys-Glu) was composed of FA linked to the peptidic chelating agent H-Gly-Gly-Cys-Glu-OH via an amide bond on the N-terminal glycine (Fig. 35). They performed *in vitro* studies in KB and HT1080 cells and *in vivo* studies in BALB/c nude mice bearing both KB and HT1080 tumors. Identical biodistribution patterns were obtained with [$^{99\text{m}}\text{Tc}$]Tc-60 and [^{188}Re]Re-60. *In vivo* HT1080 tumors showed very low uptake as expected. At 4 hours p.i., uptake of [$^{99\text{m}}\text{Tc}$]Tc-60 in kidneys was 57.04 ± 18.58 and 17.59 ± 7.40 %IA/g in KB tumor (ratio: 0.30). When [^{188}Re]Re-60 was used, kidneys and KB tumor uptake were respectively 63.71 ± 5.86 and 24.10 ± 7.28 %IA/g (ratio: 0.37). At 32 hours p.i., tumor-to-kidney ratio reached 1.04 meaning that KB tumor uptake was higher than kidney uptake. For therapy, it is mandatory to have the highest tumor-to-kidney ratio as possible, and a value above 1 is a promising result. γ -Scintigraphy using [$^{99\text{m}}\text{Tc}$]Tc-60 showed intense activity in KB tumor, kidneys and bladder at 4 hours p.i., but this activity was significantly low at 16 hours p.i. expect for KB-tumor making it the most visible tissue. SPECT/CT scan images showed intense activity in kidneys, urinary bladder and moderate activity in KB tumors at 4 and 16 hours p.i. of [$^{99\text{m}}\text{Tc}$]Tc-60 or [^{188}Re]Re-60. At last, [$^{99\text{m}}\text{Tc}$]Tc-60 displayed tumor-to-normal organ ratio at 4 hours p.i. much higher (2- to 5-fold) than those reported with [$^{99\text{m}}\text{Tc}$]Tc-23 which is currently undergoing clinical trials. Nevertheless, no preclinical study on the therapeutic effect of [^{188}Re]Re-60 have been reported.

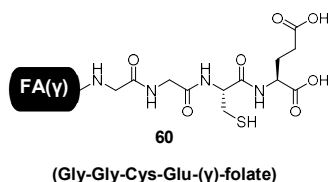


Figure 35: Chemical structure of 60 [189]

The same team worked on the radiolabelling of **18** with ^{188}Re to determine if this compound could be suitable for therapy [86]. They already worked on $[^{99\text{m}}\text{Tc}]\text{Tc-18}$ and wanted to see if the pair ($[^{188}\text{Re}]\text{Re-18}/[^{99\text{m}}\text{Tc}]\text{Tc-18}$) could be suitable as theranostic radioconjugates. Biodistribution tests were performed in KB tumor-bearing mice with $[^{188}\text{Re}]\text{Re-18}$ and $[^{99\text{m}}\text{Tc}]\text{Tc-18}$. Similar significant uptake was observed at 4 hours p.i. in kidneys (12.04 ± 0.62 vs. 18.48 ± 0.72 %IA/g, respectively), liver (2.96 ± 0.49 vs. 2.37 ± 2.85 %IA/g, respectively) and tumors (1.87 ± 0.04 vs. 2.33 ± 0.36 %IA/g, respectively). Competition tests were performed with FA and significant uptake was observed only on liver (2.00 ± 1.74 %IA/g) for $[^{188}\text{Re}]\text{Re-18}$ confirming the non-specific uptake in liver for this radioconjugate. As kidney uptake was too important and could lead to renal toxicity, the team decided not to pursue investigations with $[^{188}\text{Re}]\text{Re-18}$ for TRT.

5.4. Treatment evaluation of FR α -positive tumors using radiofolates

The therapeutic application of $[^{177}\text{Lu}]\text{Lu-54}$ was evaluated by Müller *et al.* [184] in KB tumor-bearing mice. Tumor growth inhibition was much better with a single injection of 20 MBq compared to a fractionated injection (2×10 MBq or 3×7 MBq). Using a single injection of 20 MBq, the SPECT imaging revealed the disappearance of the tumor for 4 of the 5 mice of the group. Moreover, radiotoxic side effects were not observed. The good survival rate (80%) observed for mice injected with 20 MBq in one time gave promising results to push further investigations. To conclude $[^{177}\text{Lu}]\text{Lu-54}$ was suitable for therapeutic clinical trials. Hence, the impact of the addition of an AB entity on the antitumor activity of radiofolate, and on the long-term kidney damage by comparison with $[^{177}\text{Lu}]\text{Lu-3}$ properties was achieved [187]. The addition of the AB entity increased the absorbed radiation dose for tumors by a factor of three (1.4 Gy/MBq for $[^{177}\text{Lu}]\text{Lu-54}$ vs. 0.444 Gy/MBq for $[^{177}\text{Lu}]\text{Lu-3}$) and reduced the absorbed radiation dose for kidneys by a factor of two (2.3 Gy/MBq for $[^{177}\text{Lu}]\text{Lu-54}$ vs. 4.8 Gy/MBq for $[^{177}\text{Lu}]\text{Lu-3}$). The best tumor growth inhibition was observed with $[^{177}\text{Lu}]\text{Lu-54}$ (≥ 20 MBq (≥ 28 Gy) per mouse), resulting in complete tumor remission. The long-term side effects were studied and showed a lower renal toxicity with $[^{177}\text{Lu}]\text{Lu-54}$. A threshold dose limit for the kidneys of 25 Gy in mice was obtained for $[^{177}\text{Lu}]\text{Lu-54}$ [190].

^{149}Tb -folate was used in preclinical therapeutic tests to heal FR α -positive tumor-bearing mice [191]. *In vitro* study $[^{149}\text{Tb}]\text{Tb-54}$ using KB cells were fulfilled to determine the killing potential of this radionuclide. MTT assay was performed using increasing concentration of $[^{149}\text{Tb}]\text{Tb-54}$ (0-500 kBq/mL) in cell culture media. Cell viability was reduced by 20% with 0.5 kBq/mL and by 80% with 500 kBq/mL. 3 groups of KB tumor-bearing female athymic nude mice were used for therapeutic tests. Group A was the control, group B was injected with 2.2 MBq of $[^{149}\text{Tb}]\text{Tb-54}$ and group C by 3 MBq of radiofolate. 14 days after the beginning of the therapy, blood urea nitrogen (damage of kidneys) and alkaline phosphate and total bilirubin (liver misfunction) were controlled in each group and revealed no kidneys and liver misfunction. At 21 days p.i. of radiofolate, the first mice (group A) had to be euthanized. At this time point, tumor growth was decreased by 62% and 85% for the group B and C, respectively (Fig. 36A). In the case of the survival time, it was increased by 45% and 105%, respectively (Fig. 36B). These results seemed promising as survival time was increased by 2 but a total remission would be ideal. Nevertheless, for now, other therapy studies including radiofolate showed similar results.

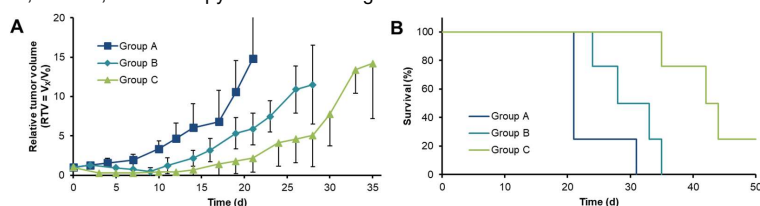


Figure 36: (A) Relative tumor volume and (B) survival rate of KB tumor-bearing mice injected with saline (group A), 2.2 MBq (group B) and 3 MBq (group C) of $[^{149}\text{Tb}]\text{Tb-54}$. Adapted from [191]

Thanks to identical chemical characteristics and complementary physical decay characteristics, ^{149}Tb and ^{161}Tb could be used to compare the effectiveness of α vs. β^- therapy for a same compound **54**. Therapy with $[^{149}\text{Tb}]\text{Tb-54}$ (2.4 MBq) and $[^{161}\text{Tb}]\text{Tb-54}$ (11 MBq) in KB tumor-bearing mice induced a marked tumor growth delay or a total remission (33% for $[^{149}\text{Tb}]\text{Tb-54}$ and 80% for $[^{161}\text{Tb}]\text{Tb-54}$) and was accompanied by a significant survival improvement (Fig. 37A,B) [81]. In a second study, *in vitro* and *in vivo* antitumor effects of

$[^{161}\text{Tb}]\text{Tb-54}$ and $[^{177}\text{Lu}]\text{Lu-54}$ in KB and IGROV-1 cells and in KB or IGROV-1-tumor-bearing mice were compared [185]. They also compared renal side effects after β^- therapy [185,192]. Biodistribution profiles of $[^{161}\text{Tb}]\text{Tb-54}$ and $[^{177}\text{Lu}]\text{Lu-54}$ in IGROV-1 tumor-bearing mice were found to be very close to those previously described on KB tumor-bearing mice (sections 4.2. and 5.2.). SPECT/CT images were performed in KB tumor-bearing mice at 24 hours p.i. of $[^{161}\text{Tb}]\text{Tb-54}$ or $[^{177}\text{Lu}]\text{Lu-54}$ and showed a clear visualization of the tumor and the kidneys in both cases. After injection of 10 MBq of $[^{161}\text{Tb}]\text{Tb-54}$ or $[^{177}\text{Lu}]\text{Lu-54}$ in KB and IGROV-1 tumors-bearing mice, higher absorbed doses in tumors and kidneys of $[^{161}\text{Tb}]\text{Tb-54}$ (3.3 and 4.5 Gy/MBq, respectively) were estimated in comparison with those of $[^{177}\text{Lu}]\text{Lu-54}$ (2.4 and 3.4 Gy/MBq, respectively). These results might explain why a more marked tumor growth delay and an enhanced antitumor effect were observed after injection of $[^{161}\text{Tb}]\text{Tb-54}$ compared to $[^{177}\text{Lu}]\text{Lu-54}$. The renal side effects (*i.e.* radionephrotoxicity) of $[^{161}\text{Tb}]\text{Tb-54}$ and $[^{177}\text{Lu}]\text{Lu-54}$ were studied in non-tumor-bearing mice at therapeutic doses (10, 20, and 30 MBq) over an 8-months period. β^- particles emission from ^{161}Tb and ^{177}Lu had the same impact on kidney function. At 15 weeks p.i. (20 and 30 MBq), a significant impairment of kidney function was observed whereas this impairment appeared after 8 months when 10 MBq was injected [192]. This study demonstrates that $[^{161}\text{Tb}]\text{Tb-54}$ and $[^{177}\text{Lu}]\text{Lu-54}$ are suitable for theranostic approach to diagnose and treat FR α -overexpressing tumors. Further biological investigations would be necessary to envision future clinical trials.

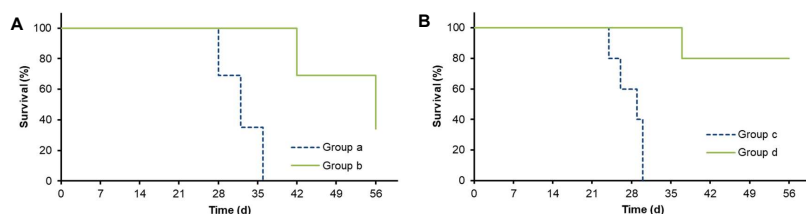


Figure 37: Survival rate of mice treated (A) with $[^{161}\text{Tb}]\text{Tb-54}$ (a: control, b: 2.4 MBq) and (B) $[^{161}\text{Tb}]\text{Tb-54}$ (c: control, d: 11 MBq). Adapted from [81]

Müller *et al.* investigated the potential of ^{47}Sc chelated to the DOTA-folate conjugate **56** with an AB entity ($[^{47}\text{Sc}]\text{Sc-56}$) for TRT [98]. Biodistribution studies of $[^{47}\text{Sc}]\text{Sc-56}$ showed a high blood uptake in the first 4 hours due to the increase in circulation time. High accumulation in KB tumor, kidneys and salivary glands (17.96 ± 2.17 , 35.4 ± 1.1 and 7.40 ± 0.97 %IA/g at 4 hours p.i., respectively) was observed with a good retention of activity for 72 hours. Consequently the tumor-to-kidney ratio reached a maximum of 0.56 ± 0.07 at 72 h p.i. SPECT/CT imaging of a KB tumor-bearing mouse at 48 hours p.i. showed an excellent visualization of tumor. Activity uptake was also clearly seen in kidneys but in a lower intensity than tumor. Preclinical therapy study was achieved on KB tumor-bearing mice. 10 MBq of $[^{47}\text{Sc}]\text{Sc-56}$ was injected in 6 mice. Compared to KB tumor-bearing mice receiving PBS injection, tumor growth was reduced by 2 and survival time was increased of 54% (Fig. 38A). Nevertheless, no remission was observed (Fig. 38B,C). For a single injection of 10 MBq of $[^{47}\text{Sc}]\text{Sc-56}$, a tumor dose of 10 Gy and a kidney dose of 20 Gy were determined. Since the kidney dose limit was approximately 23 Gy, this new therapeutic radiofolate would drastically minimize kidney toxicities. To conclude, ^{47}Sc is suitable for PET diagnostic and will be potentially used in clinical trials within a few years. These results are also promising for the use of the matched pair $^{44}\text{Sc}/^{47}\text{Sc}$ in a theranostic approach.

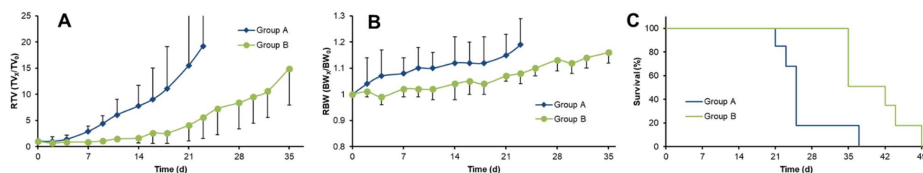


Figure 38: (A) Relative tumor volume (RTV), (B) Relative body weight and (C) survival rate of KB tumor-bearing mice injected with saline (group A) or with $[^{47}\text{Sc}]\text{Sc-56}$ (10 MBq) (group B). Adapted from [98]

Siwowska *et al.* evaluated the β^- therapeutic potential of ^{47}Sc , ^{177}Lu and ^{90}Y chelated to **56** [193]. MTT assay was performed using increasing doses (5-40 MBq/mL) of radiofolates in IGROV-1 cell culture to establish

the killing ability of these three radiofolates. Cell viability was determined 4 hours after incubation and revealed a total cell death with 20 MBq/mL of [⁹⁰Y]Y-56 whereas 20% and 25% of cell were viable with 40 MBq of [¹⁷⁷Lu]Lu-56 and [⁴⁷Sc]Sc-56, respectively. This result confirmed the killing ability of those radiofolates and particularly of [⁹⁰Y]Y-56. *In vivo* experiments were performed using IGROV-1 tumor-bearing female CD1 nude mice. For therapy study, 4 groups of mice received respectively PBS, 12.5 MBq of [⁴⁷Sc]Sc-56, 10 MBq of [¹⁷⁷Lu]Lu-56 and 5 MBq of [⁹⁰Y]Y-56. Doses were adapted regarding respective emission energy to reach the same absorbed tumor dose per radiofolate. Body weight of all mice stayed stable for the whole experiment period. A tumor growth inhibition of respectively 69%, 6% and 76% was observed leading to an increase in survival time (26%, 35% and 65%, respectively) (Fig. 39A,B). Plasma parameters like blood urea nitrogen, alkaline phosphatase and total bilirubin were evaluated. As concentration in blood was the same before therapy, non-nephrotoxicity of the three radiofolates for tested doses was concluded. These *in vitro* and *in vivo* results showed that [⁹⁰Y]Y-56 presents the best FR α -positive tumor cell killing ability. Nonetheless, regarding these promising results, all tested radionuclides could be used in TRT following the nature of the tumor.

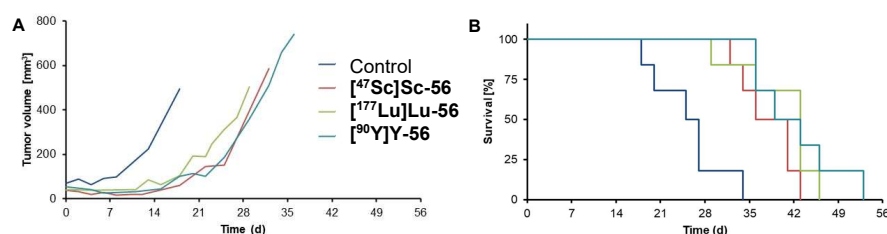


Figure 39: (A) Tumor volume and (B) survival rate of IGROV-1 tumor-bearing mice injected with PBS, [⁴⁷Sc]Sc-56 (12.5 MBq), [¹⁷⁷Lu]Lu-56 (10 MBq) and [⁹⁰Y]Y-56 (5 MBq). Adapted from [193]

5.5. Conclusion

TRT to treat FR α -positive tumor-bearing mice was experimented using mainly ¹⁷⁷Lu but also ¹⁸⁸Re, ¹⁴⁹Tb, ¹⁶¹Tb, ⁴⁷Sc and ⁹⁰Y. All results are described in Table 7. Radiofolates containing an AB entity are the most promising radiofolates to treat FR α -overexpressing tumors. Based on the tumor models and injected activities, all published radiofolates showed a positive impact on tumor regression with the addition of an AB entity even if complete remission was not achieved. As for bivalent radiofolates for nuclear imaging (Section 4.), a synergistic effect on tumor regression was also demonstrated by the combination of an AB entity and antifolate pre-injection though without attaining remission [164]. Nevertheless, these preclinical tests are quite promising as survival time was increased by 50% in average with a tumor growth reduced by 2. Depending on the radionuclide used, therapy results are different but positive. Thus, folate-tracer could be radiolabeled with different radionuclides depending on the characteristics of the tumor's patients to personally adapt treatments to patients.

Table 7: Summary of radiochemistry, *in vitro* and *in vivo* results of radiofolates depicted in this section

| Radiofolate | RCY (%) | Log P | K _i for FR α (nM) | Tumor uptake (%IA/g) (4h) | Tumor-to-kidney ratio (4h) | References |
|---------------------------|---------|-------------------------------|-------------------------------------|---------------------------|----------------------------|---------------|
| [¹⁷⁷ Lu]Lu-5 | >90 | NC | NC | 7.51 \pm 1.25 | 0.67 \pm 0.01 | [125] |
| [¹⁷⁷ Lu]Lu-3 | >98 | -4.81 \pm 0.36 ^a | NC | 7.52 \pm 1.15 | 0.10 \pm 0.01 | [168,184,187] |
| [¹⁷⁷ Lu]Lu-54 | >98 | -4.25 \pm 0.41 ^a | NC | 18.12 \pm 1.80 | 0.65 \pm 0.07 | [184,185,187] |
| [¹⁷⁷ Lu]Lu-56 | >97 | -4.40 \pm 0.23 ^a | 5.1 \pm 2.1 | 33 \pm 9 | 1.2 \pm 0.5 | [188,193] |
| [¹⁷⁷ Lu]Lu-57 | NC | -4.10 \pm 0.14 ^a | 7.4 \pm 1.2 | 19.5 \pm 3.21 | 0.61 \pm 0.10 | [188] |
| [¹⁷⁷ Lu]Lu-58 | NC | -4.10 \pm 0.20 ^a | 4.0 \pm 3.4 | 16.0 \pm 1.58 | 0.63 \pm 0.12 | [164,188] |

| | | | | | | |
|---------------------------|-----|---------------------------|-----------|--------------|-------------|----------|
| [¹⁷⁷ Lu]Lu-59 | NC | < -5.5 ^a | 4.9 ± 2.7 | 8.56 ± 2.09 | 0.12 ± 0.00 | [188] |
| [^{99m} Tc]Tc-60 | >93 | NC | 5.19 | 17.59 ± 7.40 | 0.30 | [189] |
| [¹⁸⁸ Re]Re-60 | >93 | NC | 5.19 | 21.74 ± 4.41 | 0.28 | [189] |
| [^{99m} Tc]Tc-18 | >95 | NC | NC | 2.33 ± 0.36 | 0.13 ± 0.02 | [86] |
| [¹⁸⁸ Re]Re-18 | >95 | NC | NC | 1.87 ± 0.04 | 0.15 ± 0.01 | [86] |
| [¹⁴⁹ Tb]Tb-54 | >96 | -4.25 ± 0.41 ^a | NC | NC | NC | [81,191] |
| [¹⁶¹ Tb]Tb-54 | >98 | NC | NC | 23.8 ± 2.5 | 0.87 ± 0.12 | [81,185] |
| [⁴⁷ Sc]Sc-56 | >96 | NC | NC | 17.96 ± 2.17 | 0.51 ± 0.03 | [98,193] |
| [⁹⁰ Y]Y-56 | >97 | NC | NC | NC | NC | [193] |

RCY: Radiochemical yield; Log P: Logarithm of the Partition coefficient; NC: Not communicated; dc: Decay corrected; ^a: Log D_{7.4} (Logarithm of the Distribution coefficient at pH 7.4)

6. Future Perspectives and Conclusions

The nuclear imaging approach for peritoneal carcinomatosis from ovarian cancer relies on the detection of a signal specifically emitted from metastases (chemical resolution) with no or low signal in the surrounding healthy tissue. This concept of chemical resolution can be used with folate-based radiopharmaceuticals. The intensity of the signal is high in cancer cells compared to healthy tissues because of metabolic trapping of radiofolate after their internalization by FR α [34].

Regarding radiofolate structures, some of them are now synthesized using the click chemistry approach. This effective approach allows reduction of costs of synthesis thanks to a decrease in the number of steps and an increase in yields. Moreover, some teams demonstrate that functionalization of FA at the α - and/or γ -position of the glutamate moiety have no impact on binding affinity. In this way, it is no longer necessary to use regioselective reactions that also ease the synthesis of the tracer precursors.

Depending on the imaging devices of imaging centers (SPECT or PET), radionuclide supplies will be different. These two nuclear imaging modalities depend on the isotopes used for the radiolabelling of folate derivatives. First, almost all nuclear medicine departments have a ⁹⁹Mo/^{99m}Tc generator for performing scintigraphy examinations. The low cost and high availability of this generator make possible the development and the use of ^{99m}Tc-radiofolates in clinical routine such as FolateScan ([^{99m}Tc]Tc-23) [139]. Less frequently, some nuclear medicine departments are equipped with a Ge⁶⁸/Ga⁶⁸ generator to produce gallium-68 or with a cyclotron to produce fluorine-18 used in the production of PET radiopharmaceuticals. Different radiofolates labeled with ⁶⁸Ga [72,159,160,175], ⁶⁴Cu [74,75,99,194,195] and ¹⁸F [105,147–158,173,196] have been described in the literature and the number of articles is increasing rapidly. In fact, the development of PET radiofolates is a topical issue as spatial resolution of PET imaging is better than SPECT one. The sensitivity of PET not only allows the detection of small amounts of the radiofolate (more sensitive than MRI imaging for example [197]), it is also an excellent tool for quantifying the overall absorption of the tracer and therefore to monitor the evolution of the lesions or the efficacy of a treatment, by quantifying the expression of FR α correlated with the presence of tumor cells.

Folate-based radiopharmaceuticals have been successfully developed to diagnose FR α -positive tumor cells using SPECT and PET imaging. Indeed, among all developed radiopharmaceuticals, the ones that give the most promising results are those with a high hydrophilicity, high tumor-to-liver ratio, high FR α affinity and a strong stability complex. High hydrophobicity tracers are eliminated by the hepatobiliary way that grow up the intestines, gallbladder and liver dosimetry and decrease tumor-to-background ratio entailing poor image quality and tough diagnosis, even more for peritoneal metastasis. Three radiofolates have satisfied all *in vitro* and *in vivo* specifications and have been studied under clinical trials, FolateScan ([^{99m}Tc]Tc-23) and [¹¹¹In]In-DTPA-folate ([¹¹¹In]In-4) for SPECT imaging and [¹⁸F]-Azafol ([¹⁸F]F41) for PET imaging. However, only FolateScan is currently used in clinical routine to diagnose ovarian or endometrial cancer. Promising results of [¹⁸F]-Azafol

could allow its use in clinical routine in a few years. Nevertheless, despite the constant increase in spatial resolution of imaging technology, detection of metastases of epithelial ovarian cancer remains a challenge.

Concerning TRT, one of the big challenges of its development is the good biodistribution of the tracers. Indeed, a high signal-to-noise ratio facilitates the imaging of small lesions and therefore the effectiveness of TRT. FR α is highly expressed by the tubule cells of kidneys and radiofolates are highly internalized into these cells entailing high tumor-to-kidney ratio. Due to the intense fixation and renal topology, several strategies have been developed to limit this unwanted fixation and reduce the adverse effects caused by non-specific targeting. The first strategy is a chemical modification of the radiopharmaceutical structure by adding an AB entity to increase the circulation time of the tracer and reduce renal uptake. The second one consists in the use of antifolates, more precisely pemetrexed, to reduce renal binding by competition for the FR α . Nevertheless, this does not prevent the kidneys from being exposed to radiation, but significantly reduce it as huge increase in tumor-to-kidney ratio is observed. The evaluation of the impact of the combination of pemetrexed pre-injection and the presence of an AB entity in the radiopharmaceutical structure on the tissue distribution [164] give encouraging results with a positive synergistic effect as an increase of around 30-50% of the tumor-to-kidney ratio (~1.79 which is the best ever observed). Additionally, activity intensity on SPECT imaging decreased in kidneys and increased in tumors. If the incapacity to reduce kidney fixation remains, TRT using radiofolates could be compromised. Hence, another biological markers such as anti-Müllerian hormone type II receptor (AMHRII) or Müllerian-inhibiting substance receptor type II (MISRII) are under study to target gynecological cancers. In fact, a first-in-human study is currently ongoing using GM102 mAb (NCT02978755) to diagnose gynecologic cancers by targeting AMHRII [198–200].

Moreover, the development of folate-based-radiopharmaceuticals using high energy radiation isotopes (α and β^- emission) allows their use in nuclear medicine with a theranostic approach. After diagnostic by SPECT or PET imaging concluding to the presence of FR α -positive lesions, TRT can be intended with ^{177}Lu [87,88,201], ^{161}Tb [187,190,192] or ^{149}Tb in combination with other therapies or surgery since no remission has been observed yet in preclinical trials. Nonetheless, delay in tumor growth and increase in survival rate have been observed for all TRT studies involving radiofolates which is encouraging in the development of TRT and the theranostic approach. In fact, treatment with 20 MBq of [^{177}Lu]Lu-54 and with 11 MBq of [^{161}Tb]Tb-56 showed the best survival rates. Moreover, monitoring of the injected dose and number of doses constraint nephrotoxicity. Concerning treatment monitoring, SPECT or PET radiofolates could be employed to follow the evaluation of tumor characteristics. In fact, the clinical study of the treatment of ovarian cancer using Vintafolide has been monitored with success using ^{99m}Tc -EC20 in SPECT imaging [202].

Abbreviations

%IA/g: Percentage of the injected activity per gram of tissue weight

5-Me-THF: 5-Methyl-tetrahydrofolate

AB: Albumin binder entity

Bz: Benzyl

CT: Computed tomography scan

Cys: Cysteine (C)

CuAAC: Copper-catalyzed azide-alkyne cycloaddition

Da: Dalton

DAV: δ -Aminovaleric acid

dc: Decay corrected

DFO: Deferoxamine

DNA: Deoxyribonucleic acid

DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

DO3A-EA: 1,4,7-tris (carboxymethyl)-10-(4-aminoethyl)-1,4,7,10-tetraazacyclododecane

DOTAGA: 2,2',2''-(10-(2,6-Dioxotetrahydro-2H-pyran-3-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid

DOTATOC: DOTA-octreotide

DTPA: Diethylenetriaminepentaacetic acid

EC: Electron capture

ECG: Glutamic acid-cysteine-glycine

EDA: Ethylenediamine
EDBE: 2,2'-ethylenedioxy-bis-ethylamine
EDDA: Ethylenediamine-*N,N'*-diacetic acid
FA: Folic acid
FBA: 4-Fluorobenzylamine
FDG: 2-Deoxy-2-fluoro-D-glucose
FR: Folate receptor
Glu: Glutamic acid or glutamate (E)
Gly: Glycine (G)
HBED: *N,N'*-Bis(2-hydroxybenzyl) ethylenediamine-*N,N'*-diacetic acid
His: Histidine (H)
HYNIC: Hydrazino-nicotinamide or 6-[2-(4-dimethylamino)benzaldehydehydrazono]nicotinate
IAC₅₀: Half maximal inhibitory activity concentration
IC₅₀: Half maximal inhibitory concentration
i.p. : Intraperitoneally
i.v.: Intravenous
K_d: Dissociation constant
Log D_{7.4}: Logarithm of the distribution coefficient at pH 7.4
Log P: Logarithm of the partition coefficient
Lys: Lysine (K)
MRI: Magnetic resonance imaging
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC: Not communicated
NODAGA: 1,4,7-Triazacyclononane, 1-glutaric acid-4,7-acetic acid
NOTA: 1,4,7-Triazacyclononane-1,4,7-trisacetic acid
OEG: Oligoethylene glycol
PAMA: Picolylamine monoacetic acid
PBS: Phosphate Buffered Saline
PCFT: Proton-coupled folate transporter
PDT: Photodynamic therapy
PEG: Polyethylene glycol
PEG₂: 2,2'-(Ethylenedioxy)-bis-ethylamine
PET: Positron emission tomography
p.i.: Post-injection
PMX: Pemetrexed
PSMA: Prostate specific membrane antigen
RFC: Reduced folate carrier
RGD: Arginine-glycine-aspartic acid peptide
ROIs: Region of interests
RCY: Radiochemical yield
SPECT: Single-photon emission computed tomography
TEPA: Tetraethylenepentamine
THF: Tetrahydrofolate
TPPMS: Diphenylphosphinobenzene-3-sulfonic acid sodium
TPPTS: Trisodium triphenylphosphine-3,3',3''-trisulfonate
TRT: Targeted Radionuclide therapy
TS: Thymidylate Synthase

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