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Concentration of receptor and ligand revisited in a modified receptor binding protocol for high-affinity radioligands: [³H] spiperone binding to D₂ and D₃ dopamine receptors

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Abstract

In receptor binding assays with ultra-high-affinity radioligands, it is difficult, in practice, to adhere to the golden rule that the receptor concentration in the assay should be substantially (at least 10-fold) lower than the dissociation constant (K_d) of the radioligand and inhibition constant (K_i) of compound. Especially for low specific activity radioligands (usually tritiated ligands of a couple of TBq/mmol), routinely applied in concentrations at around or below the K_d , the use of extremely small amounts of receptor protein per assay will result in low levels of bound radioactivity; the alternative use of larger assay volumes will make it difficult to apply 96-well filtration devices. For assessing the inhibition constant (K_i) of competitive inhibitors under conditions violating the above golden rule, equations are available incorporating both [receptor] and [ligand] versus K_d ; however, their application requires precise knowledge of [receptor] or initial bound/free [radioligand] ratio. In this study, we present the theoretical basis for determining the K_i for a competitive inhibitor in a new protocol at high [protein] and high [radioligand] with the simple Cheng-Prusoff correction without the need to correct for [receptor] or initial bound/free [radioligand] ratio. In addition, we present results on the binding of the ultra-high-affinity ligand [³H]spiperone to dopamine D₂ and D₃ receptors validating the K_i values calculated with the new protocol for competitive inhibitors as compared with those calculated with the most comprehensive equation available to date, that of Munson and Rodbard (1988). Binding was measured at varying [radioligand] and [receptor], test compounds (including (–)-5-OH-DPAT, (±)-7-OH-DPAT, and ropinirole) were run with varying [receptor], and simulations were done at vastly varying [radioligand] for inhibitors with vastly different K_i s. The modified high [radioligand] protocol presented here removes a major hindrance in the proper execution of binding assays with ultra-high-affinity tritiated ligands with K_d values in the subnanomolar range, allowing the use of 96-well plates with small volumes of 100–200 µl per binding assay.

Keywords

competition inhibition curve; dopamine receptor; high-affinity binding assay; spiperone; receptor concentration

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1. Introduction

Dopamine (DA) receptors are G-protein-coupled receptors broadly categorized as D₁-like (stimulating adenylate cyclase) or D₂-like (inhibiting adenylate cyclase). Within the latter category, D₂ and D₃ subtype DA receptors have been studied abundantly as targets for antipsychotic and antiparkinsonian medications, respectively (Sokoloff et al., 1992; Girault and Greengard, 2004; McCall et al., 2005; Strange 2008). Although D₂ and D₃ receptors are regulated differently, they share a similar molecular structure and pharmacological profile (Sokoloff et al., 1990; Werner et al., 1996; Levant, 1997). Site-directed mutagenesis and molecular modeling studies have provided insight into differences in the ligand-binding domain of D₂ and D₃ receptors (Mansour et al., 1992; Sokoloff et al. 1992; Lundstrom et al., 1998; Watts et al., 1998; Sartania and Strange, 1999; Strange, 2001; Dutta et al., 2002; Mach et al., 2003; McCall et al. 2005; Saur et al., 2007).

Because of the similarity between D₂ and D₃ receptors, development of compounds selective for one over the other receptor subtype is challenging. An essential component of the drug development process is the ability to test the receptor potency of compounds with a protocol that is devoid of artifactual distortions due to improper conditions for the receptor under study. In the case of D₂ and D₃ receptors, competitive inhibition of [³H]spiperone binding has been used widely to measure compound affinities. Most antipsychotics, including spiperone, bind to both D₂ and D₃ receptors with high affinity, i.e. nanomolar or sub-nanomolar K_d (equilibrium dissociation constant) (Gardner et al., 1996; Strange, 2001). A ground rule for receptor binding studies with competitive inhibitors is that the concentration of radioactive ligand in relation to its K_d must be taken into account in converting the IC₅₀ (concentration of compound to inhibit 50% of radioligand binding) to K_i (equilibrium dissociation constant of inhibitor) with the Cheng-Prusoff correction (Cheng and Prusoff, 1973). It is important to recall that in the Cheng-Prusoff equation, IC₅₀ denotes the free (not totally added) concentration of inhibitor giving 50% inhibition, and the concentration of radioactive ligand is the free (not totally added) concentration. Differences between free and totally added concentrations can be minimized - creating valid conditions for applying the Cheng-Prusoff equation - by making sure that less than 10% of the total added radioligand is actually bound (zone A binding, see Bennett and Yamamura, 1985), and that the receptor concentration in the assay is substantially (at least 10-fold) lower than the K_d or K_i of radioligand or inhibiting compound, respectively (Chang et al., 1975; Bennett and Yamamura, 1985). In order to minimize the impact of the Cheng-Prusoff correction, most researchers also choose a radioligand concentration at or below its K_d. In the case of [³H]spiperone or other tritiated ultra-high-affinity ligands that bind with a subnanomolar K_d, the above considerations necessitate the use of extremely low receptor concentrations that result in low levels of bound radioactivity with routinely used binding assay volumes of 0.2 ml or less in 96-well filtration devices combined with 96-format plate scintillation counting. With deep-well plates and some harvesters, one can increase the assay volume from 0.2 to 0.8 ml in order to get a higher amount of bound radioactivity without increasing the receptor concentration but this factor of 4 is not always enough for obtaining sufficient counts for proper counting; deep-well plates are also harder to work with in terms of making additions and ensuring proper mixing of assay components. One could, for ultra-high-affinity ligands, decide to increase the receptor concentration beyond the recommended 10% of K_d, and compute the K_i for a competitive inhibitor by applying equations that take into account the receptor concentration (Jacobs et al., 1975; Linden, 1982). This is problematic in experiments with brain tissue where the receptor density is not known unless specifically measured, and even in cell lines stably expressing receptors where receptor density can be affected by cell culture confluence, culture time and passage number, as well as culture medium conditions. The most comprehensive treatment of ligand and receptor concentration available to date is with an equation that requires the initial (without inhibitor) ratio of bound over free radioligand rather than the receptor concentration (Munson and Rodbard, 1988). The Munson-

Rodbard equation shows that high receptor concentrations by themselves (even with radioligand concentrations substantially below K_d), can result in high correction factors going from IC_{50} to K_i . For example, with an initial bound over free ratio of 5, radioligand over K_d ratio of 0.1, and $K_i \sim K_d$, the correction factor is 10. Day-to-day variation in initial bound over free will have substantial impact on the computed K_i values.

In this study, we present the theoretical basis for determining the K_i inhibitor at high receptor protein and high radioligand concentration with the simple Cheng-Prusoff correction without the need to correct for receptor concentration or initial bound over free. In addition, we present results on [3H]spiperone binding to DA D_2 and D_3 receptors validating the K_i values calculated in this manner as compared with those calculated with the Munson-Rodbard equation (1988). Simulations were run at vastly varying [radioligand] for inhibitors with vastly different K_i s for assessing the accuracy of the new protocol. The method presented here allows small-volume assays in 96-well format and can be applied to other ultra-high-affinity radioligand binding studies.

2. Materials and methods

2.1. Materials

Human embryonic kidney (HEK) 293 cells, stably transfected with rat D_{2L} and rat D_3 cDNAs, were gifts from Dr. Kim Neve (Portland VA Medical Center, Portland, Oregon). They were cultured in Dulbecco's Modified Eagle Medium, supplemented with 10% Bovine Calf Serum, 2mM glutamine and 2 μ g/ml puromycin to keep selection pressure at 37°C and 5% CO_2 . [3H]spiperone (15Ci/mmol) was purchased from PerkinElmer (Waltham, Massachusetts). (–)-5-OH-DPAT, (±)-7-OH-DPAT, ropinirole, D341, D342, D343 and D344 were synthesized by Dr. Alope Dutta (Wayne State University, Detroit, MI). Other chemicals were provided by commercial sources.

2.2. [3H]Spiperone binding assays

“Hot saturation” assays were performed to determine the dissociation constant of [3H]spiperone to DA D_2 and D_3 receptors. Crude membranes were prepared from HEK293-r D_2 and HEK293-r D_3 cells, as described before (Zhen et al., 2004). In a total reaction volume of 0.8ml, [3H]spiperone (0.3 pM to 3 nM, final concentration) and crude membranes were incubated together in assay buffer (50 mM Tris-HCl, pH 7.4, with 0.9% NaCl, 0.025% ascorbic acid and 0.001% Bovine Serum Albumin) for 1 h at 30°C in deep-well plates in a water-bath shaker. 2 μ M (+)-butaclamol was used as nonspecific binding definition for each concentration of [3H]spiperone. Assays were terminated by the addition of ice-cold 0.9% saline and filtration through a glass fiber filtermat in the Brandel-96 cell harvester. The filtermat was being washed 3 times and dried under hot air. The radioactivity was counted in a Microbeta liquid scintillation counter (PerkinElmer) after adding 10 ml of Betaplate Scint (PerkinElmer) to each filtermat. In competitive inhibition assays, appropriate concentrations of non-radioactive spiperone were included. Kinetic parameters (K_d , B_{max} , and IC_{50}) were calculated using the computer-fitting programs LIGAND (Biosoft, Cambridge, UK) and ORIGIN (Microcal Software, Inc., Northampton, MA, USA). Protein level was determined with the Lowry method (see (Zhen et al., 2004)). IC_{50} values were converted to inhibition constants (K_i) by the Cheng-Prusoff equation (Cheng and Prusoff, 1973) and Munson-Rodbard equation (Munson and Rodbard, 1988). To validate the modified protocol, the reaction volume was reduced from 0.8 ml (deep-well plates) to 0.2 ml (regular plates) with increased concentration of [3H]spiperone and protein level under conditions as described in the text.

2.3. Data simulation of competitive inhibition with random error added

Competitive inhibition was simulated to examine the accuracy of the modified binding protocol with high concentration of radioligand. The K_d was set at 1 nM; K_i values were calculated as described by Cheng and Prusoff (1973) and Munson and Rodbard (1988) with varying concentrations of receptor and radioligand. The ratio of Cheng-Prusoff K_i to Munson-Rodbard K_i was calculated to judge the error introduced to K_i when [receptor] was higher than K_i . In a different simulation set, competition curves were generated based on a one-binding site model with relative random error added as follows. In the presence of inhibitor ($K_i=1$ nM), we chose three conditions for generating binding curves, i.e. at concentrations of radioligand equal to 0.1 nM, 1 nM or 10 nM. Under all the conditions, the initial bound/free ratio was set to 0.1. The actual random values of bound equaled to the sum of the theoretic values and random errors calculated from a Gaussian distribution with a relative S.D. equal to 10% with the GraphPad Prism software (La Jolla, CA). IC_{50} s were fitted from the generated curves and averaged, for each radioligand concentration, for 3 independent simulations with the 10% scatter.

3. Results

3.1. Theoretical basis for allowing the use of [receptor] > K_d and [radioligand] >> K_d

A complex equation to convert IC_{50} to K_i that takes into account not only the radioligand concentration but also the receptor concentration has been derived by Jacobs et al. (1975):

$$IC_{50}=K_i+\frac{K_i}{K_d}\left([L_t]+[R_t]-\frac{3}{2}[LR_{max}]\right) \quad \text{eq.1}$$

in which $[L_t]$ ($=H_o$ in equation 10 in (Jacobs et al., 1975)) equals the total concentration of radioligand (in our case [3H]spiperone), $[R_t]$ ($=R_o$ in (Jacobs et al., 1975)) is the total concentration of receptor, and $[LR_{max}]$ ($=RH$ in (Jacobs et al., 1975)) stands for the concentration of receptor-radioligand complex in the absence of unlabeled ligand. In further comparing above equation 1 with equation 10 in Jacobs et al. (1975) please note that $IC_{50}=K_d'$ (app), $K_i=K_d'$, and $K_i/K_d=n$.

According to the mass law, at equilibrium and in the absence of inhibitor ($R+L \leftrightarrow RL$), the relationship of $[L]$, $[R]$, and $[LR]$ can be described as

$$K_d=[L] \times \frac{[R]}{[LR]} \quad \text{eq.2}$$

where K_d equals the dissociation constant for [3H]spiperone, $[L]$ the concentration of unbound [3H]spiperone, $[R]$ the concentration of free DA receptor and $[LR]$ the concentration of the [3H]spiperone-DA receptor complex. We can write:

$$[LR_{max}]=[LR]=[L] \times \frac{[R]}{K_d} \quad \text{eq.3}$$

$$[R]=[R_t]-[LR_{max}] \quad \text{eq.4}$$

Substituting $[R]$ in Eq.3 with its equivalent in Eq.4, we obtain:

$$[LR_{\max}] = [R_t] \times \frac{[L_t]}{L_t + K_d} \quad \text{eq.5}$$

If $[L_t] \gg K_d$, it follows that

$$[LR_{\max}] = [R_t] \quad \text{eq.6}$$

And thus Eq.1 can be rewritten as

$$\begin{aligned} IC_{50} &= K_i + \frac{K_i}{K_d} \left([L_t] + [R_t] - \frac{3}{2} [LR_{\max}] \right) \\ &= K_i + \frac{K_i}{K_d} ([L_t] - 0.5 [R_t]) \end{aligned} \quad \text{eq.7}$$

When $[L_t] \gg [R_t]$, we find that

$$IC_{50} = K_i \left(1 + \frac{[L_t]}{K_d} \right) \quad \text{eq. 8}$$

which equals the Cheng-Prusoff conversion.

In summary, provided $[L_t] \gg K_d$ as well as $[L_t] \gg [R_t]$, the complex equation of Jacobs et al. (1975) is equal to the Cheng-Prusoff correction. Thus, according to the above derivation, the complex equation taking into account [receptor] reverts to the simple Cheng-Prusoff equation if a radioligand concentration is applied that is much higher than the K_d and the total receptor concentration. Another conclusion from the above kinetic analysis is that high $[L_t]$ allows the use of higher [receptor] as long as the latter is much less than $[L_t]$.

3.2. Experimental validation for applying $[R_t] > K_d$ and $[L_t] \gg K_d$ and $[R_t]$ in the case of spiperone binding to D₂ and D₃ DA receptors

Spiperone has been reported to have sub-nanomolar affinity to D₂ and D₃ DA receptors (Chio et al., 1994; Gardner et al., 1996; Zaworski et al., 1999). We determined the K_d and $B_{\max}$ (maximal binding) under our conditions in a “hot saturation” assay with varying [³H]spiperone (0.3pM – 3nM) and 2 μM (+)-butaclamol for defining non-specific binding. The K_d was found to be 0.057 ± 0.013 nM for D₂ and 0.125 ± 0.033 nM for D₃ DA receptors (mean ± SEM for 3 experiments). The $B_{\max}$ in these experiments was 2.41 ± 0.26 pmol/mg of protein for D₂ and 1.08 ± 0.14 pmol/mg of protein for D₃ receptors (see representative experiments in Fig. 1).

If the aim is to have [receptor] < 10% of K_d (see Introduction), one can, with the above K_d and $B_{\max}$ values, calculate the need for [receptor] < 0.0057 nM for D₂ and < 0.0125 nM for D₃ assays, i.e. < 2.35 μg/ml for D₂ and < 11.6 μg/ml for D₃ assays. Our modified high-affinity radioligand protocol with [radioligand] > K_d claims that higher [receptor] can be used as long as [receptor] << [radioligand]. To test the modified protocol, [³H]spiperone binding was monitored for HEK-rD₂ and HEK-rD₃ cells as follows. An amount of 10 μg of D₂ and 40 μg of D₃ cell membrane was applied in 0.8 ml assay volume (i.e., 12.5 and 50 μg/ml), resulting in $[R_t]$ values for D₂ and D₃ receptors of 0.03 nM and 0.05 nM, respectively (see Table 1, first 3 experimental conditions for each cell line). Under these conditions, a wide range of [radioligand]s was applied from 0.16 nM to 1.2 nM. Throughout this range, $[L_t] \gg 0.5 [R_t]$ as well as $[L_t] > K_d$, and the Cheng-Prusoff conversion for inhibition by unlabeled spiperone yielded similar K_i values (~ 0.06 nM for D₂ and ~ 0.1 nM for D₃, Table 1) despite vastly

varying IC_{50} values (from 0.2 to 1.1 nM for D_2 and from 0.2 to 0.9 nM for D_3 , see Fig. 2). The effect of an even higher protein level was studied next. The assay volume was reduced from 0.8 ml to 0.2 ml, with 20 μ g D_2 and 50 μ g D_3 cell membranes (i.e., 100 and 250 μ g/ml), resulting $[R_t]$ levels of 0.24 nM for D_2 and 0.25 nM for D_3 (Table 1, last experimental condition for each cell line). Under this condition, with $[L_t] \gg 0.5 [R_t]$ as well as $[L_t] \gg K_d$, the Cheng-Prusoff conversion for inhibition by unlabeled spiperone yielded a K_i value that was similar to the values obtained with lower protein concentrations despite an extremely high value for the Cheng-Prusoff conversion factor (Table 1).

Further, we applied the modified protocol to determine IC_{50} s and K_i s of ropinirole, ($\pm$)7-OH-DPAT, (-)5-OH-DPAT and four new drugs, D341, D342, D343, and D344, under the two conditions: low protein level (i.e. 0.8 ml assay volume, 12.5 μ g/ml for D_2 and 50 μ g/ml for D_3) and high protein level (i.e. 0.2 ml assay volume, 100 μ g/ml for D_2 and 250 μ g/ml for D_3). Between the two conditions, though their IC_{50} s are varied greatly, K_i s were not affected by the protein level (Fig 3).

3.3. Simulations of competitive inhibition curves for assessing the accuracy of K_i with high radioligand concentration

We mimicked the binding of radioligand ($K_d=1$ nM) in the presence of inhibitor ($K_i=1$ nM) with [radioligand] fixed at 0.1 nM, 1 nM or 10 nM (Fig. 4). For each [radioligand], three independent simulations with 10% random scatter were done to mimic three independent experiments. For these conditions, [receptor] was kept at a level resulting in the same initial bound/free ratio ($y=0.1$) (italic, bold text in table 2). K_i s were converted from the fitted IC_{50} s with the Cheng-Prusoff equation. In the simulations with different [radioligand], IC_{50} s and K_i s were 0.967 ± 0.107 nM and 0.879 ± 0.098 nM ($[L_t]=0.1$ nM); 1.83 ± 0.28 nM and 0.913 ± 0.138 nM ($[L_t]=1$ nM); 12.7 ± 1.8 nM and 1.15 ± 0.16 nM ($[L_t]=10$ nM) (Fig.4).

4. Discussion

The theoretical analysis presented in this work shows that the complex equation of Jacobs et al. (1975) taking into account [receptor] and [radioligand] in receptor binding assays reverts to the simple Cheng-Prusoff equation if the radioligand concentration is much higher than the K_d and the total receptor concentration. Additionally, the analysis indicates that high $[L_t]$ allows the use of higher [receptor]. As long as $[L_t] \gg K_d$ and $[L_t] \gg [R_t]$, there is also no more requirement for the routinely applied rule of $[R_t] \ll K_d$ because the receptor concentration term present in the general equation 1 is absent in equation 8 derived from the general case for the conditions of $[L_t] \gg K_d$ and $[L_t] \gg [R_t]$. With these restrictions, the use of high $[L_t]$ combined with raised [receptor] allows the application of small-volume, 96-well-format binding assays to the measurement of binding of ultra-high-affinity radioligand in a manner similar to that applied to radioligands with less extreme affinities. This is especially useful for tritiated radioligands with specific activities in the range of a couple of TBq/mmol; with 125 I-labeled radioligands this is less of an issue as their higher specific activity (up to 81 TBq/mmol) allows lower bound concentrations that are still easily detectable.

In agreement with the theoretical analysis, experiments on inhibition by spiperone of [3 H] spiperone binding to D_2 and D_3 DA receptors showed that with increased radioligand concentration, though protein level was higher than K_d , K_i was not affected (Table 1). One should keep in mind that in the present experiments $K_i = K_d$ with spiperone inhibiting [3 H] spiperone binding. When the unlabeled inhibitor has a much higher affinity than the radioligand ($K_i \leq 0.1 K_d$), a 2-fold or larger error can be introduced (Munson and Rodbard, 1988). Our previous studies with the dopamine transporter showed the phenomenon of high protein concentrations increasing 4- to 5-fold the K_i of the compound (+)-R, R-D-84 (Zhen et al., 2004).

The most comprehensive analysis of all conditions affecting receptor binding is implemented by the Munson-Rodbard correction (1988). It is of interest that with our protocol, the computations of K_i values are virtually identical whether using the exact correction of Munson-Rodbard or the simple Cheng-Prusoff correction (Table 1). To assess the valid working range of our modified protocol, we simulated experimental conditions with varying [receptor], represented by the value of bound/free radioligand (defined as y_0 in the Munson-Rodbard equation), and [radioligand] (Table 2). K_d was set at 1 nM for the simulations and error ratio was defined as the ratio of K_i corrected with the Cheng-Prusoff equation over that with the Munson-Rodbard equation. With the restriction that radioligand is not depleted in the assay (bound/free radioligand ≤ 0.1), we found that the error ratio is less than 2.25 for a wide range of IC_{50} and $[L]$ values. Only when the Munson-Rodbard $K_i \leq 0.06 K_d$, error ratios of 1.79 and up were found; otherwise, error ratios were only 1.02 – 1.17 (Table 2). It should be noted this is independent of radioligand concentration (compare $[L]=10 \times K_d$, $1 \times K_d$ and $0.1 \times K_d$). The simulations for conditions where $K_i \leq 0.1 \times K_d$ also show that binding conditions can be optimized for smaller values of the error ratio by reducing bound/free radioligand, which can be accomplished by lowering receptor concentration and /or increasing radioligand concentration. It could be thought that the use of a radioligand concentration high relative to its K_d value increases the error of the Cheng-Prusoff correction. We therefore simulated data mimicking experimental conditions with random error (10%) added at a ligand concentration 10 times below the K_d , equal to the K_d , and 10 times higher than the K_d . Experimental data were simulated at various concentrations of an inhibitor with a K_i of 1 nM, and IC_{50} values were computed in the same way from these data as from actual experiments. Each IC_{50} value was converted into a K_i by the Cheng-Prusoff correction, and the error was calculated for the average of 3 simulated experiments at each radioligand concentration. With 0.1 nM, 1 nM and 10 nM of radioligand, the apparent K_i s were 0.879 ± 0.098 nM, 0.913 ± 0.138 nM and 1.15 ± 0.16 nM, respectively. No significant differences were found between the groups (one way ANOVA), and the relative error was not found to increase at higher radioligand concentration. Clearly, using a radioligand concentration 10 times over K_d , resulting in a correction factor of 11, puts great weight onto the value of K_d used in converting IC_{50} s to K_i s. It will be important to accurately determine the K_d of radioligand binding, and rigorously maintain the same assay conditions between experiments such that between-experiment variation be minimized. Since the K_d is a property of the receptor in its interaction with the radioligand, its value is a constant when assessed under identical conditions of temperature, assay buffer composition, cell line hosting the receptor, or brain region for a given animal species. In this fashion, a meaningful comparison can be made between compounds screened in the assay over different experimental days.

A final consideration is that the above analysis applies to competitive inhibitors. Compounds that inhibit by a different mechanism cannot be strictly compared on a K_i basis. For example, compounds that show an apparent noncompetitive inhibitory profile (not to be equated with actual noncompetitive inhibition of enzyme activity, see (Tomlinson, 1988)), will inhibit binding to the same extent at any [radioligand]; a general enzyme kinetics analysis of inhibition by such compounds shows IC_{50} to equal K_i (Cheng and Prusoff, 1973; for this discussion, binding terms were substituted for velocity terms). Thus, computation of their K_i at progressively higher [radioligand] by the competitive-inhibition-correction of Cheng-Prusoff (see above equation 8) will result in progressively smaller values, overestimating their affinity compared with calculation at low [radioligand] (risk of false positives in drug screening). Apparent uncompetitive inhibitors, for which Cheng and Prusoff (1973) derive that $IC_{50} = K_i (1 + K_m/S)$, will display an IC_{50} two times the K_i at a radioligand concentration close to K_d as routinely applied in many binding studies; further increasing radioligand concentration will cause IC_{50} to approach K_i . Therefore, such compounds, as apparent noncompetitive inhibitors, will also be subject to the risk of false positives in drug screening with higher ligand concentrations resulting in progressive reductions in K_i when applying the correction for

competitive inhibitors (equation 8). In drug screening projects, such false positives can be weeded out in subsequent functional assays. In addition, in assessing structure-activity relationships in a series of closely related chemicals one can expect a similar mechanism of inhibition, and, thus, K_i evaluations for such compounds will still provide valid relative comparisons. Inspection of the analysis of Cheng and Prusoff (1973) shows that, when the radioligand concentration is appreciably smaller than the K_d , the IC_{50} seriously deviates from (becomes higher than) K_i for uncompetitive inhibitors, whereas for competitive or noncompetitive inhibitors the IC_{50} is practically equal to K_i ; when $[radioligand] \sim K_d$, the IC_{50} is close to K_i (within a factor of 2) for all three inhibition mechanisms (Cheng and Prusoff, 1973). Therefore, if one has a tritiated radioligand with K_d in the nanomolar range, or an ultra-high-affinity ligand with sufficient specific activity (iodinated), it is advisable to keep the radioligand concentration close to K_d , and receptor concentration much below K_d , in order to reduce the impact of mechanism of inhibition and corrections in general.

With the bulk of inhibitors of receptor binding belonging to the class of competitive inhibitors, we believe the modified protocol presented here removes a major hindrance in the proper execution of binding assays with ultra-high-affinity tritiated ligands with K_d values in the subnanomolar range. This major hindrance is the routinely accepted requirement for low receptor concentration ($\ll K_d$), resulting in unworkable conditions for most 96-format binding assays. B_{max} values for cell lines overexpressing a given receptor can be much higher than those encountered in brain regions; in the latter case, in increasing receptor concentration in the binding assay, one needs to consider the upper limit of protein the filtration device can handle without showing clogging problems. For cells, and brain tissue with the above restriction, our modified protocol allows the use of 96-well plates with small volumes of 100-200 μ L per binding assay, removing the need for large binding assay volumes that result in large quantities of consumed expensive radioligand and filtration systems producing single filters for each assay to be counted individually in scintillation vials with expensive scintillation cocktail. The modified protocol presented here is scientifically sound and at the same time less expensive and more environment-friendly than previous methods adhering to binding rules routinely accepted as dogma.

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Appendix

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Figure 1. Saturation analysis of [^3H]spiperone binding to HEK293-rD₂ (A) and HEK-rD₃ (B)
 Data shown are from one representative experiment carried out in triplicate. Insets show Scatchard analysis. For three independent experiments, the average K_d and $B_{\max}$ values were 0.057 ± 0.013 nM and 2.41 ± 0.26 pmole/mg protein for HEK293-rD₂, and 0.125 ± 0.033 nM and 1.08 ± 0.14 pmole/mg protein for HEK293-rD₃, respectively.

Figure 2. Competition curves describing inhibition of [³H]spiperone binding by unlabeled spiperone as a function of radioligand concentration in HEK-rD₂ (A) and HEK-rD₃ (B) cells
 A rightward shift occurs upon increasing [radioligand] from 0.16 nM to 0.62 nM and to 1.2 nM. The IC₅₀ values from least squares computer analysis were converted to K_i values with the Cheng-Prusoff and Munson-Rodbard equations, shown in Table 1.

Figure 3. Comparison of potencies of test compounds obtained in low- and high- protein level assays on HEK-rD₂ (A) and HEK-rD₃ (B)

IC_{50} s (grey bars on right side of ordinate) and K_i s (white bars on left side of ordinate) in the assays of low-protein level (diagonally striped bars, i.e. in a volume of 0.8ml: 12.5 μ g/ml for HEK-rD₂ and 50 μ g/ml for HEK-rD₃ cells) and high-protein level (open bars, i.e. in a volume of 0.2ml: 100 μ g/ml for HEK-rD₂ and 250 μ g/ml for HEK-rD₃ cells). The IC_{50} values were converted to K_i values with the Cheng-Prusoff equation.

Figure 4. Simulated competition inhibition curves with three different concentrations of radioligand

Data mimicking experimental conditions with random error (10%) added, were simulated at a ligand concentration 10 times below the K_d (closed square), equal to the K_d (open circle), and 10 times higher than the K_d (open triangle). Experimental data were simulated at various concentrations of an inhibitor with a K_i of 1 nM with the K_d of the radiolabeled ligand set at 1 nM (see table 2) and receptor concentration set to give y equal to 0.1 (y is the ratio of bound over free radioligand in the absence of unlabeled ligand in the Munson-Rodbard correction). The ordinate shows radioligand binding in the presence of inhibitor divided by that in the absence of inhibitor. At each radioligand concentration, three simulations with 10% random scatter were averaged, and each data point shows the mean $\pm$ SEM (vertical bar). Inserts show the mean $\pm$ SEM of curve-fitting results of the three simulated experiments at each radioligand concentration.

Table1

Inhibition constant of unlabelled spiperone in [³H]spiperone binding assay with HEK293-rD₂ (1a) and HEK293-rD₃ (1b), obtained with Cheng-Prusoff or Munson-Rodbard conversion, under conditions of varying radioligand concentration and protein level

1a: HEK293-rD₂

[L] ₀ nM	[R] ₀ nM	IC ₅₀ nM	Bound/free radioligand	K _i by Cheng-Prusoff correction nM	K _i by Munson-Rodbard correction nM
0.16	0.03	0.255	0.129	0.067	0.064
0.6	0.03	0.616	0.047	0.053	0.053
1.2	0.03	1.149	0.024	0.052	0.052
4.6	0.24	6.897	0.05	0.084	0.085

1b: HEK293-rD₃

[L] ₀ nM	[R] ₀ nM	IC ₅ nM	Bound/free radioligand	K _i by Cheng-Prusoff correction nM	K _i by Munson-Rodbard correction nM
0.16	0.05	0.206	0.168	0.080	0.078
0.6	0.05	0.684	0.052	0.118	0.116
1.2	0.05	0.887	0.039	0.084	0.082
4.6	0.25	4.793	0.056	0.127	0.127

In the conversion from IC₅₀ to K_i, the K_d of [³H]spiperone was 0.057 nM for D₂ and 0.125 nM for D₃. Data are from single experiments carried in triplicate. For the respective corrections, see text and Cheng and Prusoff (1973) as well as Munson and Rodbard (1988).

Table 2

Comparison of Cheng-Prusoff correction with Munson-Rodbard correction in converting IC₅₀ to K_i in simulated binding assays. Value of K_d is set at 1 nM in the simulations. Corrections were calculated as described by Cheng and Prusoff (1973) and Munson and Robard (1988). Error ratio was the ratio of Cheng-Prusoff K_i to Munson-Rodbard K_i. The italicized and bolded text indicates the conditions used to generate competitive inhibition curves with random error added (Fig.4).

Bound/free radioligand	IC ₅₀ nM	[R _t] nM	[L _t] nM	K _i by Cheng-Prusoff correction, nM	K _i by Munson-Rodbard correction, nM	Error ratio
<i>0.10</i>	<i>11</i>	<i>1.01</i>	<i>10</i>	<i>1</i>	<i>0.986</i>	<i>1.02</i>
0.10	1.10	1.01	10	0.10	0.056	1.79
0.10	0.11	1.01	10	0.01	<i>*</i>	
0.01	1.10	0.11	10	0.10	0.095	1.05
0.01	0.11	0.11	10	0.01	0.005	1.98
0.001	0.11	0.011	10	0.01	0.010	1.05
<i>0.10</i>	<i>2</i>	<i>0.19</i>	<i>1</i>	<i>1</i>	<i>0.926</i>	<i>1.08</i>
0.10	0.20	0.19	1	0.10	0.050	2.01
0.10	0.02	0.19	1	0.01	<i>*</i>	
0.01	0.20	0.02	1	0.10	0.095	1.06
0.01	0.02	0.02	1	0.01	0.005	2.00
0.001	0.02	0.002	1	0.01	0.009	1.05
<i>0.10</i>	<i>1.1</i>	<i>0.11</i>	<i>0.1</i>	<i>1</i>	<i>0.873</i>	<i>1.14</i>
0.10	0.11	0.11	0.1	0.10	0.044	2.25
0.10	0.01	0.11	0.1	0.01	<i>*</i>	
0.01	0.11	0.011	0.1	0.10	0.094	1.06
0.01	0.01	0.011	0.1	0.01	0.005	2.02
0.001	0.01	0.001	0.1	0.01	0.009	1.17

* Simulation generated a negative K_i value.