Abstract: Technetium-99m has a rich coordination chemistry that offers many possibilities in terms of oxidation states and donor atom sets. Modifications in the structure of the technetium complexes could be very useful for fine tuning the physicochemical and biological properties of potential 99mTc radiopharmaceuticals. However, systematic study of the influence of the labelling strategy on the “in vitro” and “in vivo” behaviour is necessary for a rational design of radiopharmaceuticals. Herein we present a review of the influence of the Tc complexes’ molecular structure on the biodistribution and the interaction with the biological target of potential nitroimidazolic hypoxia imaging radiopharmaceuticals presented in the literature from 2010 to the present. Comparison with the gold standard [18F]Fluoromisonidazole (FMISO) is also presented.

Keywords: technetium-99m; hypoxia; nitroimidazoles; labelling strategies

1. Design of 99mTc-Radiopharmaceuticals

The design of potential 99mTc radiopharmaceuticals for molecular imaging is currently based almost exclusively on the application of the so-called “bifunctional approach” consisting of the combination of a biologically active molecule with a chelating unit for stable binding of the radiometal. Both parts are separated by a linker chain to avoid interference of the metal complex in the interaction with the target. This strategy has been successfully applied to the preparation of a variety of radiolabelled peptides [1], in particular somatostatin analogues which have been used clinically for over 20 years [2,3]. However, smaller biomolecules like carbohydrates, amino acids, receptor binding ligands or enzyme substrates represent a considerably more difficult challenge. Introduction of technetium is associated with a change in the spatial configuration of the bioactive molecule which can significantly affect the interaction with the target. Furthermore, modification of charge and lipophilicity associated with the coordination has a crucial influence in the overall biodistribution of the final compound [4]. Systematic studies of the influence of the labelling strategy in the physicochemical and biologic properties of the [99mTc]Tc radiotracers could be very useful for the fine tuning of the biological behaviour. However, this type of study is very scarce in the literature and the development of potential [99mTc]Tc radiopharmaceuticals is often based on serendipity or on trial and error and not on rational structure–biodistribution relationships.

In order to contribute to the rational design of potential [99mTc]Tc radiopharmaceuticals we proposed to review the influence of the Tc complexes molecular structure on the biodistribution and the interaction with the biological target of potential hypoxia imaging radiopharmaceuticals presented in the literature from 2010 to 2019. The review was performed in PubMed, Google Scholar and Timbó FOCO (https://foco.timbo.org.uy) using “hypoxia” and “99m-technetium” as search tags. Hypoxia
agents were chosen because of the considerable interest in the subject demonstrated by the extensive literature available and because in spite of the research performed so far no agent with adequate properties has been proposed.

2. Hypoxia Imaging Agents

Hypoxia is the pathophysiologic situation in which the supply of oxygen is insufficient to satisfy the demand of the tissues [6]. Hypoxia can be caused by a number of factors; reduced tissue perfusion due to structural abnormalities of microvessels and disturbed microcirculation are the most important in solid tumours. Tumour hypoxia is, according to the literature, associated with malignant progression and resistance to therapy and is consequently considered a very important issue in cancer treatment. Furthermore, tumour oxygenation is highly heterogeneous and difficult to measure directly and the development of non-invasive nuclear medicine methods to determine regions of hypoxia can contribute to the optimization of novel treatment options [7].

The detection of tumour hypoxia by nuclear medicine techniques was proposed by Chapman in 1979 [8] using radiolabelled 2-nitroimidazoles structurally derived from the antibiotic azomycin. The radiohalogenated derivative \[^{18}F\]FMISO has been the most successful product and is nowadays considered the gold standard for hypoxia imaging using positron emission tomography [9,10]. However, development of \(^{99m}Tc\)-labelled hypoxia imaging agents has also been pursued by many research groups due to the wider availability, especially in developing countries. The design of potential hypoxia targeting \(Tc\) radiopharmaceuticals involves the attachment of chelating groups to a bioreductive pharmacophore that is irreversibly reduced by cellular oxidoreductases in hypoxic conditions yielding reactive intermediates that are retained within the cell. This mechanism is inhibited by oxygen, thereby conferring specificity for hypoxia. Five classes of bioreductive compounds have been described [11]: nitro(hetero)cyclic compounds, aromatic \(N\)-oxides, aliphatic \(N\)-oxides, quinones, and metal complexes, from which nitro(hetero)cyclic compounds, in particular nitroimidazoles are the ones usually being applied for radiopharmaceutical development [12].

3. Nitroimidazoles as Bioreductive Pharmacophores

Nitroimidazoles are substrates of various nitro-reductase enzymes that catalyse a number of single electron reductions “in vivo”. The first step involves the formation of the nitroimidazole radical anion (Figure 1), a key intermediate in the whole process. This step is reversible in the presence of oxygen and therefore, responsible for the specificity of the uptake by hypoxic tissue. Further reduction results in the formation of hydroxylamine and amine derivatives which are trapped inside the cell through reaction with cellular macromolecules like DNA [13].

![Figure 1. Mechanism of “in vivo” reduction of nitroimidazoles.](image)

The single electron reduction potential (SERP) is very important to determine the potentiality of the different bioreductive pharmacophores for targeting hypoxia. The more positive the SERP value of a nitroimidazole, the better the chances of its reduction in cells having limited oxygen supply [14]. Table 1 shows the range of reduction potentials (relative to the standard hydrogen electrode) for 2-, 4- and 5-nitroimidazoles [15]. Although the exact value is modified by the introduction of substituents, 2-nitroimidazoles have the more adequate SERP values to differentiate hypoxic and normoxic tissue due to the reduction potential of enzymes in aerobic cells [16].
Table 1. Single electron reduction potentials (SERP) relative to the standard hydrogen electrode (extracted from [15]).

<table>
<thead>
<tr>
<th>Compound</th>
<th>SERP (in mV)</th>
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</thead>
<tbody>
<tr>
<td>2-nitroimidazoles</td>
<td>−235 to −447</td>
</tr>
<tr>
<td>4-nitroimidazoles</td>
<td>−375 to −554</td>
</tr>
<tr>
<td>5-nitroimidazoles</td>
<td>−354 to −447</td>
</tr>
</tbody>
</table>

However, penetration to the cell is governed by diffusion and consequently the lipophilicity of the molecule also plays a key role in the uptake of the potential radiopharmaceuticals. Furthermore, depuration from non-target tissue is also crucial for a radiopharmaceutical and is highly dependent of physicochemical properties of the labelled compound like lipophilicity, stability and protein binding. For these reasons 2-, 4- and 5-nitroimidazoles are worthy of being investigated as potential pharmacophores for the development of $[^{99m}\text{Tc}]$Tc-radiopharmaceuticals.

4. Labelling Strategies of Nitroimidazoles with $[^{99m}\text{Tc}]$Tc

Technetium is an element with a versatile chemistry, which has been exploited for the labelling of biomolecules. Traditionally, direct labelling through reduction of pertechnetate with stannous chloride in acidic condition was the general strategy used in the development of first-generation radiopharmaceuticals. The goal of radiolabelled compounds obtained by this method was just to follow their path within the body, and elucidation of the exact molecular structure of the carrier was not considered important. Furthermore, the structure of most first-generation $[^{99m}\text{Tc}]$Tc radiopharmaceuticals has not been elucidated and some very common radiopharmaceuticals, like $[^{99m}\text{Tc}]$Tc-bis-phosphonates are in fact a mixture of unidentified compounds [17]. Another well-known radiopharmaceutical, $[^{99m}\text{Tc}]$-diethylenetriaminepentaacetic acid ($[^{99m}\text{Tc}]$-Tc-DTPA), has never been fully characterized and there is experimental evidence indicating that a mixture of Tc III, IV, or V compounds are formed according to the specific set of reaction conditions [18]. However, when dealing with radiopharmaceuticals intended for targeting a biochemical process lack of information about the chemical structure and potential interconversion among different species “in vivo” are considered important drawbacks. In spite of this consideration a few examples of direct labelling applied to the development of radiolabelled nitroimidazoles can be found in the recent literature. Qi et al. [19] described the direct labelling of a metronidazole derivative (5-nitroimidazol) bearing a molecule of ethylenediaminotetraacetic acid (EDTA–MN; Figure 2) by reduction of pertechnetate with stannous chloride and its evaluation in nude mice bearing non-small cell lung cancer. The labelling requires a high concentration of ligand and reductant and is quite inefficient, yielding an acceptable radiochemical purity (RCP) only after HPLC purification. Structure of the technetium complex does not appear in the publication and no evidence of relationship between hypoxia and tumour uptake is presented.

![Figure 2](image-url)
Another example of labelling by direct reduction of pertechnetate is proposed by Ruan et al. [20] for the labelling of four different 2-nitroimidazole derivatives bearing an isocyanide group (Figure 3). The authors propose that, like $[^{99m}\text{Tc}]\text{Tc-MIBI}$, the structure of these complexes would be a monovalent cation with Tc in oxidation state I surrounded by six molecules of ligands coordinated through the isonitrile carbon. Structure was corroborated through the stable rhenium analogues using NMR and mass spectroscopy. The complexes were all hydrophilic, with values depending on the number of carbon atoms in the spacer chain. They all exhibited good hypoxia selectivity in cell culture (S180 cells). Biodistribution in mice bearing induced tumour showed moderate uptake, superior to other 2-nitroimidazolic complexes bearing an isocyanide group as electron donors for Tc [21] and adequate tumour/muscle ratios.

![Figure 3. Molecular structure of isocyanide derivatives of 2-nitroimidazol 2a,2b,2c,2d [20].](image)

When pertechnetate is reduced in the presence of adequate ligands it frequently loses only part of the oxygen atoms leading to monoxo ($[^{99m}\text{Tc}]\text{TcO}^+\text{O}_3$) or dioxo species ($[^{99m}\text{Tc}]\text{TcO}_2^{1+}$) of Tc(V) [22]. Most technetium radiopharmaceuticals are actually monoxo technetium complexes. The metallic centre is stabilized by tetradentate ligands containing NS or NO donor atom sets. These radiopharmaceuticals are easily obtained in high yields normally in a two-step procedure including the preparation of a suitable precursor such as glucoheptonate, citrate, cysteine, etc. The principal drawback of this concept is the lack of flexibility of the tetradentate ligands, which are usually difficult to synthesize and the presence of stereochemically active centres leading to different stereoisomers. The first $[^{99m}\text{Tc}]\text{Tc}$-labelled nitroimidazoles like BMS 181321 and BRU 59-21 derivatives were prepared using this strategy [22,23]. In the last years a number of novel $[^{99m}\text{Tc}]\text{Tc}$-labelled nitroimidazoles have been proposed (Figure 4).

Hsia et al. [24] studied the use of the tetradequate N4 chelator butylene amine oxime (BnAO) combined with 2-nitroimidazole (BnAO–NI) in the preparation of a hypoxia targeting $[^{99m}\text{Tc}]\text{Tc}$ compound. The exact structure of $[^{99m}\text{Tc}]\text{Tc}$ labelled BnAO complexes is unclear. Jia et al. [25] proposed that both five-coordinate monoxo technetium(V) species and six-coordinate trans dioxo technetium(V) species were formed upon labelling. Brauers et al. [26] suggested that the uptake in hypoxia might be related to the interconversion of these two forms. The Tc compound was obtained with high radiochemical purity (>96%), it remained stable for more than 24 h at room temperature. It is also lipophilic (log of partition coefficient between octanol and water ($P_{\text{o/w}}$) = 0.122). The accumulation in KHT cells under hypoxia was four fold higher than those under normoxic conditions indicating oxygen-dependent uptake. The distribution in KHT sarcoma bearing mice showed moderate tumour uptake at 2 h post-injection with tumour-to-blood and tumour-to-muscle ratios of 10 and four, respectively.

Huang et al. [27] studied the effect of a second redox centre in the behaviour of potential $[^{99m}\text{Tc}]\text{Tc}$ hypoxia targeting agents. A series of [Tc(V)O]$^{3+}$ complexes bearing one or two units of pharmacophore (2-nitroimidazol or 4-nitroimidazol) and a combination of 2-nitroimidazole and 4-nitroimidazol in the same molecule was prepared and evaluated “in vitro” in anoxic and normoxic conditions using murine sarcoma S180 cells. The selected donor atom set is the propylene amine oxime (PnAO). The four nitrogen atoms of the PnAO ring coordinate with the [Tc(V)O]$^{3+}$ core to form a neutral complex. Complexes bearing one or two units of 4-nitroimidazole showed no obvious anoxic/normoxic differentials while all complexes bearing 2-nitroimidazole as a bioreducible moiety displayed high anoxic accumulation.
The results also indicate that a second nitroimidazole redox centre with appropriate reduction potential might play an important role in the hypoxic accumulation. Based on these results, the compounds containing multi-redox centres are worthy of further investigations.

The same chelating unit (PnAO) was also employed in an interesting study by Zhang et al. [28] of $^{99m}$Tc complexes bearing two different redox centres in the same molecule, namely 3-nitro-1,2,4-triazole combined either with 2-nitroimidazole or 4-nitroimidazole. Comparing with the other PnAO complexes containing one or two redox centres previously reported by the same group, the number and type of redox centres were found to play an important role. Firstly, for the complexes with one redox centre, the highest to lowest hypoxic cellular uptake was 3-nitro-1,2,4-triazole > 2-nitroimidazole >> 4-nitroimidazole. Secondly, for the complexes with two redox centres, the effect of the second redox centre on hypoxic cellular uptake was 2-nitroimidazole > 3-nitro-1,2,4-triazole >> 4-nitroimidazole. Thirdly, difference in the octanol/water partition coefficient did not significantly influence cellular uptake but played an important role in the biodistribution studies since lower lipophilicity meant lower uptake in the blood, lower background, and higher tumour/blood ratio. The importance of the nature of the redox centre is also reinforced by these studies, where the complexes containing 2-nitroimidazole had higher tumour uptake than all the others.

The same group [29] also studied a series of $[^{99m}]$Tc(V)O$^{+3}$ complexes bearing one or two units of either 2-nitroimidazole or 4-nitroimidazole. The selected donor atom set is the monoamine-monoamide dithiol (MAMA) that forms neutral and lipophilic complexes with the $[^{99m}]$Tc(V)O$^{+3}$ core because three ionizable groups, two S–H and one N–H, lose hydrogen atoms upon complexation. This kind of chelators offer advantages compared with the PnAO system because it is more stable and less lipophilic and the complexes show less hepatobiliary background. Biodistribution studies were performed in mice bearing murine sarcoma S180 comparing 2-nitroimidazole with 4-nitroimidazole derivatives. Results demonstrate that 2-nitroimidazole derivatives exhibited higher tumour uptake than the corresponding 4-nitroimidazole derivatives in accordance to the SERP values of the two bioreducible pharmacophores. Another interesting feature of this study is the investigation of the effect of the incorporation of two units of bioreducible pharmacophore per molecule of $^{99m}$Tc-complex. MAMA bisnitroimidazole complexes showed better biodistribution characteristics (lower background activity in liver and fast clearance from blood) compared with MAMA–mononitroimidazole complexes leading to higher tumour/blood and tumour/muscle ratios. These features make them more appropriate for tumour targeting and imaging. Comparing also the labelling strategies the conclusion is that MAMA complexes show better biodistribution than PnAO derivatives probably due to the lower lipophilicity.

A further development by the same research group [30] is the application of a pretargeting strategy using the strain-promoted cyclooctyne-azide cycloaddition (SPAAC) between azadibenzocyclooctyne conjugated with monoamine monoamide dithiol and 2-nitroimidazole bearing a terminal azide group. The labelling reaction was evaluated “in vitro” and demonstrated adequate kinetics. Compared with the control experiments, this strategy demonstrated superior tumour/muscle and tumour/blood ratios. Another labelling strategy using $[^{99m}]$Tc(V)O$^{+3}$ complexes is selected by Li et al. [31] using hydroxyiminoamide as chelators for technetium. Hydroxamides (or N-hydroxy-carboximidamide) have been proposed by Nakayama et al. [32] who concluded that these type of molecules could form highly “in vivo” and “in vitro” stable complexes with $^{99m}$Tc and might be useful for the design of radiopharmaceuticals. The authors proposed that the chelator is bidentate, coordinates through N and O and forms two isomers upon complexation but no structural elucidation is presented. The pharmacophore is in this case 4-nitroimidazole. The biological evaluation of this complex included the “in vitro”/“vivo” stability, uptake in CHO cells and single photon emission computerized tomography (SPECT) imaging in mouse tumour models induced by inoculation of A549 Cells. $^{99m}$Tc-N4IPA accumulated significantly more in cells in hypoxic conditions than in normoxic conditions. The specificity of $^{99m}$Tc-N4IPA for hypoxia “in vivo” was confirmed by the close match between “in vivo” autoradiography and immunohistochemical analysis. Imaging studies showed clear delineation of tumours, with high tumour/blood and tumour/muscle ratios. Pharmacokinetics was more
favourable than those of $^{99m}$Tc-BMS181321 and $[^{99m}\text{Tc}]\text{Tc-BRU59-21}$, a result that was attributed to the lower lipophilicity.

Joyard et al. [33] proposed a cysteine–glycine–lysine sequence to label the 2-nitroimidazole moiety. Complexation yields a syn and anti-diastereoisomers mixture whose structure was elucidated using the analogous rhenium compounds. The ligand acts as tetradentate N$_3$S donor with the metal undergoing a dative bond with the amine function instead of a bond with the corresponding amide ion. The study includes also “in vitro” uptake studies in T98 glioma cells, biodistribution in animals bearing induced tumours using the same type of cells and SPECT images. Specific uptake in hypoxic tumour cells was observed and the tumour/muscle ratio in the animal model was favourable. The scintigraphic images showed specific tracer uptake in hypoxic areas.

Li et al. [34] designed a xanthate ligand bearing a 5-nitroimidazole pharmacophore derived from metronidazole. The structural studies using stable rhenium confirm that the coordination occurs through the formation of a $[\text{Tc(V)O}]^{3+}$ complex bearing two molecules of ligand per complex molecule. The sulphur atoms are bound to the metallic centre and the resulting complex bears a positive charge. The compound is therefore hydrophilic with a log $P_{\text{o/w}} = -1.47 \pm 0.01$. “In vitro” cell studies showed preferential uptake in hypoxic conditions and biodistribution demonstrated high tumour accumulation and very favourable tumour/blood and tumour/muscle ratios.
Zhang et al. [35] also used 5-nitroimidazol as a redox centre but conjugated the pharmacophore with asparagine through the nitrogen of the amide group. The resulting ligand has two nitrogen and one oxygen atom as potential electron donors for coordination to $^{99}$mTc. Although structural studies were not performed the authors proposed the formation of a $[\text{Tc(V)O}]^{3+}$ complex. The net charge is unclear but it is presumably positive since the compound is hydrophilic with a determined log $P_{\text{o/w}} = -0.72 \pm 0.05$. The authors also synthesized tricarbonyl complexes bearing 2- and 5-nitroimidazole moieties and performed the same experiments for better comparison. They evaluated physicochemical parameters, cell uptake in normoxia and hypoxia using lung cancer A549 cells and biodistribution in mice bearing xenografted tumours induced by the same cells. They concluded that $^{99}$mTc-5-ntm–asp exhibited relatively high stability “in vitro” and “in vivo”, as well as a favourable hypoxic/aerobic ratio, though not better than that of the tricarbonyl complex of 5 nitromidazole. $[^{99}\text{mTc}]^{99}$mTc-5-ntm–asp had better pharmacokinetics that could be attributed to its lower lipophilicity.

More recently a labelling approach was introduced based on the use of substitution-labile precursors in which part of the coordination positions are occupied by a set of ligands that is tightly bound to the metal centre generating a significant stabilization of the oxidation state [17]. The rest of the positions are occupied by weakly bound ligands that can be easily replaced by other ligands having an adequate set of donor atoms. One of the more successful examples of this concept is the use of the $[^{99}\text{mTc}]\text{fac}[\text{Tc(H}_2\text{O})_3(\text{CO})_3]^{3+}$ complex as precursor for the labelling of small biomolecules [36]. The CO is tightly bound and stabilizes low oxidation states while the three water molecules are weakly bound and can be substituted by a great variety of ligands. Figure 5 shows the structure of the imidazole derivatives that were labelled with $[^{99}\text{mTc}]^{99}$mTc using this approach.

Mallia et al. [37] applied this labelling method to the preparation of 2-nitro, 4-nitro and 5-nitroimidazole derivatives. The selected chelator was the iminodiacetic acid (IDA) which acts as tridentate coordinating through the amino and the carboxylic groups. The compound is negatively charged since two hydrogen atoms are lost upon complexation. Labelling was very efficient.
(RCP > 95%) for the three compounds. The lipophilicity was in the same order of magnitude (log $P_{o/w}$ = 0.48, 0.43 and 0.39). Evaluation in mice bearing tumours developed by inoculation of HSDMC1 murine fibrosarcoma cells showed moderate to low uptake. Variation in accumulation in tumour was in accordance with the single electron reduction potentials of the respective nitroimidazole. The 2-nitroimidazole complex with the more positive SERP showed the highest uptake followed by 5-nitroimidazole and 4-nitroimidazole. However, slow clearance of 2-nitroimidazole complex from the liver is an important drawback for potential clinical applications. In an attempt to accelerate the liver clearance the incorporation of an ether group in the spacer chain between the pharmacophore and the IDA chelator was proposed [38]. This variation slightly improved the tumour uptake and accelerated the liver clearance but the higher lipophilicity led to slower clearance from blood and muscle and consequently lower tumour/blood and tumour/muscle ratios.

The same group extended their study preparing and evaluating nine nitroimidazole derivatives (2-, 4- and 5-nitroimidazole) to obtain a group of tricarbonilic technetium complexes with variations in the SERP, overall charge and lipophilicity considering that these are the three fundamental properties responsible for their outcome as potential hypoxia targeting agents [39]. The studied complexes belong to three groups bearing different tridentate ligands, namely IDA (iminodiacetic acid), diethylenetriamine (DETA) and aminoethylglycine (AEG), linked to the N1-nitrogen atom of the imidazole ring through a propyl spacer. Tricarbonilic complexes with IDA have an overall negative charge, the ones with DETA have overall positive charge while the corresponding with AEG are neutral. In relation to the lipophilicity, the DETA and AEG $[^{99m}\text{Tc}]\text{Tc(CO)}_3$ complexes were less lipophilic (lower log $P_{o/w}$ value) than nitroimidazole–IDA–$[^{99m}\text{Tc}]\text{Tc(CO)}_3$ complexes and these values correlated with their blood clearance pattern. All compounds showed poor tumour uptake and retention when compared with $[^{18}\text{F}]\text{FMISO}$. However, the authors extracted interesting conclusions: fast clearance of radiotracer from blood had a significant impact on its uptake in tumour. DETA and AEG technetium tricarbonyl complexes that cleared faster from blood showed lower tumour uptake than the corresponding IDA–$[^{99m}\text{Tc}]\text{Tc(CO)}_3$-complexes. Consequently, the chemical structure of the chelator provides a useful tool for the manipulation of blood clearance of nitroimidazole radiotracers. By incorporating appropriate hydrophilic or lipophilic groups to the different chelators blood clearance can be accelerated or slowed down with consequences in the tumour uptake and retention. A clear example of application of this concept is the use 2-nitroimidazole–dipicolylamine (DPA) as a tridentate ligand [40]. This ligand is designed by modifying the nitroimidazole–DETA skeleton with eight extra carbon atoms to impart additional lipophilicity to the molecule. As expected the complex exhibited slower blood clearance, higher uptake and longer residence time in the tumour.

Although 2-nitroimidazoles have been preferred as redox centres in Tc complexes targeting hypoxia, 5-nitroimidazole derived from metronidazole have also demonstrated high affinity for hypoxic tumours “in vitro” and “in vivo” and have been labelled by the formation of $[^{99m}\text{Tc}]\text{Tc–(CO)}_3$ complexes. Giglio et al. [41] have prepared a cysteine-derivative that bears an NSO type-chelator unit in which the donor atom system consists of an N-primary amine, an S-thioether and an O-carboxylate. The Tc complex was neutral but the log $P_{o/w}$ was −0.75 since the incorporation of a cysteine moiety reduced the lipophilicity. Although cell studies suggested a preferential uptake by hypoxic tissue, biodistribution in mice bearing hypoxic tumours (3LL Lewis murine lung carcinoma cell line) showed low uptake probably because of hydrophilicity. However, the tumour/muscle ratio was favourable thanks to deprecation from soft tissues.

Fernández et al. [42] proposed the evaluation of the influence of ligand denticity in the behaviour of $[^{99m}\text{Tc}](\text{tricarbonyl})$ complexes bearing a 5-nitroimidazole pharmacophore. Two ligands, a bidentate one containing an imidazolic nitrogen, from a 1,2,3-triazole system, and an aliphatic amine nitrogen as electron donor atoms for Tc coordination and another having three coordinating groups, an imidazolic nitrogen, an aliphatic amine nitrogen and a carboxylate oxygen from a carboxylic acid were used to prepare the corresponding technetium complexes. The more relevant conclusion from this study is that ligand denticity significantly affects the stability and protein binding of the resulting complexes.
While the complex bearing the tridentate ligand was stable, and had a good and fast clearance from all organs and tissues, which correlates with a low protein binding, the bidentate-coordinated complex showed high protein binding and revealed a poor biological behaviour characterized by high retention of activity in blood, liver, kidneys and muscle. Performance in the tumour correlated with the above described results and tridentate coordinated complex had much higher uptake and retention than the bidentate one.

The preparation of nitrido complexes is considered another important Tc labelling strategy for the design of hypoxia imaging agents. The [Tc(V)N]^2+ core, initially developed by Baldas and Bonnyman [43] is well known for its intrinsic structural robustness. The co-ordination sphere of the metal is either built up by two molecules of bidentate σ-donor ligands (symmetric complexes) or a combination of “pseudotridentate” σ-donors π-acceptors diphosphinoamines (PNP) and bidentate σ-donor ligands (X–Y) (asymmetric complexes). Examples of the preparation of both kinds of nitrido complexes bearing nitroimidazoles appeared in the literature during the last few years (Figure 6).

**Figure 6.** Molecular structure of ligands labelled with technetium using the nitrido core [44–46].

Giglio et al. [44] and Lin et al. [45] presented symmetric technetium nitrido complexes bearing a metronidazole derived 5-nitroimidazole as pharmacophore. In the first case, the bidentate σ donor is a dithiocarbamate group while in the second it is a xanthate. The dithiocarbamate donor yielded neutral and lipophilic complexes and the xanthate donor a complex with overall negative charge and hydrophilic character. Tumour uptake was moderate in the first case and very low in the second case. The xanthate donor was also used for the preparation of a [Tc(V)O]^3+ complex which had much better performance in tumour.

Mathur et al. [46] presented the only example of an asymmetric nitrido complex for targeting hypoxia appearing in the literature after 2010. The ligand is not a nitroimidazole but a nitrotiazole derived from sanazole and having a SERP comparable to that of misonidazole. The coordination sphere was completed with σ-donors π-acceptors diphosphinoamine bis[(diethoxypropyl)phosphanyl]ethylthoxethylamine (PNP ligand). The Tc complex was lipophilic but the presence of the PNP ligand resulted in fast clearance from background organs, especially liver. However, fast clearance from blood, which was not anticipated, resulted in low uptake in the tumour.

Another interesting labelling strategy is the preparation of 4+1 Tc(III) mixed ligand complexes, formed by combination of the tetradentate tripodal NS₃ ligand 2-[bis-(2-mercaptoethyl)] aminoethanethiol. and a monodentate isocyanide (Figure 7). The non-polar isocyanide building block is stable against ligand exchange “in vivo” and provides oxo-free [⁹⁹ᵐTc]Tc complexes with the metal at oxidation state +3, with high “in vitro” stability both in aqueous solution and in plasma [47]. The bioactive function is usually incorporated in the monodentate ligand.

**Figure 7.** Molecular structure of isocyanide ligands used for labelling with technetium through the formation of 4+1 Tc(III) complexes [48,49].
Giglio et al. [48] proposed the application of this strategy to the $^{99m}$Tc labelling of a 5-nitroimidazole obtained by derivatization of metronidazole. The conjugation to the isocyanide group needed for complexation to technetium was achieved through the formation of an amide bond with two different amino derivatives of metronidazole. The amide linker was selected due to its well-known stability in biological milieu. Preferential cell uptake (using human colon adenocarcinoma HCT-15 cells) in hypoxic conditions was observed for both radiotracers. One of the compounds bearing a piperazine group in the linker exhibited higher lipophilicity and protein binding which resulted in very high liver activity. The other compound demonstrated a very favourable “in vivo” profile both in normal mice and in mice bearing induced tumours (by inoculation of 3LL Lewis murine lung carcinoma) with selective uptake and retention in tumour together with favourable tumour/muscle ratio.

Vats et al. [49] applied the same labelling strategy using isocyanide bearing 2- and 5-nitroimidazoles. Both complexes showed moderate uptake and retention in tumour (fibrosarcoma) and favourable tumour/muscle ratios with very similar absolute values for 2- and 5-nitroimidazoles.

5. Discussion

From the above described references we can extract a series of interesting conclusions. The first aspect is related to the position of the nitro group in the nitroimidazolic ring. Although the values of the SERP indicate that 2-nitroimidazoles are expected to have the best hypoxia targeting potential followed by 5-nitroimidazoles and 4-nitroimidazoles, some research groups prepared and evaluated the uptake in hypoxia of identical technetium complexes bearing 2-, 4- and 5-nitroimidazoles. The conclusion in most of the studies was the same: 4-nitroimidazoles are not selectively trapped in hypoxic cells “in vitro” while 2- and 5-nitroimidazoles show preferential uptake in hypoxia versus normoxia. The absolute percentage of uptake of both types of compounds seems to be independent of the position of the nitro group.

Some of the researchers also studied the effect of the incorporation of a second unit of bioreducible pharmacophore in the structure of the complex [28,29]. Table 2 shows the results. The authors conclude that the presence of a second redox centre enhanced the selective uptake in hypoxic cells “in vitro” and also the uptake in tumour “in vivo”. However, this improvement in uptake is not necessarily reflected in better tumour/blood and tumour/muscle ratios since the lipophilicity is also changed and affects the overall biodistribution of the compounds.

Table 2. Comparison of “in vitro” and “in vivo” uptake of $^{99m}$Tc complexes bearing one or two redox pharmacophores per molecule. (Biodistribution values are expressed in %D/g ± SD ($n = 5$)).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Log $P_{ow}$</th>
<th>Hypoxia/Oxia Ratio “In Vitro”</th>
<th>Tumour/Blood</th>
<th>Tumour/Muscle</th>
<th>Animal Model</th>
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<td>1.59</td>
<td>4.3 (S180 cells)</td>
<td>0.62 ± 0.43 (4 h)</td>
<td>4.15 ± 1.17 (4 h)</td>
<td>mice-bearing H22 tumours</td>
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<tr>
<td>Compound 3</td>
<td>1.10</td>
<td>8.7 (S180 cells)</td>
<td>1.08 ± 0.59 (2 h)</td>
<td>2.25 ± 0.59 (2 h)</td>
<td>mice-bearing H22 tumours</td>
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<tr>
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<td>0.69 ± 0.01</td>
<td>-</td>
<td>0.51 ± 0.11 (2 h)</td>
<td>1.63 ± 0.24 (2 h)</td>
<td>Kunming mice bearing murine sarcoma tumour</td>
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<tr>
<td>Compound 5</td>
<td>0.28 ± 0.03</td>
<td>-</td>
<td>0.64 ± 0.16 (2 h)</td>
<td>2.73 ± 0.47 (2 h)</td>
<td>Kunming mice bearing murine sarcoma tumour</td>
</tr>
</tbody>
</table>

In relation to the labelling strategy, although the tendency is to explore the potentiality of the new Tc cores (Tc-tricarbonyl, Tc-nitrido, Tc(III) $^{4+}$ complexes), Tc(V)-monoxo complexes still play a significant role. Furthermore, $[^{43}$Tc(V)O$]_{3}$ complexes of MAMA–nitroimidazole display more favourable properties than $^{99m}$TcO (PnAO–1-(2-nitroimidazole) due to their lower lipophilicity and better
stability [29]. Figure 8 shows the structure (experimentally determined or proposed by authors) of the different technetium complexes analysed in this review.

![Chemical structures of technetium complexes](image)

**Figure 8.** Structure of the different technetium complexes (the numbers in bold corresponding to structures experimentally determined).

The Tc-tricarbonyl core offers a variety of combinations of potential donor groups. Table 3 shows the results of a series of 2-nitroimidazolic Tc tricarbonyl complexes bearing exactly the same pharmacophore and linker and different donor atoms sets (NOO, NNN, NNO, etc.) and their comparison to the gold standard $[^{18}\text{F}]$FMISO. The most remarkable feature in the series is the variation of the lipophilicity: a higher lipophilicity is usually related to a higher absolute value of tumour uptake. However, the best tumour/muscle ratios are achieved with the less lipophilic compounds. Another interesting observation is that although tumour/muscle ratios of most of the studied compounds are in
the same range as $[^{18}\text{F}]$FMISO, the absolute tumour uptake is significantly lower for all the studied compounds. The explanation of this fact is not obvious. $[^{18}\text{F}]$FMISO is hydrophilic and consequently the cell penetration should be more difficult. In addition, the SERP value should not be very different from the other 2-nitroimidazolic compounds since according to Adams et al. [50] if the different functional groups are separated by two or more carbon–carbon bonds from the nitroimidazole ring they have minimal effect on its SERP. This fact has also been corroborated by Mallia et al. for some of their studied compounds [39].

Table 3. Comparison of “in vitro” and “in vivo” uptake of 2-nitroimidazolic $[^{99}\text{mTc}]$Tc-tricarbonyl complexes bearing the same pharmacophore and linker and different donor atoms sets. (Biodistribution values are expressed in %D/g ± SD ($n = 3$) at 1 h in Swiss mice with fibrosarcoma).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Log $P_{o/w}$</th>
<th>UPP (%)</th>
<th>Tumour Uptake</th>
<th>Muscle Uptake</th>
<th>Blood Uptake</th>
<th>Tumour/Blood Ratio</th>
<th>Tumour/Muscle Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 11</td>
<td>0.48</td>
<td>0.71 ± 0.08</td>
<td>0.50 ± 0.03</td>
<td>1.82 ± 0.08</td>
<td>0.39 ± 0.01</td>
<td>1.40 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Compound 13</td>
<td>0.28</td>
<td>0.31 ± 0.04</td>
<td>0.13 ± 0.01</td>
<td>1.28 ± 0.15</td>
<td>0.24 ± 0.05</td>
<td>2.38 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>Compound 14</td>
<td>0.06</td>
<td>0.38 ± 0.03</td>
<td>0.10 ± 0.01</td>
<td>1.04 ± 0.11</td>
<td>0.37 ± 0.08</td>
<td>3.80 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Compound 15</td>
<td>0.38</td>
<td>1.01 ± 0.11</td>
<td>0.49 ± 0.06</td>
<td>1.11 ± 0.13</td>
<td>0.91 ± 0.04</td>
<td>2.14 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>FMISO</td>
<td>-</td>
<td>3.25 ± 0.08</td>
<td>1.32 ± 0.10</td>
<td>2.18 ± 0.15</td>
<td>1.37 ± 0.20</td>
<td>2.52 ± 0.30</td>
<td></td>
</tr>
</tbody>
</table>

In spite of the above considerations this phenomenon is also observed when comparing $[^{18}\text{F}]$FMISO with a series of 5-nitroimidazolic $[^{99}\text{mTc}]$Tc complexes (Table 4). In this case, the variation is not only in the donor atom set but also in the labelling strategy (Tc-tricarbonyl, nitrido and 4+1 mixed ligand complexes). The variation of lipophilicity is very big, ranging from quite lipophilic to very hydrophilic compounds. However, the uptake in tumour is always 3–10 fold lower that the one of $[^{18}\text{F}]$FMISO, even for a complex with approximately the same lipophilicity.

Table 4. Comparison of “in vitro” and “in vivo” uptake of a series of 5-nitroimidazolic $[^{99}\text{mTc}]$Tc complexes labelled using different strategies. (Biodistribution values are expressed in %D/g ± SD ($n = 3$) at 1 h).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Log $P_{o/w}$</th>
<th>UPP (%)</th>
<th>Tumour Uptake</th>
<th>Muscle Uptake</th>
<th>Blood Uptake</th>
<th>Tumour/Blood Ratio</th>
<th>Tumour/Muscle Ratio</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 16</td>
<td>−0.75</td>
<td>15</td>
<td>0.50 ± 0.10</td>
<td>0.30 ± 0.10</td>
<td>2.20 ± 0.10</td>
<td>0.20 ± 0.10</td>
<td>1.70 ± 0.20</td>
<td>3LL Lewis murine lung carcinoma</td>
</tr>
<tr>
<td>Compound 17</td>
<td>−0.44</td>
<td>13</td>
<td>0.22 ± 0.07</td>
<td>0.09 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>0.76 ± 0.10</td>
<td>2.40 ± 0.11</td>
<td>3LL Lewis murine lung carcinoma</td>
</tr>
<tr>
<td>Compound 18</td>
<td>0.39</td>
<td>-</td>
<td>0.47 ± 0.02</td>
<td>-</td>
<td>0.44 ± 0.02</td>
<td>1.10 ± 0.07</td>
<td>-</td>
<td>Swiss mice bearing fibrosarcoma tumour</td>
</tr>
<tr>
<td>Compound 19</td>
<td>0.15</td>
<td>-</td>
<td>0.33 ± 0.05</td>
<td>0.07 ± 0.01</td>
<td>0.38 ± 0.03</td>
<td>0.90 ± 0.06</td>
<td>4.70 ± 0.10</td>
<td>Swiss mice bearing fibrosarcoma tumour</td>
</tr>
<tr>
<td>Compound 20</td>
<td>−0.53</td>
<td>-</td>
<td>0.34 ± 0.04</td>
<td>0.24 ± 0.04</td>
<td>0.40 ± 0.08</td>
<td>0.90 ± 0.07</td>
<td>1.40 ± 0.08</td>
<td>Swiss mice bearing fibrosarcoma tumour</td>
</tr>
<tr>
<td>Compound 21</td>
<td>0.63</td>
<td>31</td>
<td>1.00 ± 0.09</td>
<td>-</td>
<td>-</td>
<td>0.30 ± 0.17</td>
<td>1.29 ± 0.10</td>
<td>3LL Lewis murine lung carcinoma</td>
</tr>
<tr>
<td>Compound 24</td>
<td>0.70</td>
<td>12</td>
<td>2.20 ± 0.05</td>
<td>0.90 ± 0.60</td>
<td>3.80 ± 1.00</td>
<td>0.60 ± 0.20</td>
<td>2.40 ± 0.90</td>
<td>3LL Lewis murine lung carcinoma</td>
</tr>
<tr>
<td>FMISO</td>
<td>−0.40</td>
<td>-</td>
<td>3.25 ± 0.08</td>
<td>1.32 ± 0.10</td>
<td>2.18 ± 0.15</td>
<td>1.37 ± 0.20</td>
<td>2.52 ± 0.70</td>
<td>3LL Lewis murine lung carcinoma</td>
</tr>
</tbody>
</table>

A possible explanation of the lack of agreement between in vitro and in vivo results can be attributed to the lack of control of the real degree of hypoxia within the tumour in the animal models.
While the cell experiments were conducted in strictly controlled conditions and hypoxia was achieved by artificial oxygen deprivation, in the in vivo experiments the authors claim that the solid tumours are hypoxic although no experimental evidence is provided except for references [31] and [48] where anatomopathology studies were performed. Even if this was true, the degree of hypoxia is not strictly reproducible in different animals even if the same tumour cells are used, since the extension of necrotic areas in the solid tumour may change between animals. This fact can negatively affect the tumour uptake even if the compound is selectively taken up by hypoxic cells. Another drawback of the animal experiments is the small number of replicates (between three and five) in all the studied cases.

6. Conclusions

In conclusion, in spite of the big research efforts and the variety of potential $[^{99m}\text{Tc}]\text{Tc}$ complexes for targeting hypoxia a clear substitute for $[^{18}\text{F}]\text{FMISO}$ has not been proposed yet. Although the different labelling approaches are useful for a rational design of compounds with predictable physicochemical and pharmacokinetic properties the factors influencing the absolute value of tumour uptake remain, in our opinion, unclear and further research is still necessary to achieve a compound with acceptable properties for “in vivo” imaging of hypoxia.

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

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