Chemistry considerations for the clinical translation of Oncology PET radiopharmaceuticals

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**ABSTRACT**Positron emission tomography (PET) has proven to be an invaluable tool in the staging and management of disease in oncology; however, [18F]fluorodeoxyglucose ([18F]FDG) remains the most widely used PET radiopharmaceutical despite large financial investment in novel radiotracer development. We report our perspective and experience of translating radiopharmaceuticals into clinical studies, discussing the PET development pipeline from a chemistry perspective. We hope that by identifying potential points of attrition along the pipeline and suggesting solutions to these problems, we may help others take their pre-clinical radiotracers into human studies. This review focuses primarily on the development of fluorine-18 radiopharmaceuticals, although the broader field of radiometal chemistry is considered where the translation journey is similar.

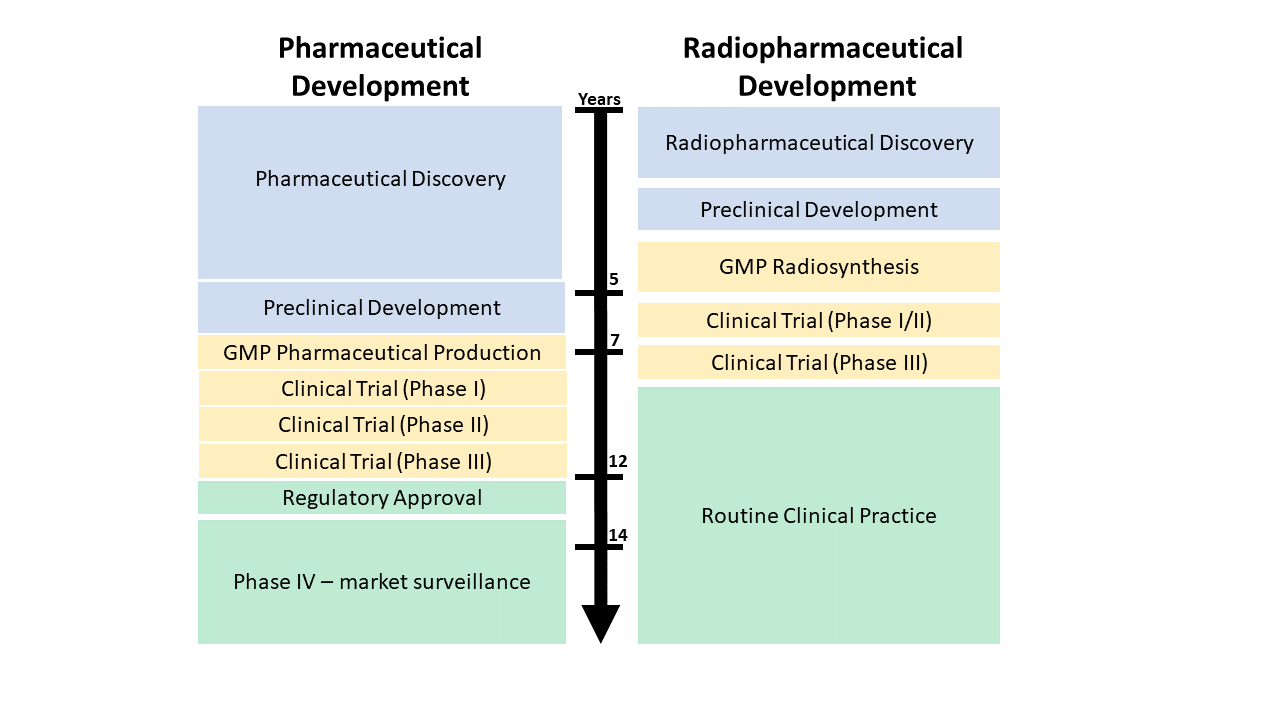
**1. Introduction**

Positron emission tomography (PET) is a powerful molecular imaging modality used in the clinic for the diagnosis, staging and management of many tumour types in oncology.1,2 PET provides functional information on cellular biology by tracing a radiopharmaceutical injected into the patient. The sensitivity of the technique is unparalleled, quantifying the *in vivo* accumulation of radioactivity at nanomolar - picomolar (nmol - pmol) concentrations. Cancer is a heterogeneous disease, within the same lesion and between distant metastases; the gold-standard for assessing tumour pathology is immunohistochemistry which is extremely high resolution and informative, but unable to capture the true phenotype of a patients disease due to the technical limitation of acquiring biopsy tissue from primary and metastatic tumours, which is impractical and unethical when the patients tumour burden is large.3,4 The clinical arena needs novel validated predictive imaging biomarkers in oncology for patient stratification and monitoring treatment response, therefore new PET radiopharmaceuticals with optimal *in vivo* performance need to be developed.5 No single imaging agent is capable of answering all biological and clinical questions in relation to a patient’s disease; despite this, the most frequently used radiotracer in oncology is [18F]fluorodeoxyglucose ([18F]FDG), a radiolabelled analogue of glucose.6 The dysregulation of energy metabolism in tumours (Warburg effect) is a well understood hallmark of cancer, so there is little surprise that imaging glycolysis with [18F]FDG has proven to be a very useful diagnostic tool for the clinic.7,8 Despite the many advantages of [18F]FDG, the biology of tumours is complex and more specialised radiopharmaceuticals are required to answer questions other than tumour energy metabolism.7,9 Acquisition of accurate and reliable imaging data from which informed clinical decisions can be made, is dependent upon the chemical structure of the radiopharmaceutical, its biological properties and the rigour in which it has been validated. The onus of responsibility for the initial phase of development lies solely with pre-clinical scientists, of which chemists, masters of harnessing chemical structure to target natures biology, are fundamental to the endeavour. Academic and industrial research institutions around the world strive to develop new imaging agents that may improve patient outcome, and some have been evaluated in humans (Table 1).

**Table 1.** A list of PET radiopharmaceuticals and their biological targets evaluated in humans.

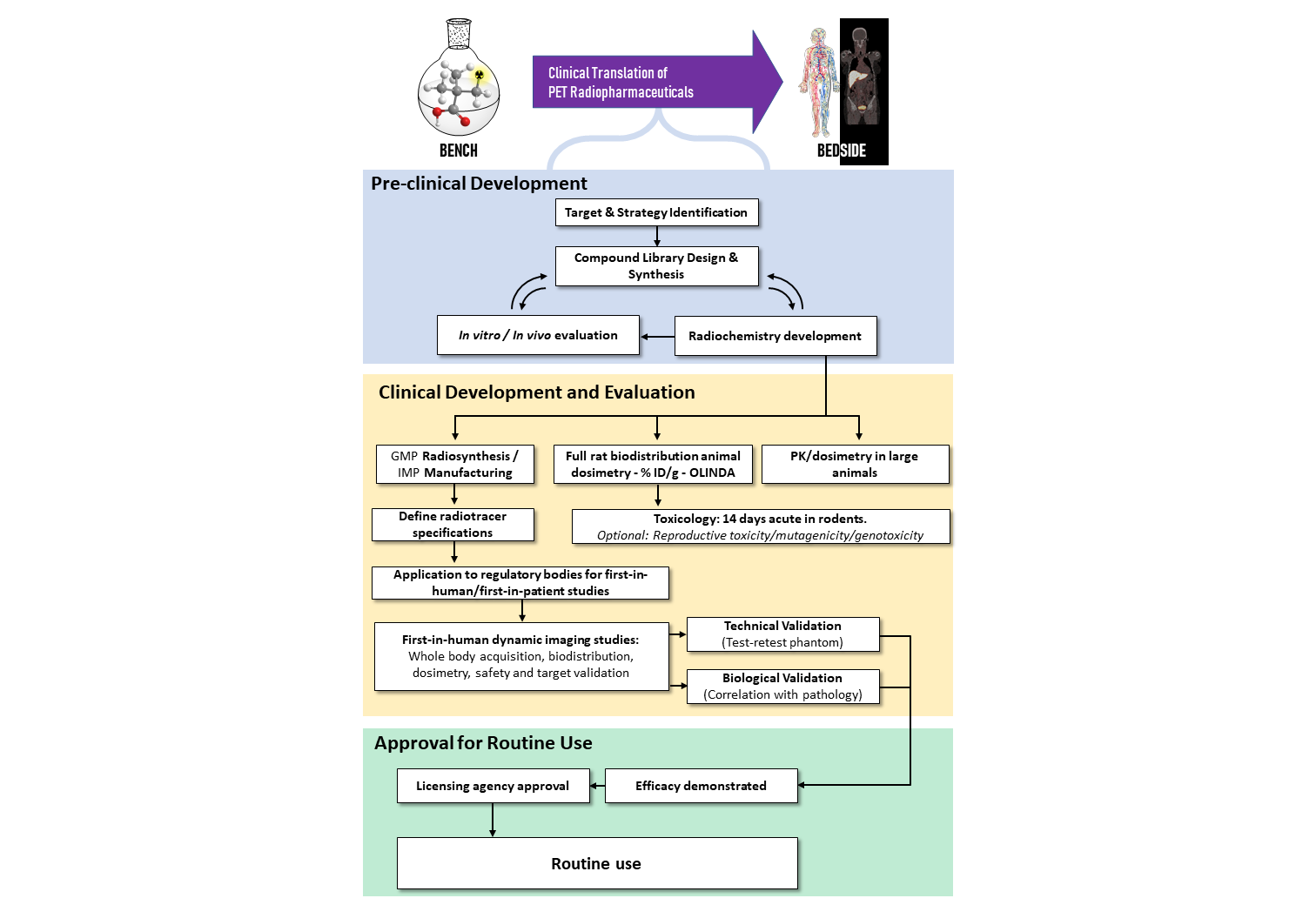
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| Radiopharmaceutical | | Radioisotope | Target | Trial Phasea | Refb |
| [18F]fluoromethyl-D4-choline ([18F]D4-FCH) | | 18F | Choline/Lipid-membrane metabolism | II | 10 |
| [18F]fluoropivalic acid (FPIA) | | 18F | Fatty acid metabolism | II | 11,12 |
| [18F]FLT | | 18F | Cellular proliferation | II/III | 13 |
| [18F]ICMT-11 | | 18F | Apoptosis (Caspase 3 / 7) | II | 14,15 |
| [18F]FET-βAG-TOCA | | 18F | Somatostatin receptor type 2 (SSTR2) | II | 16 |
| [18F]AlF-NOTA-Octreotide | | 18F | SSTR2 | II | 17 |
| [18F]FP-Gluc-TOCA | | 18F | SSTR2 | II | 18,19 |
| [18F]SiFA*lin­*-TATE | | 18F | SSTR2 | I/II | 20 |
| [68Ga]Ga-DOTA-TATE | | 68Ga | SSTR2 | II/III | 21 |
| [68Ga]Ga-DOTA-TOC | | 68Ga | SSTR2 | II/III | 22 |
| [18F]FAZA | | 18F | Hypoxia | II | 23 |
| [18F]FMISO | | 18F | Hypoxia | II | 24 |
| [18F]FES | | 18F | Estrogen receptor (ER) | II/III | 25 |
| [18F]FFNP | | 18F | Progesterone receptor (PR) | I/II | 26 |
| [18F]FDHT | | 18F | Androgen receptor (AR) | II | 27 |
| [18F]FDOPA | | 18F | Amino acid metabolism in neuro-oncology | II/III | 28 |
| [18F]Galacto-RGD | | 18F | αvβ3 integrin expression | II | 29 |
| [18F]Fluciclovine | | 18F | Energy-independent L-type amino acid transporter (LAT) | II/III | 30 |
| [18F]PSMA-1007 | | 18F | PSMA | II/III | 31 |
| [18F]DCFPyL | | 18F | PSMA | II/III | 32 |
| [68Ga]PSMA-11 | | 68Ga | PSMA | II/III | 33 |
| [18F]Tetrafluoroborate | | 18F | Sodium iodide symporter (NIS) | I/II | 34,35 |
| [18F]Fluoroethyl-L-tyrosine ([18F]FET) | | 18F | Amino acid transport in neuro-oncology | II | 36 |
| [68Ga]Ga-FAPI | | 68Ga | Fibroblast activation protein (FAP) | II | 37,38 |
| [18F]GE-226 | | 18F | Human epidermal growth factor receptor (HER2) | I/II | 39 |
| [68Ga]Ga-ABY-025 | | 68Ga | HER2 | I/II | 40 |
| [89Zr]Zr-DFO-Trastuzumab | | 89Zr | HER2 | I/II | 41 |
| [64Cu]Cu-ATSM | | 64Cu | Hypoxia | II | 42 |
| [11C]Choline | | 11C | Lipid-membrane metabolism | II/III | 43 |
| [124I]CLR1404 | | 124I | High-grade primary and metastatic brain tumours | I | 44 |
|  | aTrial phase(s) currently completed or in progress bReferences either include original clinical evaluation or a systematic review of the field | | | | | |

Despite efforts in the field to develop new radiopharmaceuticals (the Molecular Imaging and Contrast Agent Database (MICAD) lists 5359 PET agents - last updated in January 2013 - developed for oncology) the reality is that few make it into the clinic.45 This should not be the case given the advantages that radiopharmaceutical development has over traditional drug discovery in terms of a rapid progression to clinical trials.

PET radiopharmaceutical development shares similarities with drug discovery, but there are stark differences that allow a faster, safer and more cost-effective route for progressing radiolabelled molecules into human studies (Figure 1).46 One main difference is the administered dose of radioactive compounds, which is always below the pharmacologically active dose and must never elicit a biological response. As a result, PET radiopharmaceuticals only require basic toxicological assessment before progression into first-in-human studies (Phase 0/1), which significantly shortens development time and cost (ca. £100k per toxicology study).47 Side-effects are very uncommon and the concern of off-target toxicity (e.g hERG-related drug toxicity) is minimised; furthermore, physicochemical properties that are the fundamental in traditional drug discovery, such as oral bioavailability and therapeutic window, do not apply to the field of radiopharmaceutical development.   
  **Figure 1.** Comparison of pharmaceutical development and radiopharmaceutical development pipelines.

It can take 12 - 13 years to develop a new drug, progress it through clinical trials and gain regulatory approval to launch into the marketplace; PET radiopharmaceuticals can be developed, evaluated clinically and ready for routine use within 8 years at a much-reduced cost. Although PET radiopharmaceutical development is more straight-forward in terms of progressing candidates into clinical studies, investment of time, money and specialist resources are required (i.e. cyclotrons and radiochemistry laboratories). The infrastructure to support the routine use of PET radiopharmaceuticals is also a consideration in the development of novel imaging agents. Low cost of development is matched with low financial returns due to relatively few people requiring a PET scan in their lifetime (compared to the use and repeat dosing of pharmaceuticals). It may be that only radiopharmaceuticals produced in large quantities to serve a large population of patients will ever cover their development costs; this is dependent on the disease and treatment, and whether the stratification of patients by PET to receive an expensive pharmaceutical intervention results in an overall cost saving to the healthcare system.   
  
The aim of this review is to discuss the development pipeline of PET radiopharmaceuticals for oncology from its root in preclinical science. We predominantly focus on the development of 18F-radiopharmaceuticals which are arguably the workhorse of modern PET; although, we discuss the broader field of radiometal chemistry and the use of alternative, established and emerging PET radioisotopes, where the development and translation journey is similar. We provide a chemistry perspective on the radiopharmaceutical development pipeline, identifying potential problems (denoted as **⚠ *Risks***) that, in our opinion may hinder the progression of lead candidates into clinical evaluation, so that they may be anticipated and resolved without significant delay or impact to the project.

**2. Development of new radiopharmaceuticals for the clinic**

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**Figure 2.** An example of a typical route for developing and translating new radiopharmaceuticals into clinical trials and routine clinical practice.

The development of new PET radiopharmaceuticals requires close collaboration between different disciplines (e.g. chemists, biologists and clinicians) to progress probes into the clinic and may follow the path described in Figure 2. Many promising PET probes remain in preclinical development and never cross the boundary into clinical development and evaluation; this usually results from unfavourable *in vivo* pharmacokinetics (PK) including rapid metabolism which precludes translation into human studies. The attrition of PET radiopharmaceuticals may also be due to a lack of clinical interest at the host institution. It is important to establish strong clinical links early in a project, either at the host institution or with collaborators, identifying a clinical lead to progress human studies who shares similar research interests in the biological target.

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***Solution:*** *Seek collaboration with other institutions, either in academia or industry. Ensure that: 1) they have the necessary resources to perform the GMP radiosynthesis on-site; or 2) you can transport the radiopharmaceutical to support clinical studies. Make sure that a lack of clinical interest is not due to a poor choice of target, for example, developing a radiopharmaceutical to measure a biomarker that can be quickly and cheaply quantified using a simple blood test.   
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**2.1 Pre-clinical development**   
**2.1.1. Target & Strategy Identification:** PET requires the identification of a target that is present in either increased or decreased levels in diseased tissue. The biological target, when probed by an appropriate radiopharmaceutical, should answer a clinical question which is often associated with treatment planning. Clinical questions take the form of patient stratification, *will the patient benefit from receiving this treatment?* or treatment response, *is the patient responding to treatment?* Once a biological target has been selected, molecules can be identified for radiolabelling. It may be that radiopharmaceuticals have already been developed for the target of interest, which can provide a foundation of knowledge to decide if: 1) the target is worthy of further investigation; 2) the radiopharmaceutical currently developed is fit for purpose; 3) problems associated with the reported radiopharmaceutical, for example – cross-reactivity, metabolism, target specificity, specific activity – can be improved by initiating a new research project; 4) a better targeting approach is required, for example, a short peptide sequence with faster PK and short-lived PET isotopes (fluorine-18, t1/2=110 min) where previous studies have investigated a monoclonal antibodies (mAb) vector with slow PK and long-lived PET isotopes (zirconium-89, t1/2 = 78.4 h). There are numerous examples where this strategy has been employed and has resulted in new radiopharmaceuticals entering clinical trials; for instance, fluorine-18 radiolabelled octreotide peptides (e.g. [18F]FET-βAG-TOCA) to improve upon [68Ga]Ga-DOTA-TATE.16,48,49 If the biological target is novel or has not been studied in the context of PET imaging, then a “drug discovery” approach to identifying new targeting vectors from a library of compounds is required.

It is not an appropriate use of research funding to develop “me-too” radiopharmaceuticals without a clear aim of how the proposed study may lead to substantial improvements in imaging characteristics or the logistics of routine clinical use.

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***Solution:*** *Be ambitious with the design of compound libraries, radiopharmaceuticals are rarely perfect so develop innovative ways to solve current limitations (i.e. low tumour uptake, metabolism, route of clearance, low radiochemistry yields, challenging radiosynthesis, poor choice of radioisotope).   
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**2.1.2. Library Development.** The selection of an appropriate PET radionuclide is an essential consideration to be made early in the development phase. Fluorine-18 is considered an “ideal” PET radionuclide because of its favourable decay characteristics (t1/2 = 110 min, β+em = 97%), compatible with multistep radiosynthetic steps and transportation to satellite imaging facilities.50 The PET radiometal gallium-68 (t1/2 = 68 min, β+em = 89%) is often considered favourable for radiolabelling peptides *via* chelation chemistry (e.g. DOTA, NOTA).51 Carbon-11 (t1/2 = 20 min, β+em = 99%) is not ideal for developing radiopharmaceuticals for transportation and is therefore the reserve of institutions that have an on-site cyclotron, radiochemistry laboratory and scanning facilities; this review will not consider carbon-11 radiopharmaceutical development, but many of the same principles discussed are applicable. In addition, this review will not consider the clinical translation of radiolabelled antibodies and other proteins.

If a PET radiopharmaceutical for the desired biological target has not been developed, thenthe first step of the development process is to determine if the target can be bound by molecules (e.g. small “drug-like” molecules and peptides); for small molecules, it is usual to seek inspiration from medicinal chemistry and drug discovery where suitable pharmacophores with a high affinity for the target may have been identified. The budget for developing PET radiopharmaceuticals is far lower than pharmaceutical drug discovery programs (£2-5m vs. > £700m respectively) and therefore, screening large compound libraries is avoided. Evaluating existing work to determine pharmacophores and potential structural modifications to incorporate PET radioisotopes without deterioration of biological properties, significantly reduces the cost and size of libraries from which to select a lead candidate for evaluation (Figure 3). For simplicity, library development will be discussed for fluorine-18 radiopharmaceuticals, however the concept applies to all radioisotopes.

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**Figure 3.** Library development to identify A) small molecule PET probes and B) radiolabelled peptides for clinical translation; C) implementation of the proposed library development strategy for identifying the fluorine-18 radiolabelled somatostain receptor type-2 (SSTR2) targeted peptide [18F]FET-βAG-TOCA for first-in-human clinical trials; PET images: 53 year old female diagnosed with pancreatic NET and liver metastases; i) fused axial PET/CT showing liver metastases, ii) corresponding maximum intensity projection (MIP) image.16,48,49

In the first instance, structure-activity relationships (SARs) can be derived from published compound libraries in order to design and synthesise a focused library of compounds for evaluation as PET probes (Figure 3A). The focused library may be derived from one or two lead pharmacophores from the literature, however the purpose is not to expand the scope of compounds, but to probe the effect of incorporating a radionuclide into a molecule, particularly on binding affinity and target recognition/specificity. Libraries may be simplified by only including compounds for evaluation where a fluorine-18 radiolabelled derivative can be accessed through simple radiochemistry. Non-radioactive compounds may be evaluated *in vitro* to determine binding affinity and target selectivity, saving time and expense that would be required to synthesise precursors and develop radiochemistry for the full library. Promising candidates are then selected, and radiolabelling methodology developed.

Peptides selected from phage display libraries should also be investigated in a similar way to small molecule probes.52 A high affinity/specificity sequence is selected for PET radiopharmaceutical development and linker structure and radiolabelling strategy investigated to determine and modulate PK, binding affinity and metabolism to achieve optimal *in vivo* performance.48,49 This approach was exemplified in the development of a fluorine-18 radiolabelled octreotide targeting the somatostatin type 2 receptor (SSTR) expressed in neuroendocrine tumours (NETs) which was taken to the clinic (Figure 3C).16,48,49 A single core peptide structure was selected and elaborated with varying linker groups to investigate their influence on tumour uptake, overall biodistribution, PK and metabolic stability (discussed further in section 2.1.6). The use of linkers to modify overall PK is important to ensure the progression of a radiopharmaceutical that produces a favourable PET image with optimal tumour accumulation and signal-to-background ratio. The lead candidate [18F]fluoroethyltriazole-βAG-TOCA ([18F]FET-βAG-TOCA) was selected from the library based on optimal *in vivo* performance and translated into the clinic for evaluation.16 A comprehensive review of the development and translation of [18F]FET-βAG-TOCA is presented.53 It is often more convenient to evaluate radiolabelled peptides, particularly when the radiochemistry is simple (e.g. gallium-68 chelation). The use of straight forward iodine-124 radiochemistry to access radiolabel peptides for biological evaluation may also be considered with the view of adopting other radiolabelling strategies later in the project.54

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***Solution:*** *When developing a library, it is sometimes useful to simultaneously investigate potential radiolabelling strategies. This can be achieved by radiolabelling simple model compounds to get a handle on appropriate radiochemistry conditions, that can later be transferred to the desired lead compound.*

**⚠ *Risk:*** *Some peptides and linker strategies are susceptible to radiolysis, which can preclude the production of clinical doses of radiopharmaceutical.*

***Solution:*** *Use large activities where possible to investigate the influence of production scale on product stability. Include an reducing agent (e.g. ascorbic acid) when performing these radiosynthesies. Investigate a variety of linker strategies in the focused library (e.g. aliphatic chains, polyethylene-glycol PEG chains or varying length and amino acid linker groups) so that the most appropriate radioconjugate can be selected based on overall PK, metabolic and radiolytic stability.   
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**2.1.3 Radiochemistry development:** Significant advancements in PET radiochemistry methodology has resulted in an expansive toolbox of chemistries to access desired molecules.55 A summary of some noteworthy fluorine-18 radiochemistry is shown in Figure 4A.

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**Figure 4. A)** Examples of fluorine-18 PET radiochemistry; **B)** chelators for metal-based radiochemistry.55,56,65–72,57–64

A decade ago, radiochemists had relatively few fluorine-18 radiolabelling methods at their disposal when developing new radiopharmaceuticals; nucleophilic substitution (SN2) and nucleophilic aromatic substitution (SNAr) chemistry dominated the field of direct fluorination strategies.55,73–75 Although these are very effective and still used predominantly in PET radiochemistry today, there are some restrictions in where a fluorine atom can be substituted on an aromatic ring depending upon the electronic configuration of the molecule. SNAr radiochemistry requires the aromatic system to be activated towards nucleophilic attack from [18F]fluoride by the inclusion of electron withdrawing groups (EWG) *ortho* or *para* to good leaving groups (e.g. halogen, nitro or trimethylammonium moiety) which restricts the range of fluorinated compounds and substitution patterns that can be accessed using this method. These radiolabelling techniques require extensive protecting group strategies and for complex molecules, a modular approach to synthesis. Recently, new late-stage fluorination radiochemistry reactions which are tolerant to the large scope of functional groups encountered in “drug-like” molecules, allow the fluorination of positions in a molecule that were previously inaccessible.58,59,61–63,76,77 Advanced multiparametric analysis (design of experiments, DoE) optimises complex multicomponent fluorine-18 radiochemistry (e.g. copper-mediated 18F-fluorination chemistry) and offers advantages over the “one variable at a time” (OVAT) approach traditionally employed by radiochemists.78 DoE allows for faster and more efficient optimisation of radiolabelling reactions by identifying components crucial for high reaction efficiency and will undoubtedly contributed and expedite the translation of new radiopharmaceuticals that are produced using novel multicomponent fluorine-18 chemistry. Despite excellent research into new methods and further iterations of the radiochemistry to improve the accessibility of such methods to the PET radiochemistry community (simplification of precursor synthesis, handling of precursors under normal atmospheric conditions) these strategies have not yet been fully embraced in favour of traditional fluorination techniques. As these new chemistries are exemplified in the production of clinical radiopharmaceuticals, their widespread implementation is likely.

Radiochemistry methodology directs precursor synthesis which is often time consuming and challenging. Unforeseen incompatibility between functional groups in the desired molecule and the radiolabelling chemistry can hinder progression of a project. De-risking strategies can identify incompatibilities at an early stage in the development phase so that alternative strategies may be implemented. Radiochemistry reactions may be de-risked by studying the influence of fragment molecules (which contain functional groups present in the desired radiotracer) in a radiolabelling reaction that is known to work efficiently (Figure 5).79 If the desired molecule is relatively simple, it may be that incomplete fragments of the final radiotracer can be radiolabelled to glean similar information.

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**Figure 5.** Schematic representation of a de-risking strategy to identify functional groups that may potentially hinder radiolabelling.

To further mitigate the risk of precursor development and the associated costs, where appropriate, the direct radiolabelling of pharmaceuticals with fluorine-18 has been demonstrated;80–82 for example, through direct CH activation *via* Mn-salen complex catalysed oxidative benzylic 18F-fluorination.62,63 This approach permits easy access to radiolabelled drug candidates but has the limitations of variably modifying the ADMET properties of the molecule and is perhaps less GMP compatible than traditional approaches.

Library and radiochemistry development should be approached together and operate synergistically. The selection of suitable radiolabelling methods ought to be considered early in the development phase and should directly inform the library design; *is there radiochemistry available to allow the incorporation of fluorine-18 in this position on the molecule?*

Radiolabelling methods to access radiopharmaceuticals for clinical investigation should meet the following criteria:

* Rapid radiosynthesis in a good radiochemical yield (minimum RCY >10%) to support the transportation of multipatient doses to imaging facilities.
* High radiochemical purity (RCP >95%) and formulation stability beyond the time required to deliver, administer the radiopharmaceutical to a patient and complete the scan.
* High specific activity (GBq/μg) / molar activity (GBq/μmol) to ensure that the patient dose contains minimal non-radioactive compound below the levels that elicit a pharmacological response and to conform to the approved specification. This is particularly important for radioligands targeting receptors.
* Avoid the use or reagents and solvents that are not compatible with GMP radiosynthesis, according to the European Pharmacopoeia (Ph. Eur.)
* Amenable to technology transfer onto commercially available automated radiosynthesis platforms (e.g. GE FASTLab, Trasis AIO)

Radiopharmaceuticals using PET radiometals (e.g. gallium-68, zirconium-89) follows a similar development pipeline to fluorine-18 radiopharmaceuticals, despite differences in radiochemistry methodology. A summary of chelators used in metal-based radiochemistry is shown in Figure 4B. Radiometals are attractive because of their simple radiochemistry and the ease in which desirable radionuclides can be accessed, like generator-produced gallium-68, which is perfect for producing PET imaging agents in a hospital radiopharmacy.83,84 There are concerns about the scalability of gallium-68 radiopharmaceuticals where demand for PET imaging is high and therefore, the aluminium-[18F]fluoride ([18F]AlF) radiolabelling method may overcome the limitations of gallium-68;85 [18F]AlF combines the favourable decay characteristics of fluorine-18 with the simplicity of metal-based radiochemistry and is growing in popularity for the development of new PET imaging probes.86–89 Mild fluorination techniques where Si-F, B-F, Ga-F and S-F bonds are formed have been reported.68,90,91 Alternative radioisotopes for investigation as PET radiometals include scandium-44, which has a suitable half-life (t1/2 = 4 h) for radiosynthesis and national distribution to scanning facilities, as well as a high positron emission (β+em = 94%) and compatibility with existing chelators (e.g. DOTA). The favourable properties of scandium-44 compared to gallium-68 are potentially interesting for future clinical studies and routine practice when the production of scandium-44 is scalable.92,93

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**⚠ *Risk:*** *Will the production scale and availability of gallium-68 be sufficient to cope with the demand for this radiopharmaceutical if it enters routine clinical practice?*

***Solution:*** *This is not necessarily a concern for investigating radiopharmaceuticals preclinically, in early clinical trials or if clinical demand is expected to be low; however, it may be prudent to investigate alternative radioisotopes (e.g. fluorine-18) and radiolabelling strategies to develop second-generation radiopharmaceuticals if clinical studies are successful.*

**⚠ *Risk:*** *Can the necessary radiochemistry precursors be synthesised under GMP conditions if required for the clinic?*

***Solution:*** *Establishing contact with a custom synthesis company may be able to provide the answer and a route for accessing GMP grade precursor for clinical trials; this may be one of the most challenging aspects in taking a radiopharmaceutical into the clinic.   
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**2.1.4. Automated Radiochemistry:** Automated radiochemistry is key for the safe and GMP compliant production of fluorine-18 radiopharmaceuticals, therefore it is worthwhile developing a robust automated procedure for pre-clinical studies which can be adapted into a GMP compliant production.89,94–98 Automation protects production radiochemists from exposure to large doses of radioactivity as commercially available automated radiosynthesis modules are small and easily shielded inside a hot cell. Automation also allows for a reproducible synthetic method that can be initiated by any trained person with well written standard operating procedure (SOP). Different manufacturers of automated platforms result in slight differences in design; largely, platforms are cassette-based whereby all reagents and consumables (i.e. solid-phase extraction [SPE] cartridges, reaction vessels) are contained within a disposable cassette. Cassette-based systems are favourable as the single-use cassette can be disposed of after the production, avoiding a validated cleaning procedure after the radiosynthesis. Furthermore, cassette-based systems promote the national and international distribution of new radiopharmaceuticals through the commercialisation of cassette kits which contain the necessary precursors, reagents and automated sequence to produce the desired radiopharmaceutical on demand.99,100 Non-standardised radiochemistry infrastructure such as the use of different manufacturer’s automated radiosynthesis modules across clinical trial sites can be a potential barrier to multisite clinical trials; radiosynthesis methods, purification and analysis need to be developed and optimised for each type of instrument as there is no direct compatibility between modules.

It is not a necessity to fully automate the GMP radiosynthesis of gallium-68 radiopharmaceuticals, as production is often performed in the hospital radiopharmacy using low activities (<2 GBq) in kit-based radiosynthesies;101,102 certain production processes may be automated to reduce the radiation dose received by the radiochemist, for example, the automated elution of a 68Ga-generator or an SPE purification. Platform suppliers are moving towards bespoke automated modules for metal-based radiochemistry as the popularity and demand for metal-based radiopharmaceuticals increases (i.e. gallium-68, zirconium-89, lutetium-177).103 As cyclotron-produced gallium-68 demand increases in the future, and larger starting activities of the isotope are produced, automation will become imperative.103,104

There are challenges associated with automating radiochemistry which become more numerous with increasing number of radiosynthetic steps. Solvent volumes are one of the most challenging aspects as these platforms are not well suited to handling small volumes of liquid, which can often lead to incomplete, inconsistent movement and addition of reagents around a system where a high reagent concentration is requried. Furthermore, reagent vials often hold a “dead volume” which needs to be considered when automating a radiosynthesis. Solid reagents are incompatible with automated platforms which requires all reagents to solubilised which may present problems with reagent stability over the course of an automated procedure (e.g. very hygroscopic reagents). Methods to address this have been reported, including the exploration of solid-supported reagents packed into cartridges for use with automated platforms.105 Lipophilic small molecules and proteins can be retained on the plastics of the cassette which reduces reaction efficiency and can contaminate later reaction steps and purified samples; this may be addressed by treating plastics with serum albumin (BSA for preclinical, HSA for clinical production).

The development of microfluidic devices where radiochemistry, purification and formulation can be performed in flow and with small reaction volumes, may solve some of the challenges faced by traditional automated radiosynthesis platforms; for example, by scaling up radiotracer production or allowing more complex radiosynthesis protocols by careful manipulation of small reaction volumes. Compact radiosynthesis devices are easy to shield, relatively inexpensive to produce and can improve the efficiency of radiolabelling reactions and purifications.106–109 While GMP radiopharmaceuticals are not currently produced using microfluidic devices, the technology is likely to aid the translation of radiotracers in the future.110–112 Similarly, the small form-factor and simplicity of compact QC infrastructure (e.g. Tracer-QC™ by LabLogic) can also streamline the translation and routine production of radiotracers.113,114

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***Solution:*** *Investigate automated radiochemistry early in the development process. Think of innovative ways to automate complicated radiolabelling procedures using new technologies (i.e. solid-supported reagents in cartridges, reactions performed on solid-phase extraction cartridges - SPE).*

**⚠ *Risk:*** *The GMP radiochemistry facility that will ultimately produce the radiopharmaceutical does not have the same automated radiosynthesis platform.*

***Solution:*** *Automated radiosynthesis platforms from different manufacturers are very similar, collaborate with the GMP radiochemistry facility to see how simple the technology transfer is likely to be. Perhaps there are some modifications to the procedure that can be made which ultimately expedite the translation onto an automated radiosynthesis platform from another manufacturer.*

**⚠ *Risk:*** *The “dead volumes” in reagent vials and general loss of reagents/reactants during the automated radiosynthesis results in a low yield.*

***Solution:*** *Optimisation of radiolabelling conditions need to be explored for the automated platform. Increasing the reactant quantities to compensate the loss of material due to “dead volumes” can help.   
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**2.1.5. *In vitro* and *in vivo* evaluation:** Radiotracers of interest can be first evaluated using simple *in vitro* uptake experiments in cancer cell lines with an upregulated target of interest, in comparison to negative control, to report on target accumulation and specificity. The *in vitro* metabolism of the PET probe using liver microsomes (available from multiple species including rat, mice and human) may be evaluated and used to screen compounds and inform the synthesis of new metabolically stable analogues.115 Promising *in vitro* results provide confidence in progressing *in vivo* animal studies; the *in vivo* target specificity, biodistribution and metabolism are used to predict the outcome of the probe in humans. The overall outcome of the pre-clinical development phase is to identify a single, radiolabelled candidate with promising biological properties. Ideal characteristics for radiopharmaceuticals are described:

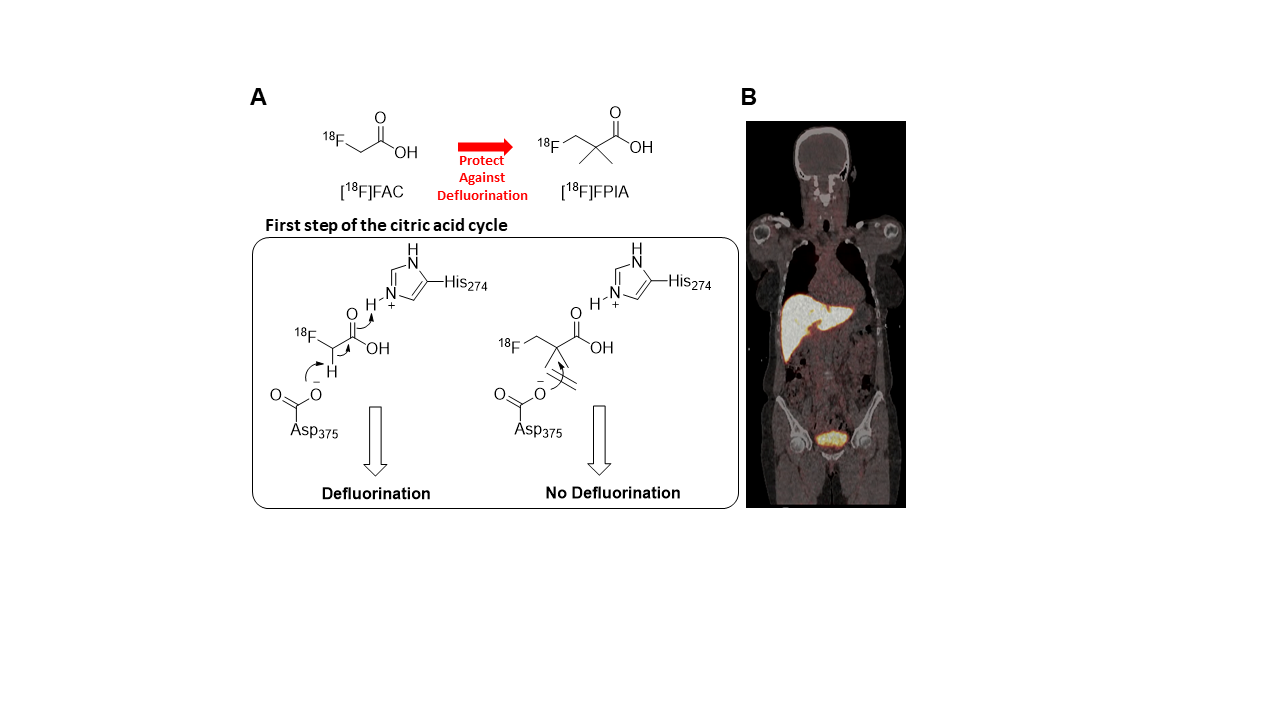
* High specificity and affinity towards the target to ensure retention of the molecule over a suitable timeframe for imaging. As well as high affinity (low nmol), suitable candidates may be selected based upon receptor kinetic profiles determined using analytical techniques such as surface plasmon resonance (SPR). Lead compounds may be selected based on their kinetic profile with desirable properties being rapid association (kon) and slow dissociation (koff) kinetics.
* Moderate lipophilicity (LogP = 0 – 3) is required for molecules that are transported into cells across lipid membranes *via* passive diffusion. There is no LogP requirement for molecules that are taken into cells through a specific mechanism (i.e. internalizing receptor, or channel).
* Metabolic stability over a reasonable imaging timeframe (e.g. 1 – 2 h). Rapidly metabolized molecules do not make good PET radiopharmaceuticals as they are cleared from the blood before sufficient target accumulation. In addition, a low affinity towards drug efflux transporters (e.g. *p*-glycoprotein, PGP) are necessary to avoid poor target accumulation.

The above requirements are important *go/no-go* considerations for taking lead candidates into clinical trials and are likely to be the main cause of attrition for the translation of PET radiopharmaceuticals into the clinic. As shown in Figure 1, a project may require several iterations of library design and *in vitro* and *in vivo* evaluation until a suitable candidate is found; it is worthwhile to spend time in the preclinical development phase to make sure the best possible compound is translated into clinical studies and that there is confidence in the candidate that is proposed for evaluation in humans.   
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***Solution:*** *A clear answer to questions regarding the selective accumulation of radioactivity in the desired target is important to inform library design and compound selection. It is imperative to remember that real life disease in patients is unlikely to mirror the pre-clinical models in terms of expression level and homogeneity, therefore care must be taken to select compounds with the most favourable PK and contrast (tumour:muscle, tumour:blood), rather than simply the highest tumour uptake.   
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**Figure 6.** Common mechanisms of oxidative defluorination in small molecules and strategies to inhibit defluorination.116

Generally, 18F-fluorinated aromatic compounds exhibit the greatest stability towards defluorination compared to aliphatic compounds due to the higher stability of aromatic C(Ar)-F bonds compared to C(sp3)-F; this should be considered when selecting possible structural locations for radiolabelling: *is there an easily accessible location on an aromatic ring system to exploit for radiolabelling, rather than an aliphatic moiety?* If the mechanism for the metabolism of a molecule is well understood, then structural modifications can be made to reduce or inhibit defluorination. A tried and tested method is to replace hydrogen atoms in the vicinity of the fluorine-18 atom with deuterium (D), the heavy (2-fold heavier) isotope of hydrogen.117 Breaking a C-D bond requires a larger activation energy than a C-H bond for chemical or biochemical reactions, with cleavage of C-D bonds occurring 6 – 7 times slower than C-H bonds.116 This approach was exemplified in the development of [18F]fluoromethyl-D4-choline which exhibited greater resistance to oxidative metabolism compared to the non-deuterated analogue; this radiopharmaceutical was evaluated in the clinic.10,118 If a molecule enters a metabolic pathway which results in the liberation of [18F]fluoride, then structural modifications can be made to the molecule to prevent full entry into the pathway. This approach was exemplified for improving [18F]fluoroacetate ([18F]FAC), a radiotracer for imaging oxidative metabolism, which undergoes significant *in vivo* defluorination catalysed by nucleophilic attack from glutathione (GHS) and entry into the citric acid cycle (Figure 7A).119 To prevent defluorination, [18F]fluoropivalic acid ([18F]FPIA), a structural analogue of [18F]FAC wad developed. [18F]FPIA contained *gem*-dimethyl substituent to prevented entry into the citric acid cycle which imparted favourable metabolic stability against defluorination.11,12 [18F]FPIA was evaluated for safety and dosimetry in healthy volunteers and is currently in phase II clinical trials (Figure 7B).120   


**Figure 7.** **A**) [18F]FAC enters the citric acid cycle and is subsequently defluorinated; whereas the gem-dimethyl substituents of [18F]FPIA prevent entry into the citric acid cycle and impart metabolic stability against defluorination. **B**) PET image (saggital): [18F]FPIA in a 55 year old healthy volunteer.

Defluorination is not the only metabolic route for 18F-radiolabelled molecules. Oxidative metabolism of aromatic ring systems can present a metabolic liability which may be inhibited by the inclusion of electronegative atoms; this strategy was employed in the design and development of [18F]ICMT-11, a caspase-3-specific PET tracer for imaging apoptosis.121 Linker moieties used in the development of radiolabelled peptide libraries may also present a metabolic liability. Preclinical investigation of the [18F]FET-TOCA library highlighted impaired metabolic stability of polyethylene glycol linkers (PEG-linkers) versus short amino acid linkers.48,49,53 In scenarios where linker strategies are required, the development of a small library with structural variation in linker moieties may provide several viable options if metabolic instability is observed.

Radiometals (e.g. 89Zr and [18F]AlF) are also subjected to *in vivo* instability typically resulting in demetallation. The deferoxamine chelator (DFO) is used clinically for the radiolabelling of antibodies with zirconium-89 and is prone to demetallation with subsequent accumulation of radioactivity in the bone; new octadentate chelators have been designed to replace hexadentate DFO.122–124 The stability of [18F]AlF complexes largely depends on the chelator used; NODA and NOTA chelators do not typically result in extensive defluorination, however defluorination can occur, especially with other chelators.125,126 Radioiodine (e.g. 124I) is prone to deiodination depending upon the substitution position of the isotope in the molecule.127 Despite extensive knowledge of drug metabolism, significant differences can occur for PET radiopharmaceuticals due to the “mass effect”. The kinetic and metabolic profiles of compounds administered to living organisms in large doses (mg/kg) differs from that of molecules administered at radiotracer levels (<1 μg/kg).128

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***Solution:*** *Apply methods to inhibit defluorination (Figure 6). When designing compound libraries, think about potential metabolic liabilities and design fall-back compounds that can be implemented if metabolism is problematic.*

**⚠ *Risk:*** *The radiopharmaceutical is rapidly metabolised into smaller organic metabolites or oxidised.*

***Solution:*** *When considering the compound library, include examples which design out potential metabolic liabilities. For example, esters are susceptible to hydrolysis by esterase in the blood – changing an ester to an amide can impart stability. If amide stability is problematic, consider the stable 1,2,3-triazole linkage as a bioisostere. Investigate the use of electronegative atoms in aromatic systems to limit oxidative metabolism.*

**⚠ *Risk:*** *Developing the precursors and radiosynthesis of complex molecules takes time, which is time wasted if the molecule is rapidly metabolised before reaching the desired target.*

***Solution:*** *If the metabolic stability of a radiolabelled moiety is of concern, investigate the metabolic stability of a similar, simple model compound; this will either alleviate the concern, or allow alternative metabolically stable molecules to be investigated.   
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**2.2 Clinical development and evaluation**If a suitable lead candidate exhibits good *in vivo* performance in pre-clinical studies, then radiation dosimetry and nonclinical toxicology in male and female rats is considered. Studies into off-target activity may also be evaluated (e.g. kinase screening). An essential requirement for the clinical evaluation of a radiopharmaceutical is a GMP compliant radiosynthesis.

**2.2.1. GMP radiosynthesis & production:** to support clinical studies, a GMP radiosynthesis is developed to produce the radiopharmaceutical, ideally in multipatient doses in a facility approved by the Medicines and Healthcare Products Regulatory Agency (MHRA). Fluorine-18 radiopharmaceuticals require a GMP compliant automated protocol. This not only protects the radiochemist from exposure to large radioactive doses, but provide a standardized approach (standard operating procedure, SOP) for the radiochemistry to promote batch reproducibility. There are some important considerations and bottlenecks in scaling up a radiosynthesis from the typically low activity work of preclinical studies (<100 MBq product) to the higher activities required for clinical studies (>1 GBq product for the transportation of multipatient doses).

***Radiolysis:*** the degradation of molecular structures by reactive oxygen radicals formed at high levels of radioactivity. The degree of radiolysis is dependent upon the molecular structure, but typically, peptides are more prone to radiolysis than small molecules. To prevent radiolysis, the incorporation of a radioprotectant (antioxidant) to scavenge the reactive radical species is required; these include ascorbic acid, gentisic acid or ethanol.129,130 The stability of a radiopharmaceutical over time in the dispensed formulation is important and validation is required.

***Radiation safety:*** is an important consideration for GMP production. There are many ways to reduce the exposure of production personnel to radioactivity, including shielded hot cells and automated radiosynthesis methods, however radioactive volatiles are a concern for radiopharmaceutical production facilities. Radiosynthetic methods that use distillation, for example the purification of the 2-[18F]fluoroethylazide ([18F]FEA) prosthetic group, can pose a challenge for GMP scale up.131 This challenge is not insurmountable, as shown in the GMP radiosynthesis of [18F]FET-βAG-TOCA which required the distillation of [18F]FEA.16 Alternative production techniques (i.e. solid phase extraction *vs.* distillation) may reduce volatile release and should be investigated.97

***Quality control:*** as well as a validated GMP compatible radiosynthesis, a validated quality control (QC) method of the final patient formulation is necessary to ensure the impurity profile is compliant with the necessary regulatory body [European Medicines Agency (EMEA) or Food and Drug Administration (FDA)]. In-house QC determines the quantity of minor impurities and their identity, along with the sterility of the formulation, absence of pyrogens and that the formulation is within a suitable pH range for administering to patients. The impurity profile of a radiopharmaceutical is a direct result of the chemistry used to synthesise product. Therefore, it might be necessary to change the chemistry to modify the impurity profile of the radiopharmaceutical. This approach was exemplified in the GMP radiosynthesis of [18F]ICMT-11 where direct nucleophilic substitution with [18F]fluoride was selected over a [18F]FEA copper catalysed azide-alkyne cycloaddition (CuAAC) prosthetic group approach, to achieve a more favourable impurity profile.98

***Instrument validation:*** validation and qualification of processes for the manufacture and QC of PET radiopharmaceuticals is essential to produce safe and reproducible patient doses. While this is beyond the scope of this review, we have cited comprehensive publications on instrument validation and establishing GMP compliant radiochemistry facilities.132,133

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***Solution:*** *Investigate the use of reactive oxygen species scavengers early in the development phase. There inclusion may also be necessary in HPLC eluents (e.g. 1% EtOH) to prevent degradation on the column. Automated synthesise that concentrate radioactivity into a small volume may be more susceptible to radiolysis.*

**⚠ *Risk:*** *The purity profile of the radiopharmaceutical is not good enough for clinical use.*

***Solution:*** *Changing the radiolabelling chemistry (if possible) may produce a different impurity profile that allows for better separation of the radioactive compound from non-radioactive impurities. If this is not possible, investigate alternative purification methods (e.g. different solvent systems, different HPLC columns) to try and separate the impurities; commercial supplies of chromatographic equipment and consumables often have separation science experts at hand to help identify where their products may help in your project – contact their representatives who may offer suggestions and a “try before you buy” service for their products.   
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**6. Conclusions**

As a community of scientists, we stand at the forefront of personalised medicine and hold the key to understanding an individual’s disease phenotype, how to effectively treat their disease and minimise debilitating side-effects associated their treatments. Tremendous advancements in the development of radiopharmaceuticals have directly impacted the lives of patients around the world; however, despite great investment in the field, relatively few radiopharmaceuticals have entered routine clinical practice. The translation of novel radiopharmaceuticals into the clinic for evaluation in humans and ultimately, routine use, is not without its challenges; therefore, the attrition rate of PET radiopharmaceuticals may be due to unforeseen challenges during the development phase. This review examined the development pipeline for PET radiopharmaceuticals, highlighting the progression of radiolabelled molecules through pre-clinical evaluation and into clinical studies; insight into the process, regarding the challenges one might face in selecting lead candidates, suitable radiolabelling methods and radioisotopes, investigating and improving metabolic stability, will hopefully provide a useful resource for the PET community. It is not always possible to predict the road ahead and the complexity of natural biology ensures that we are unable to grasp a firm handle on predicting processes like metabolism; but it is always worthwhile to look back on previous studies to try understand the challenges we face, anticipate similar scenarios and how best solve problems to promote new radiopharmaceuticals entering the clinic.

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