Small Molecules as Radiopharmaceutical Vectors

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A wide repertoire of small molecule PET radiopharmaceuticals has been successfully developed over the last 30–40 years [1]. Both the acceleration of the discovery of novel targets and advances in our understanding of pathophysiological mechanisms have created a rapidly increasing demand for new radiotracers. Alongside the traditional academic pursuit of new radiotracers, the resources of the pharmaceutical industry have been increasingly engaged in creating novel radiotracers for the development of new therapeutics and companion diagnostics.

The radiotracers used today have a wide variety of origins. Some are based on existing ^{3}H- and ^{14}C-labeled compounds. The ubiquitous 2-[^{18}F]-fluorodeoxyglucose, for example, is a descendant of 2-[^{14}C]deoxyglucose, a ^{14}C-labeled compound developed by Sokoloff *et al.* [2]. Over the years, rational approaches to the development of radiopharmaceuticals have evolved. This chapter will describe some 'tried and tested' approaches that have been used to select, design, and evaluate successful PET radiotracers. The chapter is divided into two parts: 'design parameters' and 'test criteria'.

In the first section, we will cover several critical 'design parameters' for the creation of effective small molecule radiopharmaceuticals (Table 1, top). More specifically, we will discuss an eclectic set of physicochemical and pharmacological properties, guidelines, tolerances, and 'rules of thumb' that—when considered together—can assist in the identification of molecules that are more likely to produce successful radiotracers. These criteria can be considered prior to performing any physical experiments, using data that may be gleaned from a variety of sources, including literature reports, databases, and *in silico* tools. It must be remembered, however, that some of these criteria are not 'hard and fast' rules but rather guidelines. Simply put, exceptions can and will be found. This underscores the fact that we admittedly do not yet fully understand the molecular and pharmacological requirements for radiotracers. Some examples of these exceptions are provided in the commentary.

Table 1 Design and test criterion for the discovery and development of small molecule radiotracers

Design criteria
Choosing and appropriate target
High affinity and selectivity for the target
Ease of radiosynthesis
Maximizing target accessibility while minimizing non-displaceable binding
Test Criteria
Good signal-to-noise ratio *in vivo*
Good *in vivo* pharmacokinetics
In vivo distribution and pharmacology consistent with literature reports
Low levels of radiolabeled metabolites in the region of interest
High sensitivity towards the target

In the second section, we describe a set of quantitative metrics for radiolabeled tracers that can be obtained via a series of *in vitro* and *in vivo* experiments to determine a radiotracer's potential utility (Table 1, bottom). There are many possible variations for these tests, often depending on the resources and infrastructure available. Yet if these test criteria are experimentally verified in one way or another, the chances of producing a useful radiotracer for *in vivo* imaging are greatly increased. It is important to note, however, that simple 'usefulness' is—for better or worse—not the endgame here. Indeed, even after an effective radiopharmaceutical has been created, there are further translational hurdles that must be negotiated before a radiotracer is considered suitable for *in vivo* imaging in humans. These will also be discussed.

Throughout the chapter, concepts are illustrated and reinforced graphically using a set of cartoon representations of an idealized *in vivo* environment (Fig. 1).

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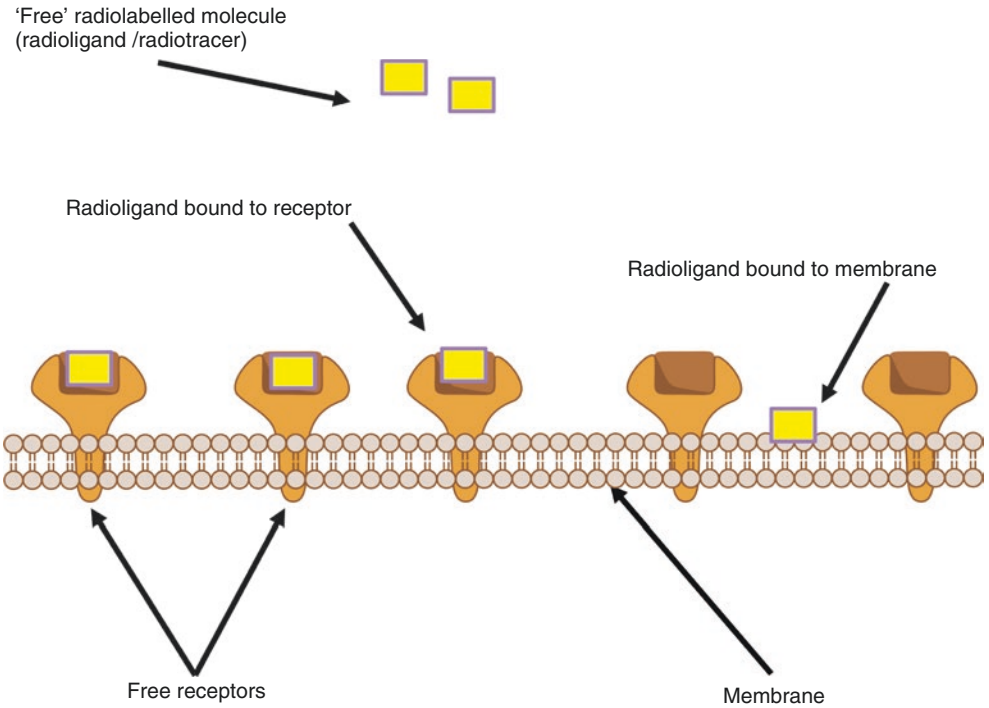


Fig. 1 Representation of some key concepts in the development of PET radiotracers

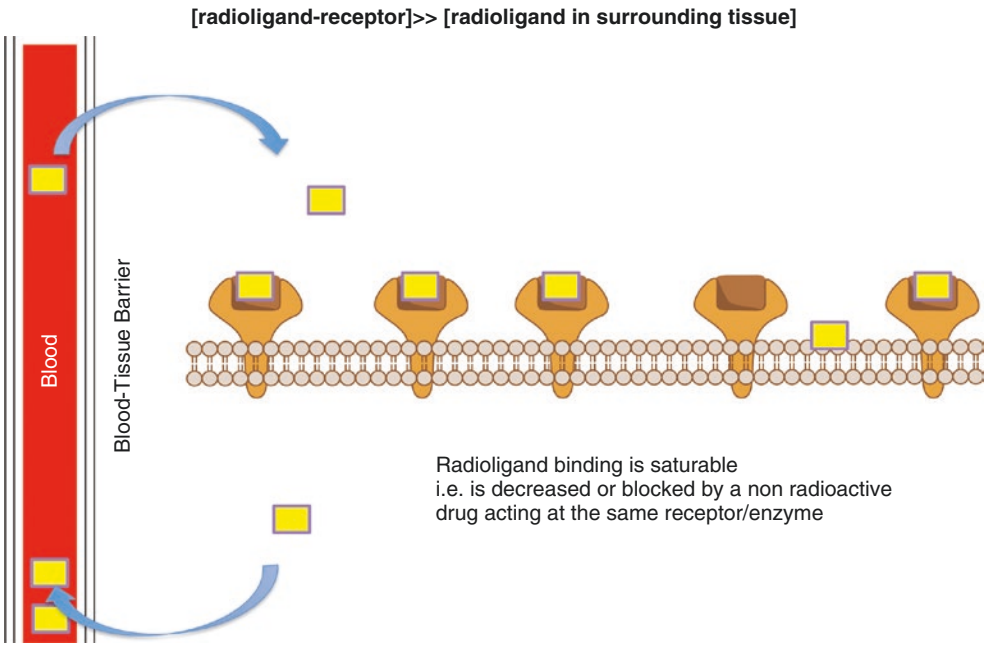


Fig. 2 Representation of the properties of an ideal CNS radiotracer: [radioligand-receptor] >> [radioligand in surrounding tissue]

An example schematic—a representation of the properties of an ideal radiotracer—is shown in Fig. 2. After the radiotracer is introduced to the blood via intravenous administration, it is able diffuse across the blood-tissue barrier, where it initially occupies the aqueous environment between cells. The radiotracer can subsequently bind to its molecular target, bind to non-target membranes or proteins, or diffuse across the blood-tissue barrier back into the bloodstream. For an ideal radiotracer (at a suitable time point after administration), there is significantly greater binding to the target than the surrounding compartments (*i.e.* [radiotracer bound to target receptors] >> ([free

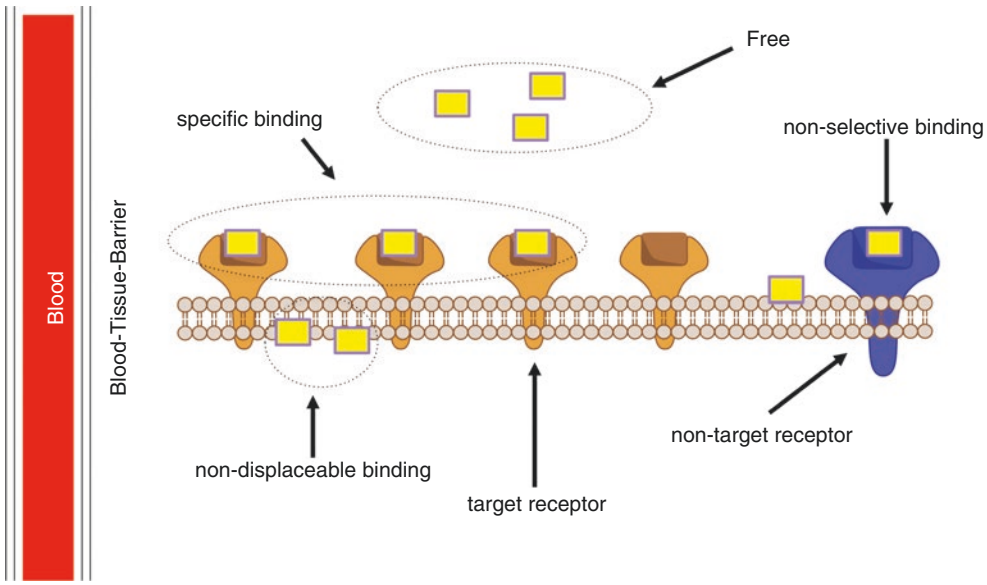


Fig. 3 Specific binding, non-selective binding, and non-displaceable binding

radiotracer] + [radiotracer bound to the membrane] + [radiotracer bound to non-target receptors])). Binding to (unwanted) non-target receptors is termed 'non-selective binding' (Fig. 3).

One brief aside before we begin in earnest: the term 'non-specific binding' is often used in the literature to describe the binding of a radiopharmaceutical to *all* non-target entities, including non-target receptors, serum proteins, membranes, *etc.* Strictly speaking, this is a misnomer. The binding of a radiopharmaceutical to non-target receptors is best described as 'non-selective binding', and the indiscriminate binding of a radiopharmaceutical to serum proteins and membranes is best described as 'non-displaceable binding'. To avoid this confusion, the terms 'non-selective binding' and 'non-displaceable binding' are recommended and are used consistently throughout this chapter.

Part 1: Design Criteria for Small Molecule Radiotracers

Requirement 1: Choosing an Appropriate Target

The first requirement when selecting an appropriate target for a radiotracer discovery program is knowing the concentration of the target protein (B_{max}) in the region of interest (ROI) (*e.g.* the tumour, thalamus, cerebellum, *etc.*). A quick poll of radiotracers developed over the past three decades shows—with only a few exceptions—that the lowest B_{max} that has been successfully imaged is around 1 nM. Target concentrations are typically expressed in units of nM, mol/g, or mol/cm^3 tissue.

Dopamine D_2 receptors (for which several radiotracers have been developed) are present in the 10–20 nM range. If the target protein is present in sub-nanomolar concentrations, very special efforts are required to develop an effective radiotracer. A pictorial representation of a tissue with a low target concentration is shown in Fig. 4. Compared to the idealized case shown in Fig. 2, the number of target proteins is too low to provide significant signal contrast. To overcome this, a radiotracer must have higher affinity for its receptor or reduced propensity for non-displaceable and non-selective binding.

The appropriateness of a target also depends on the physical size (or volume) of the ROI in which the target is located (Fig. 5) [3]. The size/volume of the ROI is typically expressed in units of micrometer, mm, mm^3, cm^3, *etc.*

In this regard, there are a number of criteria to consider, including motion correction (for moving organs such as the heart or lungs), co-registration with an anatomical atlas, and camera sensitivity and resolution. If the molecular target of interest is present in sufficient concentration and in a ROI with a volume exceeding the resolution of the scanner (*e.g.* the striatum, thalamus, cerebellum, large tumour, *etc.*), it is usually relatively easy to accurately quantify the concentration of the tracer within the target. However, ROIs that have sizes similar to or less than the resolution of the camera (*e.g.* the raphe nucleus, substantia nigra, microtumours, etc.) can appear 'blurred' or 'smeared out'. Furthermore, the radioactivity concentrations in these regions can be underestimated, making it difficult to identify and accurately quantify the binding of the radiotracer to the target.

The 'smearing out' of the tissue signal is termed the partial volume effect—the loss of apparent radioactivity concentration in small ROIs because of the limited resolution

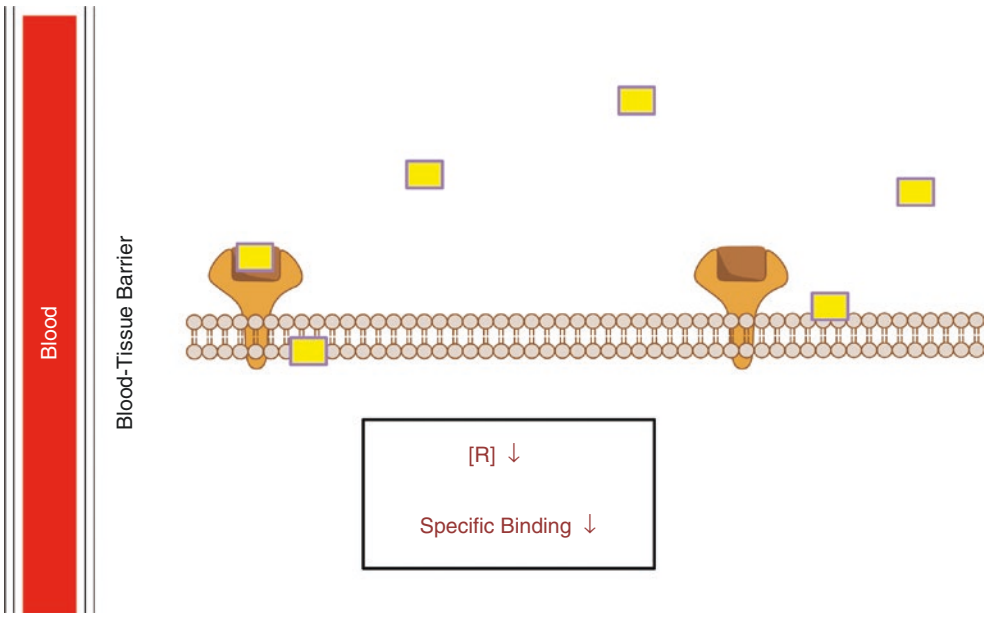


Fig. 4 Representation of a cell with low target expression

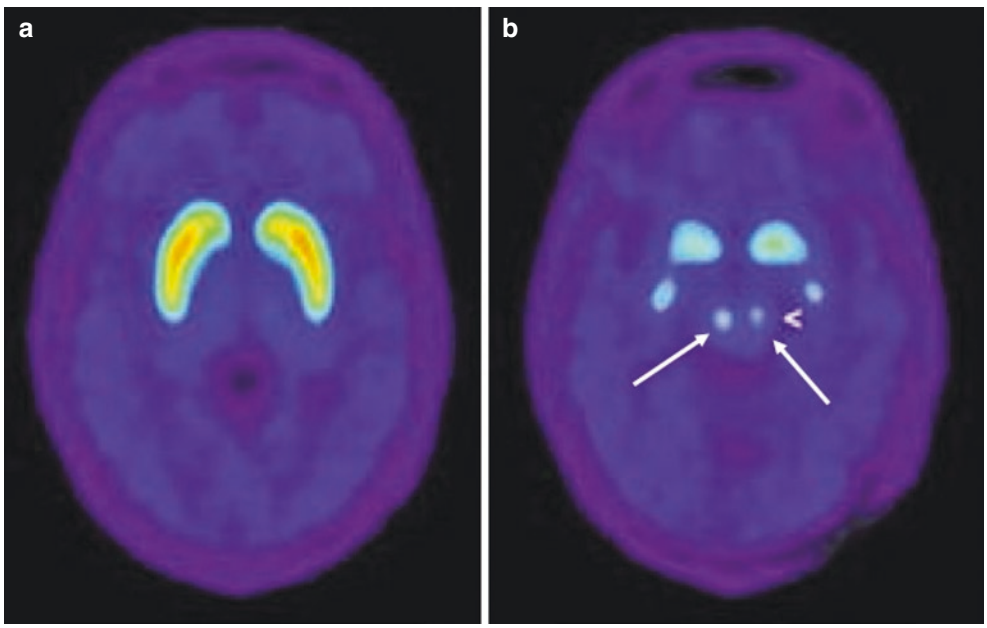


Fig. 5 Visualizing large vs. small target regions: (**a**) imaging of large target regions, the striatum; (**b**) imaging of small target regions, the substantia nigra nuclei (see arrows). (Adapted from Varrone *et al.* [3], with permission)

of the scanner. At present, the practical resolution of clinical PET scanners is typically in the range of 2–5 mm. If the ROI about the same size as the resolution of the scanner, the activity concentration of the structure appears to 'spill out' into the surrounding tissue and is thus underestimated compared to the true value (Fig. 6). If the ROI in which the target resides is very small, the radioactivity concentration is almost impossible to quantify. Great efforts are being made by the imaging community to improve the resolution and sensitivity of PET scanners. Readers are referred to reviews on this subject for in depth treatment of the subject.

Requirement 2: High Affinity and Selectivity for the Target

Affinity For a particular molecular target, the magnitude of B_{max} and the physical size of the ROI in which the target resides influence the affinity needed for an effective radiotracer. To illustrate this, let us consider the targeting of three individual monoamine transporters: the dopamine transporter (DAT), the serotonin transporter (SERT), and the norepinephrine (noradrenaline) transporter (NET). The expression levels of these proteins in the central

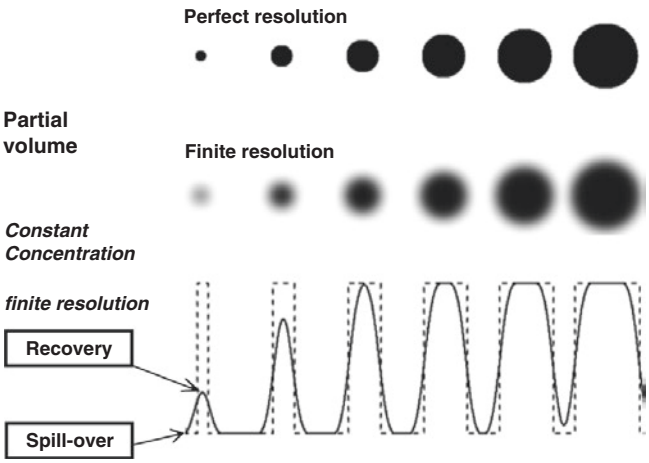


Fig. 6 Illustration of the partial volume effect

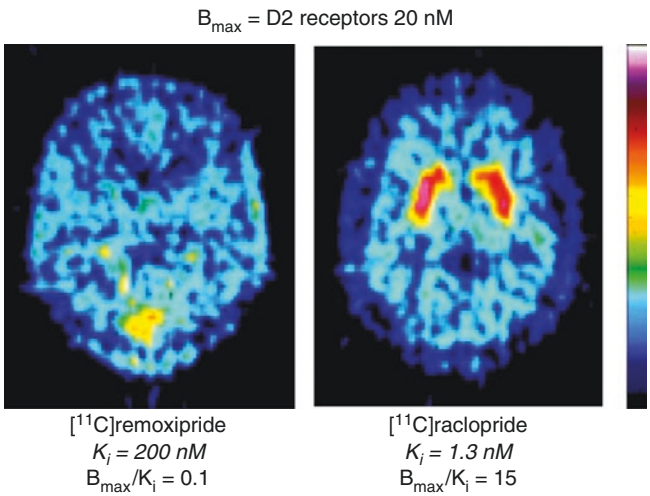


Fig. 7 PET brain scans of two dopamine D2-targeted radiotracers with different affinities. [^{11}C]Remoxipride has a lower affinity of 200 nM and does not give a noticeable PET signal in the striatum (a region rich in D2 receptors). [^{11}C]Raclopride, in contrast, has a much higher affinity (in the low nanomolar range), enabling adequate visualization of striatal D2 receptors. (Courtesy of Drs. Lars Farde and Christer Halldin, Department of Clinical Neuroscience, Karolinska University Hospital, Stockholm, Sweden)

Table 2 Required affinity for radiotracers to image DAT, SERT, and NET

Target	B_{max}	Required affinity (K_d)	Radiotracers
DAT	>100 nM	10 nM	>20 radiotracers
SERT	20 nM	2 nM	3–4 radiotracers
NET	5 nM	0.5 nM	No good radiotracer

DAT dopamine transporter, *SERT* serotonin transporter, *NET* norepinephrine (noradrenaline) transporter

nervous system (CNS) are quite different with B_{max} values DAT > SERT > NET (Table 2).

For imaging DAT, a radiotracer with an affinity (K_d) of between 10 and 100 nM is required. Producing small molecules with affinities in this range is not too challenging for modern medicinal chemistry, and several DAT radiotracers have been successfully developed. The SERT, however, is expressed at ~5 times lower concentrations than the DAT. As a result, a radiotracer with an affinity 5 times greater is required to image SERT with similar target-to-background ratios. This requirement puts greater demands on medicinal chemistry, and it is perhaps not surprising that only a few radiotracers have been developed for SERT to date. Finally, the NET is present in even *lower* concentrations than DAT and SERT, necessitating a radiotracer with a sub-nanomolar affinity for the target. Despite some decent attempts, there are currently no really effective radiotracers for NET.

More generally speaking, if the affinity of a radiotracer for its target is too low, sufficient target-to-background contrast ratios will not be achieved. This phenomenon is illustrated in Fig. 7: specific binding decreases as the affinity of the radioligand decreases.

Over the years, some researchers have developed a rule of thumb for estimating the minimum affinity required to target a certain expression level of protein (B_{max}). The binding of a radiotracer to a target is related to its affinity for the target and the target concentration. A useful way to quantify this was developed by Eckelman *et al.* [4] using a derivation of the Scatchard plot (Fig. 8) and Eq. 1 (the Scatchard equation):

$$\frac{[\text{Bound radiotracer}]}{[\text{Unbound radiotracer}]} = \frac{B_{max}}{K_d} - \frac{[\text{Bound radiotracer}]}{K_d}$$

For a radioligand to behave as a tracer, the amount bound radioligand approaches zero, and the equation reduces to the following (Eq. 2):

$$\frac{[\text{Bound radiotracer}]}{[\text{Unbound radiotracer}]} = \frac{B_{max}}{K_d}$$

Eckleman *et al.* proposed that a minimum ratio [Bound radiotracer]/[Unbound radiotracer]—or signal/noise ratio—of 10 is needed for a successful radiotracer. In other words, the ratio of the B_{max} of the target to the K_d of the radiotracer must be at least 10 in order to achieve adequate signal-to-background contrast ratios *in vivo*. If this equation is used to plot a series of isocontours representing different protein expression levels, the graph in Fig. 9 can be generated (Gee, previously unpublished data). The ratio [Bound radiotracer]/[Unbound radiotracer] is shown on the *Y* axis, and the affinity of the radiotracer (K_d) is shown on the *x*-axis. This graph clearly shows that achieving signal-to-noise ratios of 10 is contingent upon both B_{max} and K_d. For example, a target with

a B_{max} of 100 nM requires a radiotracer with a K_d of 10 nM in order to obtain a B/F ratio of 10. However, a radiotracer with a K_d of between 1 and 0.1 nM is needed to image a target with a B_{max} value in the 3 nM range.

Patel *et al.* demonstrated the correlation between B_{max}/K_d ratios and signal-to-noise ratios for a small number of radiotracers with different affinities and molecular targets at different expression levels (Table 3) [5].

As we have noted, high affinity for a target is typically a good thing. However, it is important to note that sometimes the affinity of a radiotracer can be *too* high. In this case, the distribution of the radiotracer may become dependent on blood flow and transport rather than the expression level of the target itself. Furthermore, high affinity radiotracers often exhibit 'irreversible' kinetic behaviour, making them unsuitable for reliable quantification because quantitative *in vivo* pharmacokinetic analyses typically assume 'steady-state' or 'pseudo-equilibrium' conditions.

Selectivity The selectivity of a radiotracer depends on a number of factors, including its affinity for the target, the B_{max} of the target, and the presence of 'interfering' target. The latter can be illustrated by differences in the expression of dopamine and serotonin neurons in the brain as shown in Fig. 10.

There are some regions of the brain where dopamine and serotonin neurons are co-expressed and other regions where serotonin receptors or dopamine receptors are predominant. We have already stated that the affinity needed for a radiotracer is dictated by the expression level—or B_{max}—of the target. However, if the radiotracer is 'non-selective' (*i.e.* binds to more than one target), both the location and the B_{max} of the interfering target need to be considered. For example, if our goal is to develop a radiotracer to image the SERT in

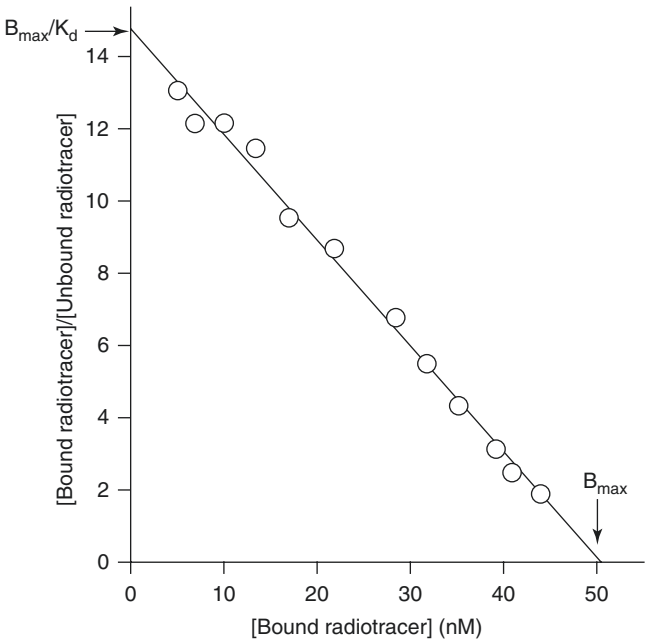


Fig. 8 The Scatchard plot. (From Patel and Gibson [5], with permission)

Table 3 B_{max}/K_d ratio of common CNS radiotracers

Target	Radiotracer	B_{max} (nM)	K_d (nM)	B_{max}/K_d
DAT	[^{11}C]cocaine	150	130	1.2
DAT	[^{18}F]β-CFT	180	11	16
D2	[^{11}C]raclopride	19	3.5	5.4
D2	[^{11}C]*N*-methylspiperone	19	0.1	190
CB1	[^{18}F]MK9470	14	0.3	47
m-AChR	[^{123}I]/[^{11}C]QNB	150	0.2	750
m-GluR5	[^{18}F]FPEB	50	0.2	250
m-GluR5	[^{18}F]PyrPEB	50	16	3

From Patel and Gibson [5], with permission
DAT dopamine transporter, B_{max} the concentration of the target protein, K_d affinity

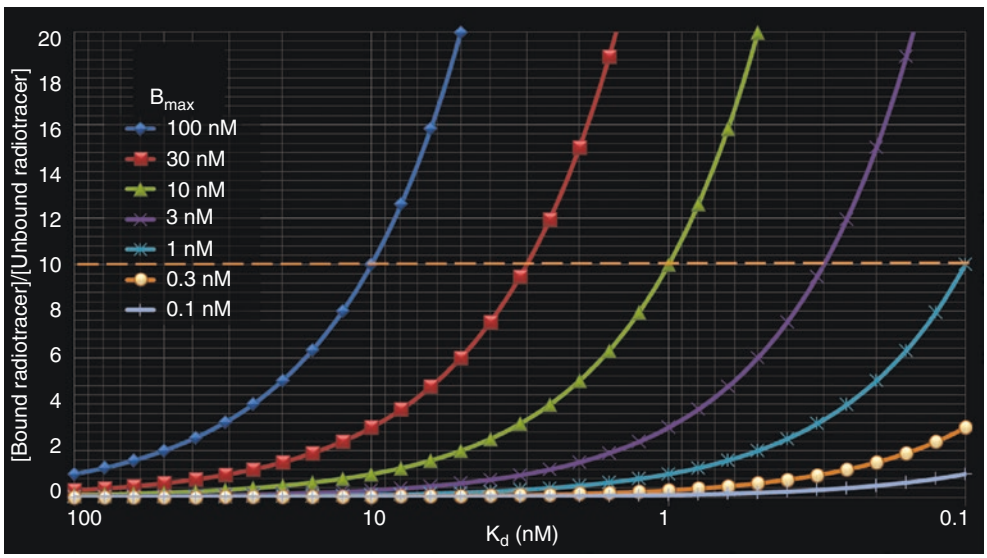


Fig. 9 'Plot' illustrating the [bound radiotracer]/[unbound radiotracer] ratio as a function of B_{max} and K_d

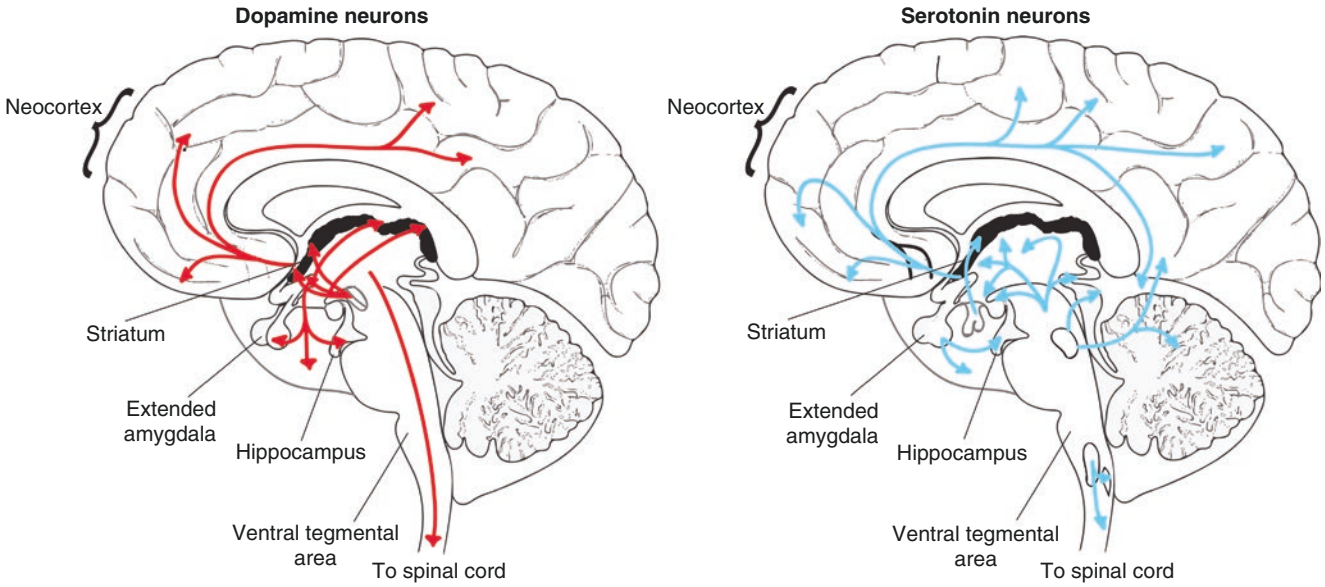


Fig. 10 The required selectivity of a radiotracer depends upon the affinity (K_d) of the radiotracer for its target, the presence of interfering targets, the B_{max} of the target, and the localization of the target. (Adapted with permission from Neuroscience & Graphic Design for the Centre for Neuroimaging Sciences at King's College London. https://neuroscience-graphicdesign.com/, courtesy of the artist Gill Brown)

the striatum, the radiotracer will require selectivity for SERT vs. DAT of ca. 100 or greater. This is because the B_{max} of DAT in the striatum is much greater than the B_{max} of SERT in the same area (see Table 1). However, if the goal is to image the DAT in the striatum, then we can live with a non-selective radiotracer, because the expression of DAT is so much higher than that of the SERT the same region.

Requirement 3: Ease of Radiosynthesis

Other chapters within this book cover methods for the radiosynthesis of tracers with radionuclides ranging from ^{11}C to ^{225}Ac. Many of these methods have proven to be robust and have been applied to a wide number of radiopharmaceuticals. Other radionuclides are used less frequently, possibly due to their complexity, their need for a narrow set of structural or synthetic criteria, or the lack of availability of appropriate labeling methods. When designing a new radiotracer, the selection of a robust approach to radiolabeling should be a priority. Accessibility is key. Even the most fantastic radiotracer can be rendered irrelevant if it can only be produced one time in ten attempts. Moreover, radiosynthetic strategies to produce radiotracers bearing short half-life radionuclides must be rapid.

That said, if a difficult-to-make radiotracer has nonetheless demonstrated the ability to address an unmet need, radiopharmaceutical chemists can develop more robust synthetic pathways. As this book demonstrates, the field of radiopharmaceutical chemistry is active and evolving, and significant progress has been made in recent years to create a wide variety of robust labeling methods.

Requirement 4: Maximizing Target Accessibility While Minimizing Non-displaceable Binding

To be able to image a target protein, a radiotracer must be able to reach that target. For most radiotracers (see exceptions below), this means having the ability to diffuse from the blood into the tissue of interest through the plasma membrane via a passive transport. The inability to access the tissue of interest is one of the most frequent reasons for radiotracers to fail. There are many factors which contribute to a radiotracer's ability to diffuse passively across a membrane. Admittedly, many of these are poorly understood; however, the lipophilicity of a molecule seems to be one of the physicochemical parameters that is significantly correlated with tissue penetration.

The most common method to measure lipophilicity is the octanol-water 'shake-flask' method. In this assay, the radiotracer is introduced into a system containing equal volumes of octanol and water, shaken vigorously, and allowed to partition between the octanol and water phases. The 'lipophilicity' is measured as the log of the partition coefficient of the non-charged molecule between the two phases (Fig. 11).

If the pH of the aqueous layer adjusted to physiological pH (7.4), the measure is termed a LogD value and accounts for the partition of both charged and non-charged species at

physiological pH. If a tracer is not sufficiently lipophilic—*i.e.* is too hydrophilic—it will not be able to diffuse across cell membranes from the blood pool in order to reach its target (Fig. 12).

Great efforts have been made to develop *a priori* rules for predicting a molecule's ability to penetrate into tissue. Lipinski's 'rule of five' is a notable example of a multiparametric *in silico* approach to predicting the tissue permeability of small molecule drug candidates. Empirical screening procedures—including assays using artificial phospholipid bilayers, cell monolayers, and even chromatographic methods—are also adopted occasionally to probe the interplay between lipophilicity and passive permeability. It is important, however, that molecules that cross membranes via active or facilitated transport (*e.g.* amino acid transporters, glucose transporters, P-glycoprotein, efflux pumps, *etc.*) are exempt from these membrane diffusion and lipophilicity discussions.

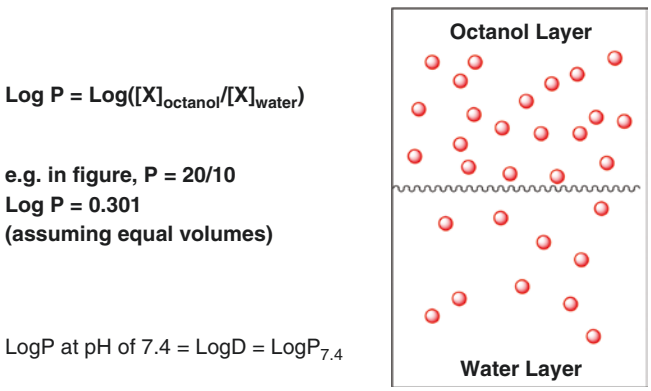


Fig. 11 Illustration of the estimation of an octanol-water partition coefficient

The importance of lipophilicity also extends to non-displaceable binding (NDB). Non-displaceable binding is the affinity of the radiotracer for all non-saturable components of tissue, such as lipids, phospholipids, membranes, *etc.* In other words, the radioligand-tissue binding cannot be displaced or blocked by macroscopic quantities of non-radioactive blocking agents. NDB is independent of the target, and it increases linearly with increasing lipophilicity of radiotracer (Fig. 13). In nuclear imaging, the NDB can be considered the 'noise' or 'background 'signal', while the specific binding is the 'contrast'. Indeed, high non-displaceable binding is probably the primary reason for the failure of many radiotracers. The correlation between NDB and the lipophilicity of a radiotracer is strong. Generally speaking, higher values of LogD produce more non-displaceable binding.

All of this leaves us with a bit of a conundrum. If the lipophilicity of a radiotracer is too low, it will not be able to access the target. However, if the lipophilicity of a radiotracer is too high, it will have high NDB. This begs the question: *what is the optimal lipophilicity for a radiotracer?*

The optimal lipophilicity value for a radiotracer is a balance between a number of parameters:

- *Target accessibility*: a degree of lipophilicity is required to help the radiotracer diffuse across cell membranes.
- *Non-displaceable binding*: High NDB is observed if the lipophilicity is too high.
- *Plasma protein binding:* The bloodstream contains numerous proteins (*e.g.* albumin). Plasma protein binding is increased with increasing molecule lipophilicity; if the plasma protein binding is too high, very little radiotracer is 'free' to find its target.

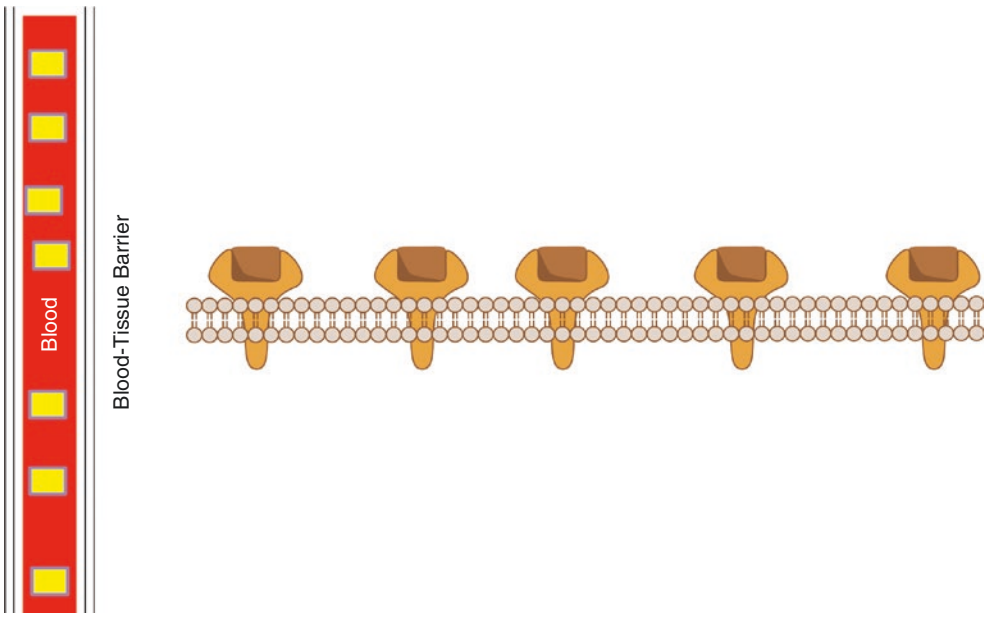


Fig. 12 Radiotracers which are not sufficiently lipophilic may struggle to cross membranes to reach their targets

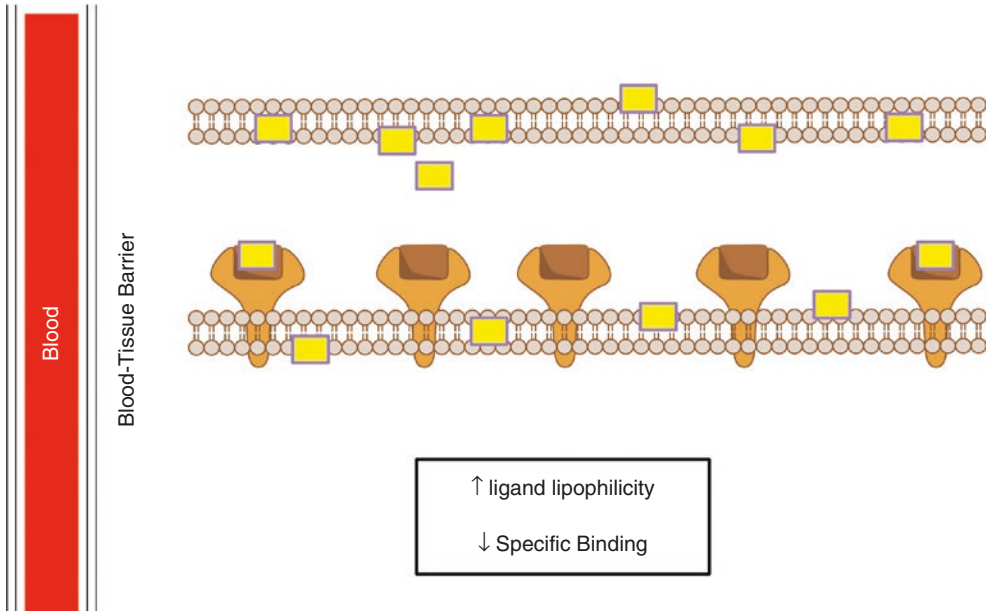


Fig. 13 High lipophilicity can result in high non-displaceable binding (NDB)

- *Affinity:* Because receptors and enzymes are themselves proteins, the binding affinity of radiotracers to many molecular recognition sites actually often increases with increased lipophilicity!

Researchers have typically found that LogD (or LogP) values of 1–3 are optimal to balance the competing factors discussed above (see Waterhouse 2004 for a discussion on this topic) [6].

The importance of properly measuring the LogD values of radiotracers was discussed by Wilson *et al.* along with a recommendation of how to accurately measure this parameter. There are also many computer programmes that can be used to calculate LogP and LogD values from structures. However, these should be treated with caution, as they are often inaccurate. While these may be useful for comparing the lipophilicity of analogues across a series of compounds, it is strongly recommended that *experimentally determined* LogD and LogP values be used whenever possible.

While the '1–3 LogP' rule is very useful, there are (inevitably) notable exceptions. For example, WAY100635 has a LogD value of 3.1, but [^{11}C]WAY100635 has negligible non-saturable binding and rapid tissue washout *in vivo* (Fig. 14). Conversely, CFT has LogD values of around 0.6 but [^{11}C]CFT exhibits high non-displaceable binding *in vivo* accompanied by slow washout from tissues.

The fact that there are many exceptions to the 'LogP 1–3' rule suggests that lipophilicity is not the only factor contributing to non-saturable binding and that the molecular basis of non-displaceable binding as a whole is poorly understood. In this regard, advances in our understanding of NDB could be very helpful in refining the selection criteria used during the discovery of radiotracers, which to

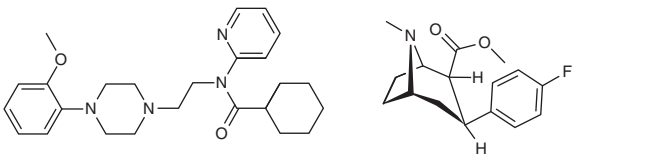


Fig. 14 Notable exceptions to the 'LogD = 1–3 rule'

date has a high attrition rate. Researchers have proposed hypotheses regarding the molecular mechanisms of non-saturable binding. Baciu *et al.*, for example, have proposed that non-displaceable binding may be linked to the ability of a molecule to hydrolyse the fatty acid chains of membrane phospholipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)) *via* an autocatalysed acid hydrolysis mechanism [7] (Fig. 15).

The investigators observed that the rate of membrane digestion caused by cationic amphiphilic drug-like (CAD) was *inversely* correlated the magnitude of its *in vivo* NDB.

According to this hypothesis, molecules—such as CAD—that rapidly hydrolyse membrane phospholipids exhibit low NDB because they are able to rapidly translocate across the membrane to reach their target (Fig. 16). The authors further hypothesized that the translocation might be facilitated by the formation of micelles around the molecule, facilitating its transport in the aqueous intracellular environment, the so-called membrane munching hypothesis of NDB. Conversely, molecules that only slowly hydrolyse the membrane—or do not do so at all—exhibit high NDB because they become 'stuck' at the polar-apolar interface of the phospholipid membrane (Fig. 17).

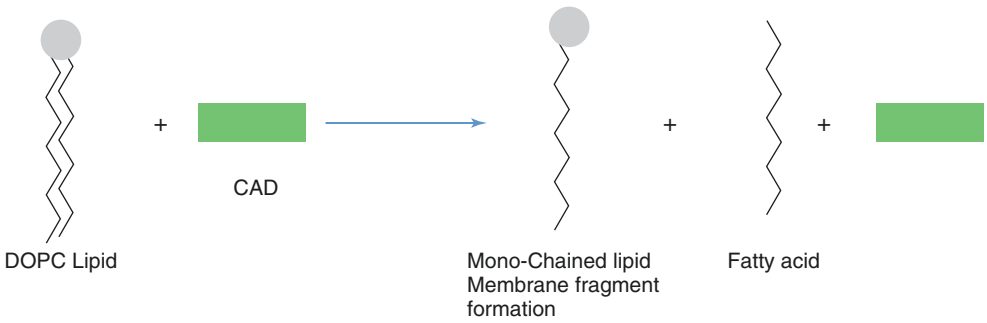


Fig. 15 Acid hydrolysis of the ester linkage of DOPC

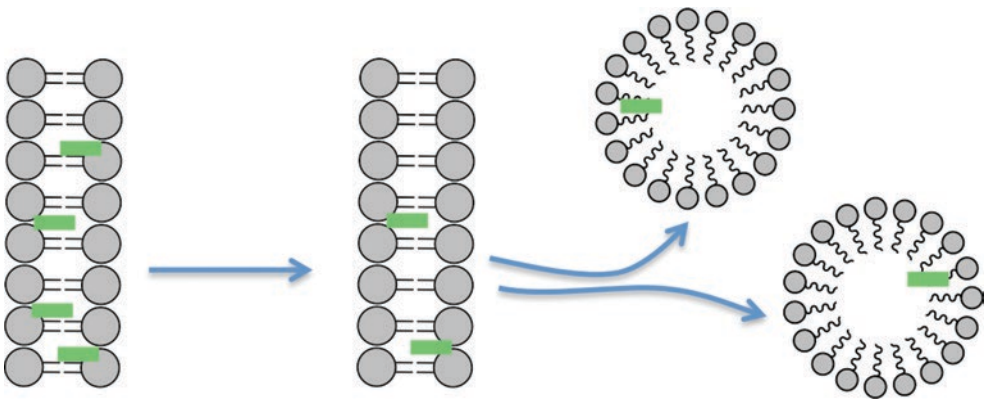


Fig. 16 Molecules (green rectangle) that rapidly hydrolyse membrane phospholipids exhibit low NDB because they are able to rapidly translocate across the membrane to reach their target

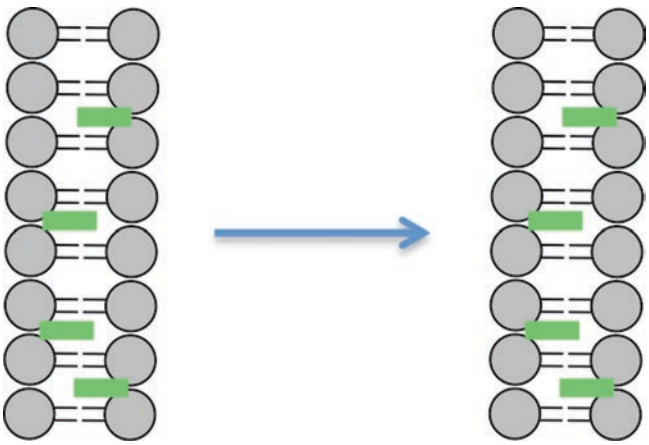


Fig. 17 Molecules (green rectangle) that only slowly hydrolyse the membrane—or do not do so at all—exhibit high NDB because they become 'stuck' at the polar-apolar interface of the phospholipid membrane

Further evidence for the insufficiency of LogP as an accurate indicator of non-displaceable binding came from ab initio quantum mechanical calculations of the interaction energy of between drugs and lipids. The interaction energy between drugs and lipids was shown to be a better predictor of *in vivo* NDB than estimates obtained from experimentally determined LogP values. Not surprisingly, calculated Log P values have even poorer correlations with the degree of *in vivo* NDB! Ultimately, further research into the molecular mechanisms of non-displaceable binding and transmembrane relocation will sharpen our understanding of these phenomena as well as our ability to design molecules that have low NDB while effectively targeting proteins of interest.

Starting Points for the Development of Novel Radiotracers

There are a several starting points that one can use when beginning the development a novel radiotracer. These include publications on structure-activity relationships, existing pharmaceuticals, and existing radiotracers (as well the patent literature). Each of these sources can contain information that will allow for at least an initial assessment of a molecule's potential to fulfil the design criteria discussed above without having to perform a single experiment. Empirical rules are also being developed—particularly by drug companies—to facilitate the identification of promising platforms for radioligands by screening compound libraries for candidates with the molecular hallmarks of successful radiotracers (Table 4).

A recurring trend within the field is the creation of 'second-generation' radiotracers following the initial report of a 'first-generation' radiotracer. The 'first-generation' tracer may be promising but suboptimal with respect to some key trait (*e.g.* kinetics, selectivity, metabolism, high non-displaceable bind-

Table 4 Minimum starting points for the development of a small molecule radiotracer

Property	Ideal situation	Minimum starting points
B_{max}	Known B_{max} and location of the target in animals and humans	Know approximate location and B_{max}—at least in an animal (model)
Affinity	Known	Known for analogues at least
Selectivity	Known	Known for analogues at least
Metabolism	Known	Rarely known
Log P	Known	Easily calculated values but inaccurate; measurable if compound or radiotracer is available
Radiosynthesis	Robust and simple radiochemistry methods for labeling Commercially available precursors/reagents Commercially available automated synthesis apparatus	Possible synthetic route to the target compound (may need significant resources for development for a new radiosynthetic route, with associated higher risk of failure)

ing, or radiolabel). In this case, this 'first-generation' radiotracer can be an inspiration for 'second-generation' iterations. There are numerous examples of this phenomenon, including radiotracers that target the DAT, TSPO, amyloid, PSMA, and somatostatin receptors.

While many of these 'second-generation' radiotracers provide useful incremental improvements (or create intellectual property), the overall process can frequently represent an unnecessary duplication of effort and resources. Furthermore, it can actually hinder the translational progress of class of agents when each group develops its 'own flavour' of a particular radiotracer. Clearly, embarking on the development of 'me-too' radiotracers should be carefully considered in order to assess if the investigation is an effective use of resources.

Other Pathways to Radiotracer Discovery

A useful starting point in the development of novel radiotracer is the use of tritium-labeled compounds. The creation of these radiopharmaceuticals does not require the use of a cyclotron, and a single batch is often sufficient to conduct many experiments. Furthermore, the evaluation of tritiated radiotracers is not constrained by the short half-lives of many radionuclides, making *in vitro* binding studies easy. More recently, techniques such as MALDI imaging and mass spectroscopy are starting to be utilized as alternatives to using radioactive compounds as starting points for the discovery of radiotracers, opening doors for even more efficient discovery efforts in the future.

Part 2: Test Criteria

Assuming the initial design parameters have been met for a candidate radiotracer and its molecular target, the next step is to evaluate a prototype radioligand against a set of test criteria to see whether or not it can be developed into a successful radiotracer. The five test criteria are as follows:

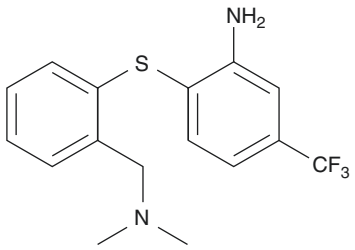
- Good signal-to-noise ratio *in vivo*
- Good *in vivo* pharmacokinetics
- *In vivo* distribution and pharmacology consistent with literature reports
- Low levels of radiolabeled metabolites in the region of interest
- High sensitivity towards the target

There are many different ways that these test criteria can be addressed. The choice of methods often comes down to the resources available, for example, the availability of instrumentation for *in vitro* studies or access to small animal PET scanners. Each set of strategies has its own set of pros and cons. Yet ultimately, irrespective of differences in the experimental approach, the same test criteria need to be addressed. The examples illustrated below are based upon the *in vivo* evaluation of radiotracers in rodents as well as the use of *ex vivo* tissue dissection and counting methods.

To illustrate the testing phase for a candidate radiotracer, a reconstruction of the test criteria used in the development of the ultimately successful SERT radiotracer, DASB, is described. The published details of this work by can be found in Wilson *et al.* [8]. The SERT is a protein expressed in the CNS which recycles the neurotransmitter serotonin after being released as a result of neuronal firing. The released serotonin is transported back into the presynaptic serotonin nerve terminals for repackaging in neurotransmitter vesicles. The SERT is the site of action for many antidepressant drugs (*e.g.* Prozac, paroxetine, fluoxetine, *etc.*).

The starting point for the SERT radiotracer development campaign (resulting in the discovery of the DASB radiotracer) was found in the patent literature, specifically a class of compounds that had not previously been explored as an imaging agent but met the design criteria described above (Fig. 18).

Encouraged by the discovery of SERT-5, Wilson et al. set out to label this lead compound via methylation with ^{11}C-methyl iodide to test whether the compound could be labeled. This was indeed found to be the case (Fig. 19) [8].



SERT-5

- Reported in patent WO 97/17325 (1996)
- IC_{50} for inhibition of reuptake of serotonin - 0.02 nM
- Claimed to be selective for SERT over DAT and NET
- Suitable site for radiolabeling with C-11

Fig. 18 SERT-5—the lead compound for serotonin transporter radiotracer development

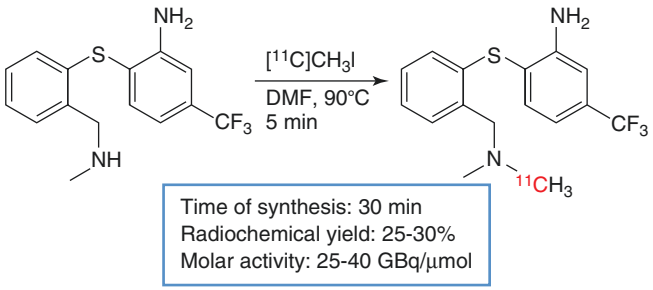


Fig. 19 Radiosynthesis of [^{11}C]SERT-5

Test Criterion 1: Good Signal-to-Noise Ratio *In Vivo*

Following the confirmation of the successful labeling of SERT-5, the prototype radiotracer was administered to rats which were sacrificed at selected time points post-injection. Blood samples were collected at the time of sacrifice, selected brain regions were dissected, and the activity levels and weights of selected tissues were determined (Fig. 20).

The activity concentrations in each tissue were determined by expressing the tissue activity as 'activity/tissue weight' or MBq/g tissue. The dissected brain regions were selected based on known *in vitro* expression levels in the rat brain (SERT expression: hypothalamus > thalamus = striatum > cerebellum). As the cerebellum contains almost no SERT, it can be used as a control tissue for comparison with other SERT-rich regions.

The selection of a range of time points allows for the creation of a plot of the activity concentration of the tracer in different tissues over time: the y-axis represents the radioactivity concentration in a given tissue, while the x-axis represents time. These graphs are commonly known as 'time-activity curves' or 'TACs' (Fig. 20). In this way, a comparison between the TACs of regions rich in the target with those that lack the target can provide an indication of

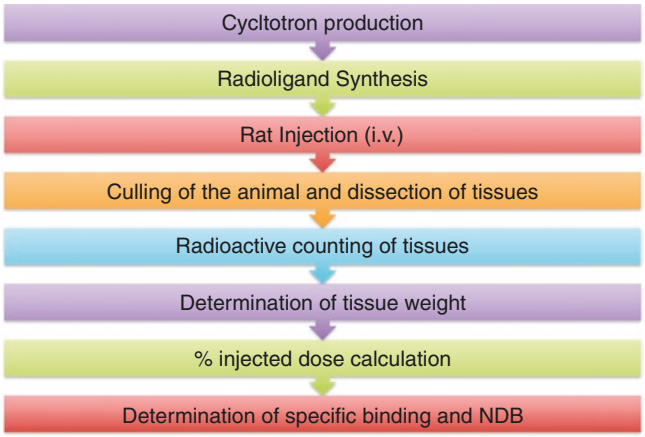


Fig. 20 *In vivo* rat experimental protocol

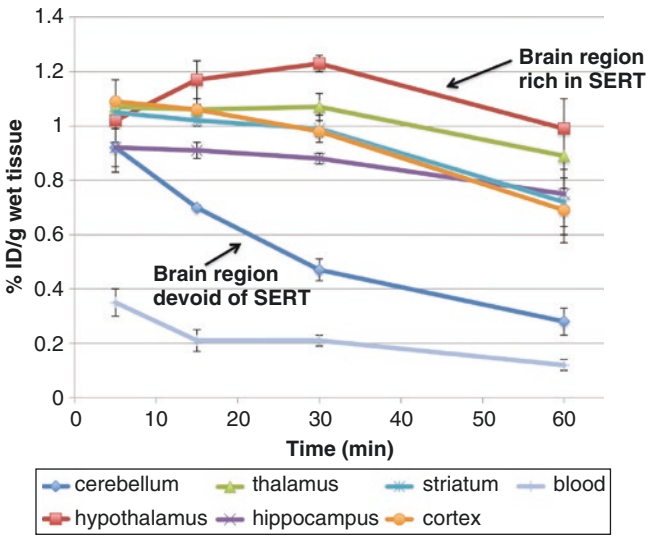
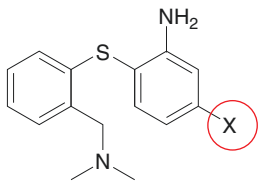


Fig. 21 Biodistribution of [^{11}C]SERT-5 in rat brain

the magnitude of the SERT-specific signal of the radiotracer. The TACs of nontarget tissues will also give an indication of the non-displaceable background signal.

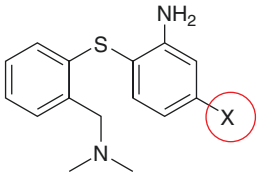
This TAC-based analysis answers two critical questions: (1) 'Does [^{11}C]SERT-5 access the target organ?' and (2) 'Does [^{11}C]SERT-5 bind to the SERT with adequate contrast *in vivo*?' The TACs in Fig. 21 clearly show that the activity concentration in the hypothalamus (which is rich in SERT) at 20–60 min post-administration is much higher than that of the cerebellum (which is low in SERT). In addition, regions containing intermediate levels of SERT have intermediate radioactivity concentrations.

Taken together, the data thus far confirmed that the lead compound could be labeled with ^{11}C and that the resulting radiotracer produces an *in vivo* distribution in the brain that is consistent with the known literature distribution of SERT. Furthermore, it also shows that the radioactivity washout from regions devoid of SERT is more rapid than



Name	X	SERT (K_i, nM)	NET (K_i, nM)	DAT (K_i, nM)
SERT-5	CF_3	0.33	1205	2038
SERT-21	Cl	0.27	230	115
SERT-24	OCH_3	1.89	1990	2650
DASB	CN	1.10	1350	1420

Fig. 22 Binding affinity of 2-(phenylthio)arylamine derivatives to cloned human transporters



Name	X	LogD
SERT-5	CF_3	3.77
SERT-21	Cl	3.55
SERT-24	OCH_3	2.83
DASB	CN	2.71

Fig. 23 LogP of 2-(phenylthio)arylamine derivatives

the washout regions which contain significant levels of SERT. Furthermore, the TACs also show that the radiotracer reversibly binds to SERT within the timescale of the experiment.

As [^{11}C]SERT-5 showed promising *in vivo* SERT-targeting characteristics, a number of close analogues of the prototype—containing chlorine, methoxy, or nitrile substituents in place of the CF_3 moiety—were subsequently synthesized to determine if the properties of the prototype could be further improved (Fig. 22).

The affinity of the SERT-5 analogues were determined using cloned human cell lines expressing the serotonin transporter. These data show that all of the analogues have high affinity for SERT as well as hundred- to thousand-fold selectivity for the target over NET and DAT. The LogP values of the compounds were also measured using the octanol-water 'shake-flask' method, revealing that [^{11}C]SERT-5 had the highest log P value (3.77) of the compounds assayed (Fig. 23).

According to the 'logP 1–3' rule of thumb, the candidates with methoxy and nitrile substituents appear to have optimal lipophilicities for imaging the CNS. The regional distribution of these ^{11}C-labeled analogues of [^{11}C]SERT-5 was subsequently compared using an approach identical to that used in the initial evaluation of the parent radiotracer (Fig. 24).

The signal-to-noise ratios of each of these ^{11}C-labeled analogues were compared using the hypothalamus-to-cerebellum activity concentration ratios (Fig. 25) (Table 5).

In this analysis, the cerebellum—which has low expression of SERT—is treated as a reference region (the noise), while the hypothalamus, which has high expression of SERT, is the 'target-rich' region (the signal). In comparison to SERT-5, the data shows that DASB has the superior signal-to-noise ratio at time points 30–60 min post-injection. SERT-24 and SERT-21 also have good signal-to-noise ratios throughout the period studied compared to the prototype tracer.

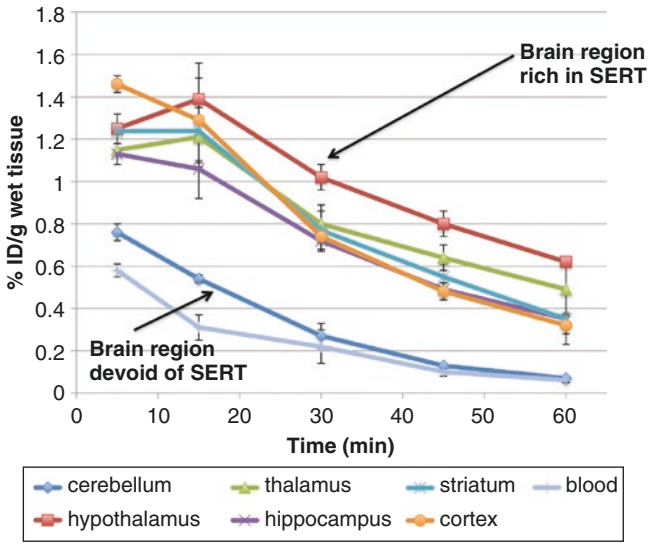


Fig. 24 Biodistribution of [^{11}C]DASB in rat brain

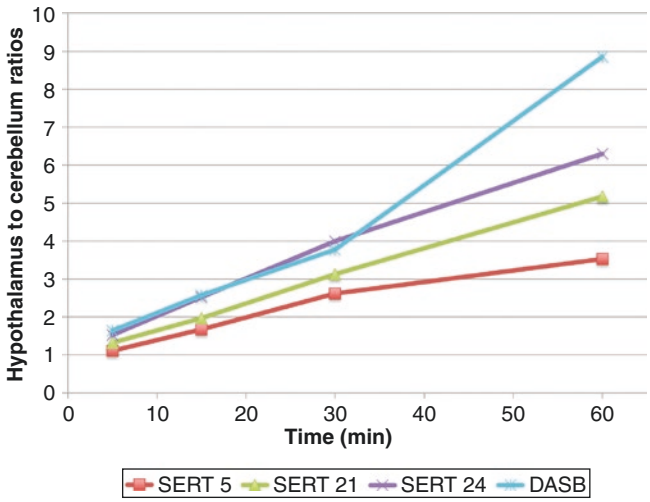


Fig. 25 Comparison of SERT radiotracers: signal-to-noise ratios in rats over 60 min

Table 5 Relationship between logD values, cerebellar clearance washout rates, and signal-to-noise ratios

Name	LogD	Cerebellar clearance rate (t_{half} min)	Signal-to-noise ratio
SERT-5	3.77	32	2.5
SERT-21	3.55	25	4.2
SERT-24	2.83	19	6.3
DASB	2.71	16	7.9

SERT serotonin transporter

Test Criterion 2: Appropriate *In Vivo* Pharmacokinetics

The TAC analysis also allows for an assessment of the pharmacokinetic profile of the radiotracer. More specifically, these data facilitate the determination of whether the kinetics of the tracer are reversible or irreversible over the time period studied. In all cases, [^{11}C]SERT-5 and its ^{11}C-labeled analogues displayed reversible kinetics *in vivo*. That is to say, their activity concentrations reached a plateau in the target tissue and then subsequently washed out of tissue during the timescale of the experiment). At this stage, in the evaluation procedure, it has been confirmed that the radiotracers are able to enter the brain, have appropriate regional distributions, have suitable pharmacokinetic profiles (*i.e.* are compatible with the half-life of carbon-11), and produce good signal-to-noise ratios *in vivo*. The most promising of these compounds—[^{11}C]DASB—was selected for further characterization.

Test Criterion 3: Appropriate *In Vivo* Pharmacology

To assess the appropriateness of [^{11}C]DASB *in vivo* pharmacology, rats were pretreated with a non-radioactive 'blocking agent' prior to the administration of the radiotracer. Tissue and blood TACs were then generated in a manner similar to that described above in order to explore whether or not there are any significant alterations to the radiotracer's signal-to-noise ratio alterations upon blocking.

The choice of blocking agents depends on a variety of factors. In the case of a SERT radiotracer, the selectivity of the radiotracer for SERT over DAT and NET is critical given the structural similarities between these proteins and the known off-target pharmacology of SERT compounds for these transporters. As a result, experiments in which animals are pretreated with selective SERT-, DAT-, and NET-targeting blocking agents are important (in addition to a 'vehicle'-only control experiment, of course). In addition, it is also useful to perform 'self-block' experiments in which a non-radioactive variant of the tracer is administered as a blocking agent (Fig. 26).

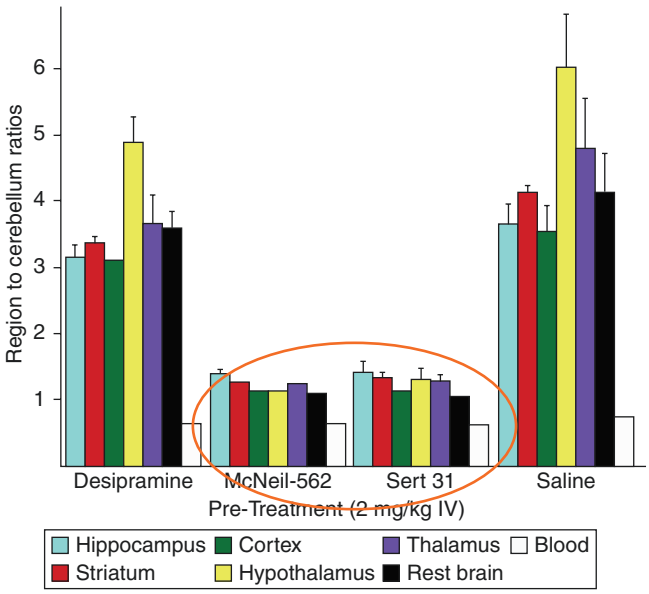


Fig. 26 Region-to-cerebellum activity concentration ratios of [^{11}C]DASB in rats pretreated with desipramine, McN-5652, SERT-31, and saline

These data reveal that [^{11}C]DASB administration with saline 'vehicle' results in the accumulation of the radiotracer in the hypothalamus as well as regions with intermediate expression of SERT. In contrast, blocking with non-radiolabeled DASB and McN-5652 (a known selective SERT blocker) dramatically reduces the radioactivity concentration in the hippocampus. In addition, the administration of desipramine (a reasonably selective NET blocker) does not significantly perturb the accumulation of the radiotracer in SERT-rich regions.

Similar experiments can be performed using inhibitors of the DAT as well as any other receptors enzymes that the radiotracer may be binding in a non-selective manner. In this case, pretreatment experiments using GB12909 (a DAT inhibitor), haloperidol (a dopamine D2 blocker), WAY100635 (a serotonin 5-HT$_{1A}$ blocker), ketanserin (a 5-HT$_{2A}$ blocker), and raclopride (a dopamine D2/D3 receptor blocker) produce no significant reductions in the regional distribution of [^{11}C]DASB. Based on this evidence, [^{11}C]DASB appears to have appropriate pharmacology for imaging SERT in the CNS, though the off-target binding of the radiotracer to other receptors/transporters cannot be absolutely ruled out unless tested.

Test Criterion 4: Radiolabeled Metabolites in the Region of Interest

After their administration, most radiotracers are subject to enzymatic breakdown or metabolism *in vivo*. This frequently results in the generation of radiolabeled metabolites that differ in structure from the parent compound. To test this possibility, [^{11}C]DASB was administered intravenously, and at selected

time points post-administration, blood samples and brain tissue were collected. The blood was analysed using radioHPLC to determine the amount of intact [^{11}C]DASB as well as the presence of ^{11}C-labeled metabolites. Figure 27 shows a profile of the radiometabolites of [^{11}C]DASB in the blood at 30 min post-administration, revealing that only 25% of the [^{11}C]DASB remains intact in the blood at this time point.

If any of these radiolabeled metabolites are capable of penetrating the brain, they can lead to background signal that can obscure the specific binding signal of [^{11}C]DASB. Yet conversely, if the metabolites cannot penetrate the brain, their influence on the signal in the area of interest is minimized.

A similar analysis of brain tissue at 30 min after the administration of [^{11}C]DASB can reveal if these blood-borne metabolites can cross into brain tissue. To this end, the brains of rats that had been administered [^{11}C]DASB were collected 30 mins following the administration the tracer, homogenized, and analysed via radioHPLC.

Figure 28 shows that over 95% of the radioactivity in the brain at 30 min postinjection corresponds to [^{11}C]DASB. Although 5% of the radioactivity corresponds to radiometabolites, the majority of the signal in the brain is due to parent compound. As a result, [^{11}C]DASB was considered suitable for further evaluation as a SERT-targeted imaging agent.

Test Criterion 5: Sensitivity Towards the Target

The ultimate purpose of a radiotracer is to test sensitivity of the imaging agent towards its target. In the case of [^{11}C]DASB, this was explored via an experiment aimed at determining whether the radiotracer could follow the differential occupancy of the SERT by an antidepressant drug and follow the drug's natural washout from the SERT over time. To this

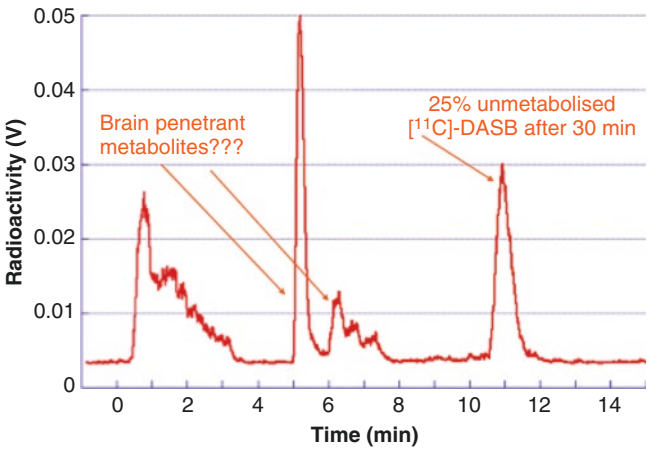


Fig. 27 HPLC analysis of rat plasma after the administration of [^{11}C]DASB

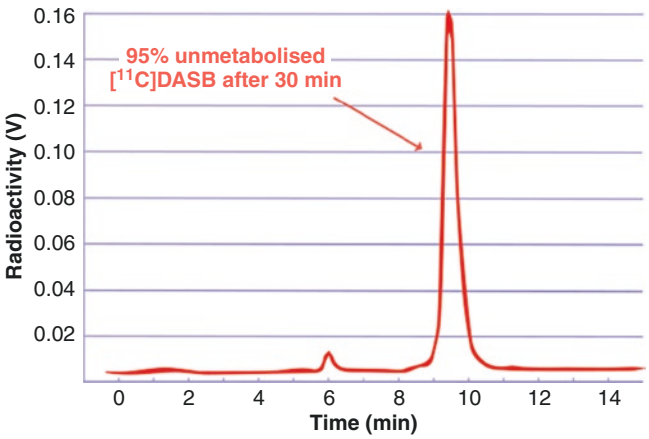


Fig. 28 HPLC analysis of rat brain extract after the administration of [^{11}C]DASB

end, rats were pretreated with paroxetine (a known SERT blocker) at various time intervals (1, 7, 24, and 28 h) and subsequently administered with [^{11}C]DASB. Brain regions were analysed for the presence of [^{11}C]DASB at 60 min post radiotracer administration (Fig. 29) [9].

The tissue (hypothalamus, striatum, cortex, and thalamus)-to-cerebellum activity concentration ratios show that, compared with the baseline condition, the paroxetine quickly occupies the SERT at around 1 h post-administration [9]. At 7 h post-administration of paroxetine, the occupancy of the SERT by paroxetine has slightly decreased (due to washout). And at 24 h following the administration of the paroxetine, the tissue-to-cerebellar activity concentration ratio of [^{11}C]DASB has returned back to the original (control) levels, clearly demonstrating that the radiotracer is sensitive to changes in the occupancy of the transporter by paroxetine [9].

Translation

Some radiotracers that look promising in preclinical studies can be unsuccessful upon translation to humans. [^{11}C]DASB, however, was successfully translated as a radiotracer for *in vivo* human SERT imaging: the clinical data revealed reversible pharmacokinetics and good regional distribution in humans (Fig. 30).

Additional clinical experiments confirmed the selectivity and sensitivity of the radiotracer for imaging SERT *in vivo*, and the tracer has since become a valuable tool for studying the function of SERT in humans (Fig. 31) [10].

Of course, the translation of imaging agents from animals to humans is not always as successful, as with the given example of [^{11}C]DASB. An example of a troublesome translation to humans is provided by the development of the serotonin 5-HT$_{1A}$ radiotracer [^{11}C]WAY100635 (Fig. 32).

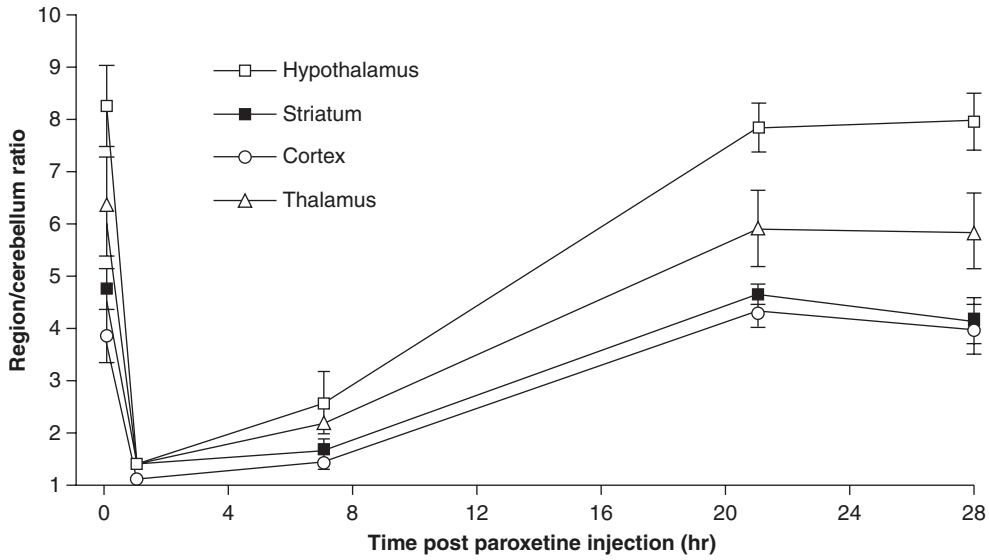


Fig. 29 [^{11}C]DASB: Recovery of binding after pretreatment with paroxetine (2 mg/kg, IV) (From Wilson et al. [9], with permission)

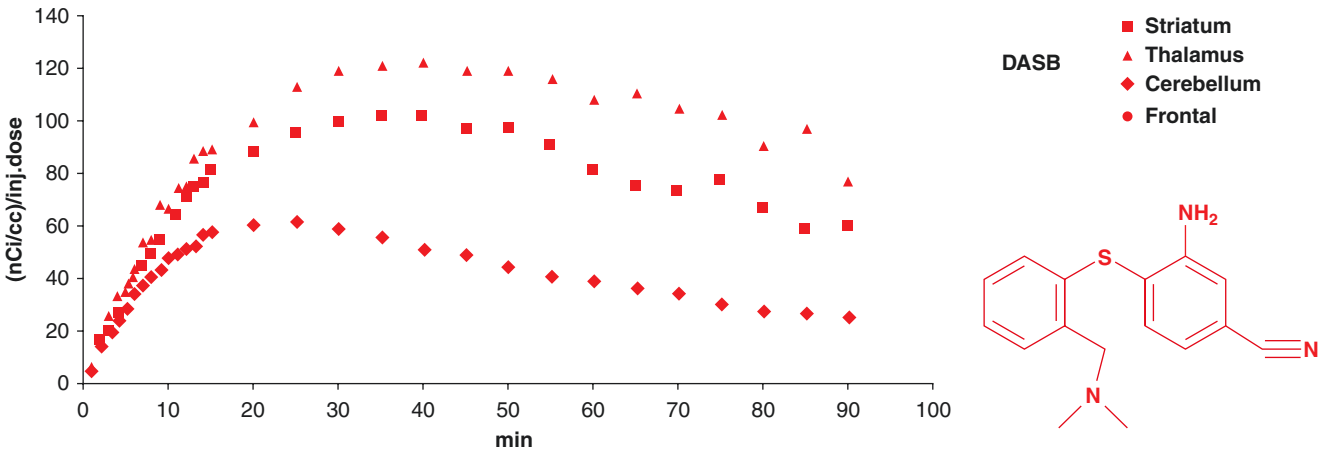


Fig. 30 Time-activity curves of [^{11}C]DASB in the CNS

When evaluated in rats, [^{11}C]WAY100635 demonstrated selective binding for 5-HT$_{1A}$ as well as an excellent signal-to-noise ratio. When first used in humans, however, the radiotracer suffered from poor signal-to-noise ratios, preventing the visualization of 5-HT$_{1A}$ receptor expression. Further analysis showed that there is a difference in the liver metabolism of this compound in humans compared to rats. In humans, an enzyme breaks down the parent molecule to a brain-penetrant metabolite which obscures the binding of the parent molecule to the receptor (Fig. 33a).

This problem was circumvented by labeling the molecule in a different position (Fig. 33a). In the case of [carbonyl-^{11}C]WAY100635, metabolism in the liver produced non-brain-penetrant metabolite instead, allowing this new radiotracer to become a very successful tool for the imaging of 5-HT$_{1A}$ receptors in humans. There are many other excellent examples of the development of radiotracers in the literature, including the creation of amyloid-targeted imaging agents and PSMA-targeting radiotracers.

Summary

In summary, when embarking on the development of a novel radiotracer, time should be taken to assess the design parameters discussed above in 'Part 1'. Only when these have been

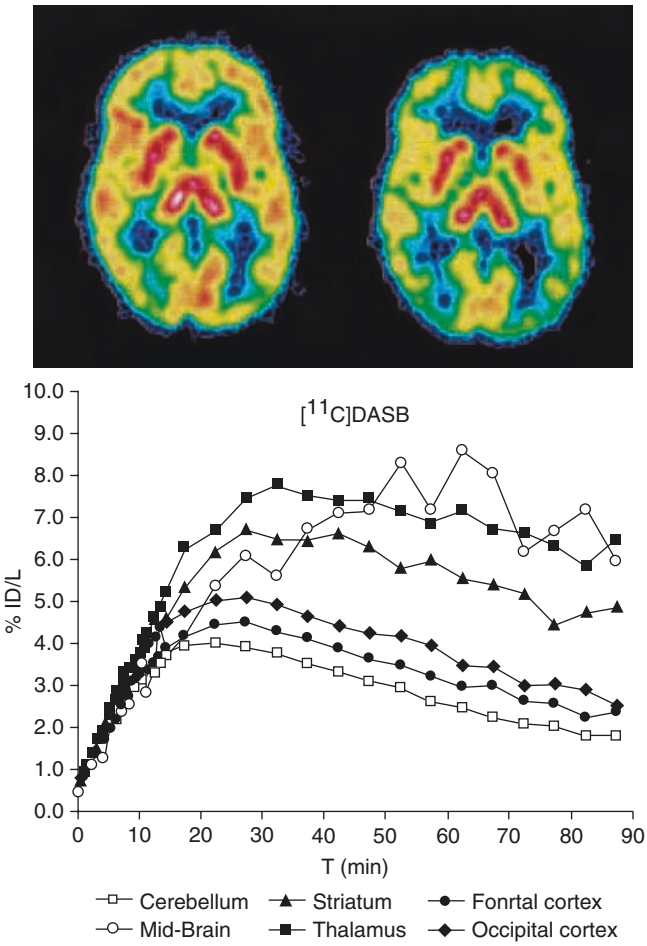
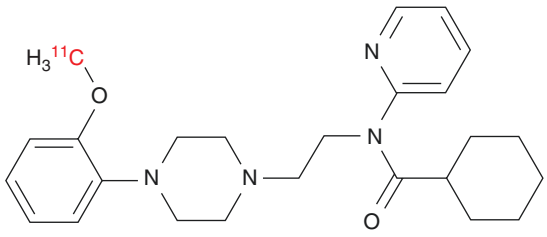


Fig. 31 Effect of citalopram (pretreatment versus post-treatment) upon [^{11}C]DASB scan. Treatment was with 20 mg of citalopram/day for 4 weeks. Images represent summated frames normalized to mean summated cerebellum value. (From Houle *et al.* [10], with permission)

satisfactorily addressed should a prototype compound be labeled and tested. Subsequently, the evaluation of this prototype radiotracer should include the test criteria addressed in 'Part 2'. Problems can arise at any point during the development of a radiotracer. Solving these problems may require revisions to the structure of the prototype radiotracer. In some cases, it may be possible to use an imperfect radiotracer for a particular application. In other cases, however, the project may have to be terminated or pursued using a new starting point or molecular scaffold.



[^{11}C]WAY100635

- High affinity antagonist
- Selective for the 5-HT1A receptor
- Facile radiosynthesis with carbon-11
- Readily crosses the BBB
- Excellent signal-to-noise ration in rats

Fig. 32 Properties of [^{11}C]WAY100635

The Bottom Line

- There are a number of test and design criterion which should be used to maximize the chances of developing a successful radiotracer.
- The following design criteria should be assessed ahead of any experimental work:
  - The choice of an appropriate target
  - High affinity and selectivity for the target
  - Ease of radiosynthesis
  - Maximizing target accessibility while minimizing non-displaceable binding
- A minimum set of test criteria should be examined during the evaluation of a prototype radiotracer:
  - Good signal-to-noise ratio *in vivo*
  - Good *in vivo* pharmacokinetics
  - *In vivo* distribution and pharmacology consistent with literature reports
  - Low levels of radiolabeled metabolites in the region of interest
  - High sensitivity towards the target
- The translation of radiotracers from animals to humans is not always straightforward.

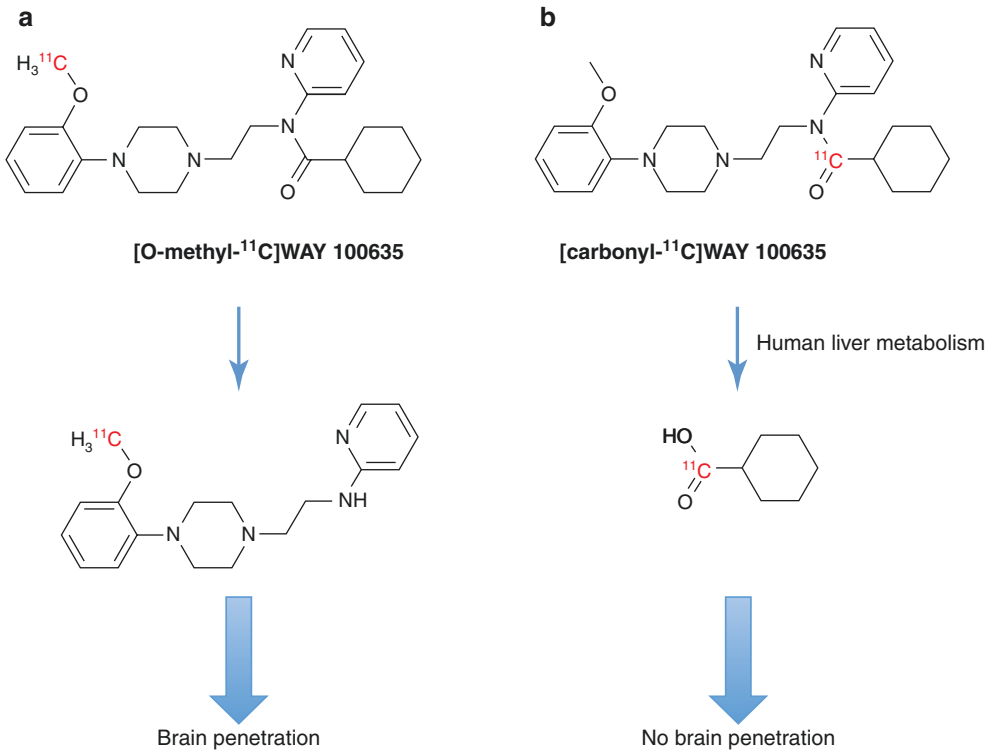


Fig. 33 Radiolabeling of [11C]WAY100635 at methyl and carbonyl positions

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