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Label-free assay for the assessment of nonspecific binding of positron emission tomography tracer candidates

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ABSTRACT

Positron emission tomography (PET) is a valuable non-invasive technique for the visualization of drug tissue distribution and receptor occupancy at the target site in living animals and men. Many potential PET tracers, however, fail due to an unfavorably high non-specific binding (NSB) to non-target proteins and phospholipid membranes which compromises the sensitivity of PET. Hence, there is a high demand to assess the extent of NSB as early as possible in the PET tracer development process, preferentially before ligands are radiolabeled and elaborate imaging studies are performed. The purpose of this study was to establish a novel Lipid Membrane Binding Assay (LIMBA) for assessing the tendency of potential tracers to bind non-specifically to brain tissue. The assay works with unlabeled compounds and allows the medium-throughput measurement of brain tissue/water distribution coefficients, logD_{brain} (pH 7.4), at minimal expense of animal tissue. To validate LIMBA, logD_{brain} (pH 7.4) values were measured and compared with NSB estimates derived from *in vivo* PET studies in human brain (n = 10 tracers, literature data), and *in vitro* autoradiography studies in rat and mouse brain slices (n = 30 tritiated radioligands). Good agreement between logD_{brain} (pH 7.4) and the volume of distribution in brain of non-specifically bound tracer in PET was achieved, pertaining to compounds classified as non-substrates of P-glycoprotein ($R^2 \geq 0.88$). The ability of LIMBA for the prediction of NSB was further supported by the strong correlation between logD_{brain} (pH 7.4) and NSB in brain autoradiography ($R^2 \geq 0.76$), whereas octanol/water distribution coefficients, logD_{oct} (pH 7.4) were less predictive. In conclusion, LIMBA provides a fast and reliable tool for identifying compounds with unfavorably high NSB in brain tissue. The data may be used in conjunction with other parameters like target affinity, density and membrane permeability for the selection of most promising compounds to be further investigated *in vivo* as potential novel PET tracers.

1. Introduction

Positron emission tomography (PET) has emerged as a valuable tool in the drug development process to study the biodistribution and receptor occupancy of drug candidates at the target site in living animals and men (Fernandes et al., 2012). The value of PET as a non-invasive imaging technique is of particular interest in the field of CNS drug discovery where large discrepancies may arise between plasma and biophase pharmacokinetics and, moreover, where animal models may not adequately reflect the complexity of human disease (Danhof et al., 2007).

In order to exploit the full potential of PET for drug development, drug candidates and the corresponding PET tracers for the target site of interest should ideally be developed in parallel. However, there is still a lack of suitable PET tracers for many targets, and their innovation is challenging given the large number of sometimes conflicting requirements to be fulfilled, particularly for imaging agents that bind to specific targets in the brain (Honer et al., 2014; Laruelle et al., 2003). A high binding sites density is desirable and only the unmetabolized radiotracer should pass the blood–brain barrier to bind with high affinity and specificity to the target site of interest (specific binding, SB) (Ametamey and Schubiger, 2006). In contrast, non-specific binding (NSB) to non-target proteins and lipids should be low to ensure a high SB to NSB ratio. The higher the ratio, the higher the sensitivity of PET to monitor changes of available binding sites due to drug-related receptor occupancy (Laruelle et al., 2003; Pike, 1993). Effective radiotracers should achieve SB/NSB ratios in the range of SB/NSB = 3–10 to be successfully applied *in vivo* (McCarthy et al., 2009; Pike, 1993), yet, Laruelle et al. (2003) summarized that "most of the failures in ligand development result from an unfavorable combination of target density, ligand affinity and nonspecific binding".

The full characterization of a PET tracer is time-consuming, expensive and requires expert knowledge of a radiotracer imaging team as

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well as special technical facilities (Wong and Pomper, 2003). Less resource-demanding *in vitro* assays that allow the selection of compounds with optimal properties are therefore applied to facilitate the development of PET tracers and to focus resources on the most promising compounds. *In vitro* autoradiography (incubation of radioligands with tissue slices), for example, can be performed to obtain a primary measure of the SB/NSB ratio, without the need to incorporate positron emitting nuclei into the ligands. Tracers are rejected when the SB/NSB is too low, *i.e.* when a major part of the total binding (TB = SB + NSB) in the brain region of interest (ROI) is originating from an unfavorably high NSB, as assessed in a reference region (R_{REF}) devoid of receptors. Yet, failing at this point of the tracer development process is costly given that autoradiography studies are time-consuming, require radiolabeling of the ligand and the sacrifice of animals.

To provide a faster and less resource-intensive estimation of NSB, *in vitro* methods working with the unlabeled compound are highly desirable. The octanol/water distribution coefficient, logD_{oct} (pH 7.4) is still the commonly used parameter for assessing the NSB of potential PET tracers, albeit with limited prediction accuracy for structurally unrelated compounds (Dickson et al., 2011; Laruelle et al., 2003). The unbound fraction of a ligand in brain, $f_{u,brain}$, available from equilibrium dialysis with brain homogenate (Kalvass and Maurer, 2002; Liu et al., 2009; Summerfield et al., 2007), provides a physiologically more relevant NSB-estimate which has successfully been applied for the parameterization of a comprehensive radiotracer kinetic model (Guo et al., 2009). Likewise, Fridén et. al (Friden et al., 2014) proposed using the unbound volume of distribution ($V_{u,brain}$) available from the brain slice uptake technique (Friden et al., 2009) to predict NSB in PET.

However, a drawback of the homogenate dialysis and the brain slice uptake technique is the high consumption of tissue and the relatively low throughput, respectively, which impedes the screening of larger datasets. Recently, a vesicle electrokinetic chromatography (VEKC) method working with an artificial anionic detergent (Sodium bis(2-ethylhexyl) sulfosuccinate, AOT) has been developed for the prediction of NSB showing a good correlation between chromatographic retention factors and *in vitro* NSB derived from equilibrium dialysis. The VEKC method has also been shown to provide a useful tool for the classification of either successful or terminated PET ligands tested *in vivo*, however, on a qualitative level only (Jiang et al., 2011).

Quantitative *in vivo* NSB estimates, expressed as the volume of distribution of non-specifically bound tracer in brain, have been derived from kinetic analysis of PET scans and were used for the validation of a computational NSB prediction method. A good correlation with calculated drug-lipid interaction energy was found (Dickson et al., 2011; Rosso et al., 2008), yet, the *ab initio* quantum mechanical method was computationally expensive and relied on the interaction of the ligand with 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine (DOPC) neglecting the full complexity of a biological membrane.

The purpose of the present study was to test whether a novel *in vitro* assay, referred to as Lipid Membrane Binding Assay (LIMBA), proves useful for estimating the NSB of potential PET tracers. LIMBA requires only small amounts of rat brain tissue for the label-free assessment of tissue binding. The medium-throughput assay was recently developed in our laboratory and yields brain tissue/water distribution coefficients, logD_{brain} in excellent agreement with $f_{u,brain}$ (homogenate dialysis) (Assmus, 2015; Assmus et al., in preparation-b), even when brain tissue was replaced by a microemulsion of brain polar lipids (Belli et al., in preparation). In the course of this study, we evaluated the ability of LIMBA for predicting NSB on a set of i) 30 publicly available and proprietary tritiated radioligands for which NSB-estimates in brain were available from *in vitro* autoradiography studies (in-house data, in rat and mouse), and ii) 10 PET tracers for which the volume of distribution of non-specifically bound drug related to the free plasma concentration (V_{NS}/f_p) was retrieved from *in vivo* PET studies (literature data, in human).

2. Materials and methods

2.1. Materials

Fallypride and 3-amino-4-[2-[(di(methyl)amino)methyl]phenyl] sulfanylbenzonitrile (DASB) were purchased from ABX (Radeberg, Germany). Diprenorphine, SCH 23390 and SR 222200 were obtained from Tocris (Bristol, UK). Flumazenil and raclopride tartrate were purchased from Sigma (Steinheim, Germany). All other drugs were available through our in-house Compound Depository Group as proprietary compounds. TRIS, TAPSO and sodium chloride were obtained from Fluka (Buchs, Switzerland). Octanol and dodecane were purchased from Sigma (Steinheim, Germany). DMSO was obtained from Acros (Geel, Belgium). Water and acetonitrile for HPLC were supplied from Merck (Darmstadt, Germany) and were of HPLC-grade.

2.2. Determination of n-octanol/water distribution coefficients

Unless otherwise stated, logD_{oct}(pH 7.4) values were measured with a miniaturized shake flask technique. In brief, a DMSO stock solution of the test compound (0.5 mM) was dispensed in aqueous buffer (50 mM TAPSO/pH 7.4, final DMSO content 5%) which was pre-saturated with octanol. The aqueous drug solution (25 μM) was filtrated and an aliquot of the filtrate was transferred to a 96-well PCR plate (Eppendorf). After adding buffer-saturated octanol on top of the filtrate, the PCR plate was sealed and incubated for 2 h at room temperature (RT), while shaking. The plate was left undisturbed overnight and then centrifuged for 10 min at 3000 rpm (Eppendorf, Centrifuge 5810R). The aqueous drug solution was sampled with a robotic pipetting system (Tecan) and the equilibrium aqueous drug concentration, C_{aq}^{eq}, was determined with a UV plate reader (SpectraMax). In parallel, a reference experiment was carried out under the same conditions but without octanol in order to obtain the initial aqueous drug concentration, C_{aq}^{0}. LogD_{oct} (pH 7.4) values were obtained by mass balance according to

$$\log D_{oct} = \log\left(\frac{\left(C_{aq}^{0} - C_{aq}^{eq}\right)\cdot V_{aq}}{C_{aq}^{eq}\cdot V_{oct}}\right), \quad (1)$$

where V_{aq} and V_{oct} denote the volume of the aqueous and the octanol phase, respectively.

2.3. Determination of brain tissue/water distribution coefficients with LIMBA

Brain tissue (rat) /water distribution coefficients, logD_{brain} (pH 7.4), were determined with the in-house LIMBA assay as described below. Briefly, drug-naive female Wistar rats were sacrificed in a CO_2 chamber and the whole brain was sampled and immersed in assay buffer (50 mM TRIS/114 mM NaCl/pH 7.4) to remove adherent blood. The rat brain was weighted and homogenized on ice in 2 volumes (w/v) of n-dodecane using an ultrasonic probe (Branson sonifier, G. Heinemann, Schwäbisch Gmünd, Germany). Experiments were conducted in accordance with the current Cantonal and Federal legislation on the welfare of experimental animals. For the preparation of the aqueous phase, test compounds were introduced into the assay buffer (50 mM TRIS/114 mM NaCl/pH 7.4) as DMSO stock solutions (10 mM). The resulting aqueous drug solution (25 μM, DMSO content 0.25%) was filtrated (NUNC 278752) and aliquots of the filtrate (V_{aq} = 50 μL) were transferred into an in-house made Teflon plate. In parallel, the rat brain homogenate (V_{br} = 1.2 μL) was coated on a PVDF filter attached to the bottom of the customized and commercially available DIFI tubes (Weidmann Plastics Technology AG, Rapperswill, Switzerland, 23358) (Fischer et al., 2006). The pipetting was conducted with a Hamilton robotic system using new pipetting tips (disposable, 10 μL) for each coating step and a sealed 96 well PCR plate (Eppendorf) for the storage of brain