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The use of PET and knockout mice in the drug discovery process

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Although [^{15}O]H_2O, 2-[^{18}F]fluoro-2-deoxyglucose (FDG) and other radioligands for low-density receptors and enzymes have been used in drug discovery and drug development, the impact on the pharmaceutical industry, to date, has been anecdotal. As new chemical entities are developed, radiotracers that aid in characterizing these drugs need to be developed rapidly to have an impact on the development process. The combined use of positron emission tomography (PET) and gene-manipulated animal models to validate radioligands quickly holds great promise for accelerating the discovery process.

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▼ Positron emission tomography (PET) is an external imaging technique with high sensitivity and resolution that is best suited for monitoring biochemical reactions *in vivo* during the development of a new pharmaceutical. Two 511 keV gamma-rays are emitted at ~180 degrees as a result of the collision between a positron emitted from a radionuclide and a nearby electron. It is the two 511 keV gamma-rays, and not the positron, that are detected by external coincidence circuits. Positron-emitting radionuclides include C-11 ($t_{1/2}$ = 20 min), F-18 ($t_{1/2}$ = 109.7 min) and Br-76 ($t_{1/2}$ = 16 h), all of which are produced in a cyclotron using a nuclear transformation and possess an average specific radioactivity of 500 – 5000 Ci $mmol^{-1}$ at the time of intravenous injection. Thus, the small amount (approximately nanomole) of mass that is injected intravenously is insufficient to significantly affect the steady-state of the biochemical process under investigation. Therefore, the advantage (if not a unique characteristic) of PET, is its ability to measure low-density binding sites without perturbing the biochemistry of the system. Single photon-emitting computed tomography (SPECT) employs radionuclides such as I-123 and Tc-99m, which also possess high specific radioactivity, but the imaging devices are less sensitive and are of a lower resolution. PET scanners are being produced with a 5–6 mm resolution range for human studies and a 1–2 mm resolution range for small animal scanners [1]. Other modalities are being employed for external imaging of low-density sites but have not yet attained the level of sensitivity and quantitative accuracy of PET [2].

The ability of PET to accelerate the drug discovery process

To prove the value of PET in drug discovery there must be a clear paradigm to accelerate the drug development process. The present drug approval process can be as long as 15 years, with the longest periods attributed to the discovery phase and Phase III testing [3]. The greatest impact of external imaging will probably be in the discovery phase if imaging approaches such as PET can reduce the attrition rate in drug discovery by: (1) validating the mechanism of drug localization; (2) establishing the transport efficiency of a drug to the target; (3) establishing the drug occupancy of the saturable receptor sites; or (4) determining the half-time of occupancy of the drug. PET is not useful for detecting potential toxicity because of the discrepancies in the dose required to produce a toxic effect compared with the high specific activity of PET radioligands.

Readily available PET radiopharmaceuticals

To be effective, PET must be able to deliver information in a timely fashion consistent with the timeline of drug development. To date, the development of an appropriate radioligand has not consistently met this demand, especially when a new ligand is required. Information concerning the pharmacokinetics

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(PK) and pharmacodynamics (PD) has traditionally been obtained in humans from the concentration of drug in the blood or in other body fluids, such as urine or cerebrospinal fluid (CSF) [4]. The additional information obtained from the target tissue by PET scanning should increase the reliability of the PK analysis. Although there are presently no comparisons of the reliability of PK data obtained from blood-only analysis and PK data obtained from blood and target tissue analysis using PET, data from the PK analysis of both the target tissue and the blood should be of greater value.

Table 1. Drug candidates studied using [^{15}O]H_2O[a]

Drug	Mechanism	Parameter studied
Lorazepam	Benzodiazepine receptor agonist	Pharmacokinetics, dose–response
U91356a	Dopamine D2 receptor agonist	Dose–response
SKF82958	Dopamine D1 receptor agonist	Dose–reponse, biological half-life

[a]Data from [5].

The most common approach in animals and human subjects is the use of [^{15}O]H_2O, which is used to measure blood flow [5], or 2-[^{18}F]fluoro-2-deoxyglucose {[^{18}F]FDG}, which is a glucose analog [6]. These two radiopharmaceuticals are available in the majority of PET centers and FDG can be shipped to imaging centers from central radiopharmacies. Because of the coupling of regional cerebral blood flow (rCBF), as measured by [^{15}O]H_2O, and metabolism to local neuronal activity, rCBF can be used as a surrogate to assess the effects of drugs on brain activity. This approach has been applied to investigations of drug action in the brain (Table 1) [5]. Transport across the blood–brain barrier is essential for centrally acting drugs and can be investigated by analyzing central PK or PD effects using either of these radiopharmaceuticals.

Although a drug might interact with a particular neurotransmitter system, it can be the downstream consequences, as measured by [^{18}F]FDG, that are of relevance to its pharmacological effect. One example of the importance of [^{18}F]FDG as a downstream probe of drug action is the predictive utility of an FDG scan immediately after the start of Gleevec™ chemotherapy [7]. A radiotracer that binds directly to the tyrosine kinase subtype blocked by Gleevec™ would enable direct measurement of enzyme inhibition but this is not available at present. Nevertheless, the indirect analysis using [^{18}F]FDG is a sensitive probe of Gleevec™ efficacy.

Readily available radioligands for low-density sites

The use of a radioligand with appropriate binding site selectivity and specificity can be used to study potential drug candidate binding to low-density sites. Many universities have filed investigational new drug (IND) applications for a variety of radioligands, most of which are receptor binding radiotracers [8]. A list of these radiotracers can be found on the Society of Non-Invasive Imaging in Drug Development home page (http://www.SNIDD.org). However, an available radioligand implies a known binding site and, therefore, the development of a second-generation drug. Given that newly identified binding sites are the primary goal of pharmaceutical development, these radiotracers might not be generally useful. However, in the case of new drugs that bind to a new combination of multiple receptor binding sites, these radiotracers would be invaluable. One example of this is the testing of the putative dopamine D2 and 5-HT_{2A} receptor antagonist M100907 for binding to the 5-HT_{2A} and the D2 receptor [9]. M100907 is a putative antipsychotic, a class of drug thought to act through the D2 receptor. Using the D2 and 5-HT_{2A} receptor ligand N-[^{11}C]methylspiperone (NMSP), Offord and co-workers used PET to show that M100907 inhibited NMSP binding to the 5-HT_{2A} receptor in the cortex, but did not inhibit binding to the D2 receptor in the striatum and was therefore an atypical antipsychotic agent that did not operate through the D2 receptor [9]. Because N-[^{11}C]methylspiperone binds specifically to the 5-HT_{2A} receptor in the frontal cortex, the authors were also able to determine receptor occupancy by the decrease in C-11 binding. The biological half-life of the drug in the frontal cortex was also determined by carrying out an occupancy study as a function of time. In the single-point occupancy study and the time-dependent occupancy study, the investigators were able to correlate these data with plasma concentrations.

Indirect measurement of neurotransmitter concentration

Another approach is to measure the effect of drugs intended to increase neurotransmitter concentration. For example, the binding of [^{11}C]raclopride (a D2 receptor antagonist) will be decreased in the presence of an increased concentration of synaptic dopamine, such as that observed after an amphetamine bolus injection. This approach has not been used to test neurotransmitter drugs *per se* but the application to drugs that increase dopamine levels is obvious. This approach has been used to investigate dopamine release in schizophrenia. Administration of amphetamine caused a greater release of dopamine in schizophrenia

Table 2. Examples of the use of site-selective radiotracers in drug development[a, b]

Radioligand	Drug	Information	Refs
[^{11}C]Raclopride	Ketamine	Small effect of blocking NMDA receptor on dopamine release	[39]
[^{11}C]WAY100635	Robalzotan	5-HT$_{1A}$ receptor occupancy	[40]
[^{11}C]NMSP	Risperidone	5-HT$_{1A}$ and D2 dopamine receptor occupancy	[41,42]
[^{11}C]Raclopride	Clozapine	5-HT$_{1A}$ and D2 dopamine receptor occupancy	[41,42]
[^{11}C]SCH23390	SDZMAR327	D1 and D2 receptor occupancy	[43]
[^{11}C]Cocaine	Cocaine	Dopamine transporter occupancy	[44]
[^{11}C]Carfentanil	Nalmefene naloxone	Occupancy half-life	[45]
[^{18}F]Setoperone	Clomipramine	5-HT$_{2A}$ receptor occupancy	[46]
[^{11}C]Raclopride	Remoxipride	D2 receptor occupancy	[47]

[a]Abbreviations: NMDA, N-methyl-D-aspartate; NMSP, N-methylspiperone.
[b]Refs 36–38.

patients than in control subjects [10]. Physostigmine, which is an inhibitor of acetylcholinesterase, has also been used to increase the concentration of acetylcholine and decrease the binding of 3-(3-(3-[^{18}F]fluoropropyl)thio)-1,2,5 thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methyl-pyridine {[^{18}F]FPTZTP}, which is a selective muscarinic M_2 acetylcholine receptor ligand [11]. Other drug candidates thought to increase the concentration of synaptic acetylcholine could be studied using the same approach.

In summary, there are relatively few examples of the use of PET in drug development. With the readily available PET radionuclides C-11 and F-18, PK and biodistribution analyses are limited because of the short physical half-life of these two radionuclides and the relatively long biological half-life of drugs that are taken once or twice a day. However, short-term factors such as blood–brain barrier transport, drug disposition in the lung [12] and the early distribution phase of drugs can be measured. The major use of PET has been in receptor occupancy studies, either at a single time point or as a function of time after drug dosing (Table 2). These parameters are important to the successful development of a proprietary pharmaceutical. However, the development of new radiotracers for new drug targets should enable the increased use of PET in drug discovery and development in the future.

New radiotracers for new drug targets

Proteomics should have a major impact on the development of new pharmaceuticals. The development of Gleevec™ is an impressive example [7], and early data show this to be a very effective chemotherapeutic agent. The ideal approach to monitoring the effectiveness of chemotherapy would be to radiolabel a probe and directly assess the interaction of the chemotherapeutic agent with the low-density binding site. A potential probe for radiolabeling must be chosen, but the classical approach to validating a new radiotracer is a time-consuming process and not compatible with the drug development process. Therefore, there might not be enough time in all situations to proceed systematically through the series of validation steps described here. The radiotracer must be developed in concert with the drug discovery and development timeline.

Use of a model to predict the maximal target to non-target ratio for potential binding site-specific radiopharmaceuticals

Having chosen a biochemical system relevant to a disease state, the use of a mathematical model to choose potential binding site-specific radiopharmaceuticals is key. A simple model was suggested by Eckelman [13] and Katzenellenbogen [14] as a first approximation. At high specific radioactivity, the maximal bound:free ratio (B:F) ratio will be equivalent to the receptor concentration (Bmax) / ligand affinity (K_d). Distribution factors, protein binding and metabolism will decrease the maximal B:F ratio. Therefore, this approach is necessary but not sufficient. This estimation is particularly important when targeting low-density (picomolar levels) binding sites because this will necessitate choosing radiotracers with sub-picomolar binding affinities.

Use of [^{3}H]-labeled compounds to determine distribution in vivo

With the number of [^{3}H]-labeled compounds now available commercially, the use of [^{3}H]-labeled compounds in rodents is an efficient method to determine if a particular binding site ligand is suitable for radiolabeling with a gamma-emitting radionuclide.

The preparation of the non-radioactive analog and determination of in vitro *and* in vivo *stability*

The preparation of a non-radioactive analog avoids the problems associated with high specific activity until the substituted ligand is shown to be a true tracer for the unsubstituted ligand, particularly in those cases where the substitution of the radionuclide introduces a substantial perturbation. This step also produces the necessary reference compounds for the radiolabeled compound.

Most compounds are either chemically unstable and/or metabolized *in vivo*. Therefore, the stability of the new derivative should be determined in plasma and in liver. The most convenient model for the enzymatic activity of the liver is commercially available cryopreserved hepatocytes. One difficulty with using the non-radioactive compound for stability testing is caused by the large concentrations of compound used, which could result in a second-order enzyme reaction whereas the high specific radioactivity ligand will most likely be in a pseudo first-order reaction. This problem has been solved with the use of liquid chromatography–mass spectrometry (LC–MS) analysis where picograms of material can often be analyzed [15,16].

The evaluation of various physical parameters of the non-radioactive derivative (structure–distribution relationship)

Just as structure–activity relationships have been the foundation of classic drug design, structure–distribution studies are the backbone of radiopharmaceutical development. The use of both theoretical and experimental parameters can direct the choice of radioligand.

Preparation of the radioactive derivative and use of pre-injection, co-injection or post-injection to decrease effective specific radioactivity of the radioactive derivative

One important design criteria for a new series of drugs is the incorporation of a moiety that will allow easy radiofluorination or C-11 methylation. Animal distribution studies using the radiolabeled ligand are the most important experiments. It is a requirement that the radioactivity be present in the target organ with a target:non-target ratio of approximately two for PET, and a higher ratio is necessary for SPECT and planar imaging. Radioligands for saturable binding sites must also show specific binding. In general, this is carried out by using pre-, co- or post-injection of a known binding biochemical or drug. The distribution of the radioligand must meet the criteria of high affinity, specificity (including stereospecificity), saturability and correlation with biological activity. There are situations where these criteria cannot be tested. These occur if the distribution of the binding site is homogeneous throughout the target organ or if there are no specific ligands for that binding site from a different chemical class.

Measurement of sensitivity of the radioligand, most often in non-human primates

Kinetic sensitivity has been defined as the ability of a physiochemical parameter to alter the time–activity data of a radiotracer [17]. In the context of radiotracers for high-affinity sites, the time–activity curve in the target organ should be sensitive to a change in binding site density. To use the more practical single-scan technique [18], the change must be recorded at a single time point. This information is often normalized to the metabolite-corrected plasma concentration of the radiotracer or an equivalent reference organ, and either the original target organ concentration or these target:non-target ratios are compared to the receptor concentration obtained by analyzing tissue samples *in vitro*.

This approach to validation is thorough but time-consuming. In order for the timeline on new radioligand development to match the timeline required in drug development, a streamlined approach is necessary. In this case, the use of post-genomic technology, in the form of the gene-manipulated mice, is a possible solution.

Knockout mice

Knockout mice have proven invaluable in validating new radiotracers as ligands for a specific receptor. The biodistribution of the proposed binding site-specific radioligand is determined in groups of wild-type and knockout mice homozygous (–/–) for the target of interest. The difference in binding of the radioligand in wild-type and knockout mice can be attributed to the absence of the specific binding site, all other variables being equal. This is important given that doses of drug to inhibit binding at the binding site of interest are rarely specific, especially at the doses needed to saturate the binding site. Therefore, knockout mice represent a solution to the time-consuming process of validating new radiotracers that is quantitative and requires minimal experimentation.

PET in Alzheimer's disease

The early detection of Alzheimer's disease using PET imaging has thus far not been possible, although several radiotracers are being evaluated. The post-genomic approach to Alzheimer's disease detection involves the development of drugs and radioligands for the major genetic changes in presenilin-1 and -2, amyloid precursor peptide and the epsilon4 allele of apoE [20]. Traditional approaches to the detection of Alzheimer's disease involve the targeting of drugs or radioligands at sites of characteristic

neuropathology, such as neurofibrillary tangles and amyloid plaques. Amyloid plaques have been investigated using radiolabeled analogs of Congo Red, which pathologists have used for some time to identify amyloid plaques *in vitro* [21]. Another approach is to use receptor density changes from autopsy studies as a guide [22–24]. For muscarinic receptors, the M_4 subtype does not appear to be an important target because of the lack of change in receptor density as a function of Alzheimer's disease. Likewise, the M_3 receptor subtype is expressed at a low density and would not provide a sensitive target. However, both the M_1 receptor and the M_2 receptor show significant changes in receptor density as a function of Alzheimer's disease. From autopsy studies, there are indications that there is selective loss of M_2 receptors in the hippocampus and a trend towards a decrease in the cortex. In addition, an increase in the density of the M_2 receptor subtype in the striatum was observed. The M_1 and M_2 receptor subtypes might be suitable targets for investigating Alzheimer's disease, although the autopsy studies were small in number and carried out on patients who had been treated with various drugs. Agonists measure both the receptor density and the affinity state of the receptor, whereas antagonists measure only receptor density. Therefore, agonists are likely to be more useful radiotracers {e.g. the M_2 receptor agonist [^{18}F]FPTZTP} [25].

The use of knockout mice in Alzheimer's disease

Based on experiments involving the radiolabeling and validating of [^{18}F]FPTZTP and [^{18}F]paclitaxel (FPAC), the knockout mouse appears to be the most expeditious method for radiotracer validation [26,27]. The pharmacological approach is time-consuming, and might not give a definitive answer [28]. A smaller number of experiments are involved in radiotracer validation using knockout mice compared with validating a new tracer by testing saturability, specificity and distribution characteristics. The knockout mouse approach is therefore more suitable for drug development. Knockout mice represent a clearly defined biochemical change whereas pharmacological intervention rarely represents a simple biochemical change. Many binding site-specific molecules are not specific at the doses needed to block the binding site in order to prove saturable binding.

Regional brain localization of [^{18}F]FPTZTP in M_2 receptor knockout mice compared with wild-type mice, M_1 receptor knockout mice, M_3 receptor knockout mice and M_4 receptor knockout mice clearly shows the preference of [^{18}F]FPTZTP for the M_2 receptor subtype [27]. With the availability of knockout mice, these validation experiments can be completed in a matter of weeks rather than the months necessary for the full pharmacological approach.

Knockout mice and glycogen storage disease

Knockout mice have been used to show that [^{18}F]FDG is a sensitive probe of changes in 6-glucose phosphatase (G6Pase) levels [29]. The monitoring of gene therapy of glycogen storage disease type 1a in a mouse model was achieved using [^{18}F]FDG and a dedicated animal scanner. The G6Pase knockout mice were compared with knockout mice infused with a recombinant adenovirus containing the murine gene encoding G6Pase (Ad-mG6Pase). Serial images of the same mouse before and after therapy were obtained and compared with wild-type mice of the same strain to determine the uptake and retention of [^{18}F]FDG in the liver. Image data were acquired from heart, blood and liver 20 min after injection of [^{18}F]FDG. The retention of [^{18}F]FDG was lower for the wild-type mice compared with the knockout mice. The mice treated with adenovirus-mediated gene therapy showed [^{18}F]FDG retention similar to that found in age-matched wild-type mice [29]. These studies show that FDG can be used to monitor G6Pase concentration and, therefore, the progress of glycogen storage disease.

Knockout mice in multi-drug resistance (MDR)

[^{18}F]FPAC is a sensitive probe for P-glycoprotein (P-gp), a protein responsible for multidrug resistance. Paclitaxel (Taxol®) is a clinically important chemotherapeutic agent. [^{18}F]FPAC shows high uptake into and rapid clearance from tissues in rats [30]. Pre-administration of paclitaxel in rats significantly increases the retention of [^{18}F]FPAC in blood (33.0% increase in retention), heart (32.0%), lung (37.6%) and kidney (142.4%). Biodistribution and radiation dose estimates for [^{18}F]FPAC have been obtained in monkeys, and the effects of a P-gp blocker, XR9576 (Xenova, http://www.xenova.co.uk), on FPAC kinetics [31] have also been studied. Liver uptake of FPAC was significantly affected by XR9576.

Studies with mdr1a/1b (–/–) knockout mice showed significant increases in the uptake of [^{18}F]FPAC in the heart, lungs, femur, muscle and brain compared with wild-type mice. Changes in the uptake of [^{18}F]FPAC resulting from pre-injection of unlabeled paclitaxel were significant only in the lung and kidney of wild-type mice. P-gp-mediated uptake of paclitaxel in the lung is suggested by three results: (1) the differential retention of radioactivity in the lungs of wild-type compared with knockout mice; (2) the increased lung uptake of paclitaxel in wild-type mice after pre-injection of paclitaxel; and (3) the increase in [^{18}F]FPAC uptake observed with

paclitaxel pre-injection in rats. The large increase in uptake observed in the brain of mdr1a/1b (–/–) knockouts compared with wild-type mice confirms that [^{18}F]FPAC is excluded from the brain by P-gp. Therefore, [^{18}F]FPAC is a substrate for P-gp and might be useful for *in vivo* imaging of P-gp-mediated efflux. Knockout mice have been used to screen potential drug candidates as probes for P-gp but no candidates have been proposed for further clinical development [32]. Slapak and co-workers used P-gp knockout mice to validate ^{99m}Tc sestamibi as a P-gp substrate [33] and [^{11}C]verapamil has been validated using the same technique [34].

Applying preclinical information to human studies

To apply the information obtained from knockout mice to clinical studies, experiments must be carried out to confirm that the PK and PD are similar. Metabolic differences are the most likely confounding factor. With the availability of mouse and human hepatocytes and the improved sensitivity of LC–MS, metabolite identification in both species can be easily ascertained. Therefore, the metabolic profile of FPTZTP was studied in rat and human hepatocytes using LC–MS and compared with independently synthesized standards, where these were available [15]. In both human and rat hepatocytes, the major metabolite is produced by oxidation of the nitrogen in the 1-methyltetrahydropyridine ring. However, the N-oxide was detected at lower levels in humans compared with rat hepatocytes. Other metabolites arise from sulfur oxidation, demethylation of the tertiary amine and oxidation of the tetrahydropyridine ring. The metabolism of FPTZTP *in vivo* in rats is similar to metabolism in rat hepatocytes [15]. In this case, one could expect similar behavior in terms of blood–brain barrier transport and metabolite profile. Other parameters such as specific binding and drug occupancy depend on the relative density of the targeted binding site between rodents and humans. This is often obtained from *in vitro* data.

In another study, the metabolic profile of N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridyl) trans-4-fluorocyclohexanecarboxamide (FCWAY) in rat and human hepatocytes was different. When FCWAY was studied in rat hepatocytes, the oxidation product of the orthomethoxyphenylpiperidine was the major metabolite. In human hepatocytes, the major product was the result of the hydrolysis of the amide bond, which produced a cyclohexanecarboxylic acid and the phenylpiperidine ethylamine moiety [16]. In this case, the issues relating to the toxicity and biodistribution of metabolites is different for rats and humans and must be taken into account.

Conclusions

Time is of the essence in drug development and shortening the 'bench to bedside' timeline can be a tremendous advantage to a pharmaceutical company [35]. If the power of *in vivo* imaging is to be used to its full potential, the radiolabeled probe must be developed in parallel with the development of the new chemical entity. Radionuclide imaging has been used in all phases of drug discovery and development, but might be applied most effectively in the latter phases of preclinical screening when the number of candidates is less than ten. The requirements for radiolabeling would not permit this approach to be applied to significantly greater numbers of drug candidates. The value of evaluating new drug candidates using knockout mice comes from the rapid evaluation of drug PK and PD, in addition to validation of drug binding to the specific target. This approach is particularly advantageous when compared with the classical pharmacological approach because of the increased specificity and the decreased time to perform the key experiments. The knockout mice can be used to determine blood–brain barrier penetration, binding to the specific target and target occupancy if the target density is similar between rodents and humans. The use of LC–MS and hepatocyte preparations allows the differences in metabolism between species to be assessed. In humans, the major use of PET has been, and will continue to be, in occupancy studies, either at a single time point or as a function of time after drug dosing. Driven by many advances in technology and the use of knockout mice and LC–MS, PET imaging is rapidly becoming a major force in drug discovery.

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