Analytical Biochemistry 419 (2011) 117–122

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# Optimal design for the dose–response screening of tight-binding enzyme inhibitors

Petr Kuzmič

*BioKin Ltd., Watertown, MA 02472, USA*

ARTICLE INFO

*Article history:*
Received 27 April 2011
Received in revised form 26 May 2011
Accepted 7 June 2011
Available online 25 July 2011

*Keywords:*
Enzyme kinetics
Theory
Mathematics
Statistics
Regression
Enzyme inhibition
Screening
Optimal experimental design

ABSTRACT

Optimal experimental designs for the dose–response screening of enzyme inhibitors were studied within the framework of the Box–Lucas theory. If the enzyme concentration $E$ is considered as a fixed constant, an exact two-point $D$-optimal design consists of a pair of inhibitor concentrations equal to $I_1 = 0$ and $I_2 = E + K$, where $K$ is the apparent inhibition constant. If the enzyme concentration is treated as an adjustable parameter, an empirical three-point $D$-optimal design consists of three inhibitor concentrations equal to $I_1 = 0$, $I_2 = E + 3K$, and $I_3 = 0.7E$. These results were applied to design optimized, irregularly spaced concentration series for routine inhibitor screening. A heuristic Monte Carlo simulation study confirmed that the optimized dilution series is significantly more efficient than the classic series characterized by a constant dilution ratio. An online calculator to create optimized dilution series is freely available at http://www.biokin.com/design/.

© 2011 Elsevier Inc. All rights reserved.

This study used the statistical theory of optimal experiment design [1,2], specifically the Box–Lucas $D$-optimality criterion [3], to design optimized experiments for the dose–response screening of enzyme inhibitors.

Similar ideas have been used previously to design optimal experiments for the determination of the Michaelis constant [4], $K_M$, or for the determination of ligand–receptor dissociation constants [5], $K_d$, by biophysical methods. These previously published reports assumed that there exists an approximate estimate of the given parameter of interest (either $K_M$ or $K_d$). This initial estimate then serves as the starting point for the optimized choice of substrate or ligand concentrations to be used in an experimental study. We have extended these ideas to the situation where, instead of a single initial estimate of the apparent inhibition constant [6,7], $K$, we have a range of possible values that characterize enzyme inhibitors in the screening pool.

We derived two specialized formulas for the optimal design of dose–response experiments depending on whether the enzyme concentration, $E$, is known exactly or whether it needs to be estimated from the data. When the enzyme concentration is treated as a fixed constant, the optimal design consists of two measurements of initial reaction rates: one measured in the absence of the inhibitor ($I_1 = 0$) and the other measured at $I_2 = E + K$. When the enzyme concentration must be treated as a parameter optimized simultaneously with the apparent inhibition constant [8], the optimal design consists of three measurements. The first is the control data point at zero inhibitor concentration. The two approximately optimal inhibitor concentrations are $I_2 = E + 3K$ and $I_3 = 0.7E$.

Given a range of inhibition constants of interest, we applied the above formulas to a particular dose–response screening scenario to generate an optimally designed dilution series. Unlike a conventional dilution series, where adjacent inhibitor concentrations differ by a constant increment or ratio, each optimally designed dilution series is irregularly spaced. The efficiency of these optimized, irregularly spaced dilution series was tested in Monte Carlo simulations, and was shown to be superior to the conventional experimental design. As an illustrative example, an optimized dilution series allowed a reliable determination of apparent inhibition constants ranging from micromolar to picomolar values using a compact design involving only eight data points.

## Theory

### *Rate equation and sensitivity functions*

According to Eq. (1), the initial rate of an enzyme-catalyzed reaction, $v$, depends on three factors: the inhibitor concentration $I$, the enzyme concentration $E$, and the apparent inhibition constant $K$ [6,7]. In Eq. (1), $V$ is the control velocity observed in the absence of inhibitors ($I = 0$):

*E-mail address:* pksci01@biokin.com

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.06.013

$$v(I) = \frac{V}{2E}\left(E - I - K + \sqrt{(E - I - K)^2 + 4EK}\right) \quad (1)$$

$$\frac{\partial v(I)}{\partial V} = \frac{v}{V} \quad (2)$$

$$\frac{\partial v(I)}{\partial K} = \frac{V}{2E}\left(\frac{E + I + K}{\sqrt{(E - I - K)^2 + 4EK}} - 1\right) \quad (3)$$

$$\frac{\partial v(I)}{\partial E} = \frac{V}{2E}\left(\frac{E - I + K}{\sqrt{(E - I - K)^2 + 4EK}} + 1\right) - \frac{v(I)}{E}. \quad (4)$$

The Box–Lucas statistical theory of optimal experimental design (see below) uses the corresponding sensitivity functions, shown in Eqs. (2) to (4), which are partial derivatives of the rate equation with respect to optimized model parameters.

*Box–Lucas method*

The Box–Lucas theory of optimal experimental design [3] had been previously applied in enzymology to Michaelis–Menten saturation kinetics [4] and to ligand–binding experiments [5]. It is a representative example of optimal design methods based on the *D*-optimality criterion [2], essentially maximizing the determinant of the Fisher information matrix.

In the following derivations, we distinguish two separate scenarios that differ in the number of optimized model parameters (either two or three). In the first scenario, only the control rate $V$ and the inhibition constant $K$ are treated as adjustable model parameters, whereas the enzyme concentration $E$ is treated as a fixed constant. In the second scenario, not only $V$ and $K$ but also $E$ is treated as an adjustable model parameter. A previous report [8] discussed in detail the experimental conditions under which either the two-parameter model ($V$, $K$) or the three-parameter model ($V$, $K$, $E$) is most appropriate for any given dataset.

*Optimal design for fixed enzyme concentration E*

As we demonstrated in a previous theoretical study based on Monte Carlo simulations [8], if the apparent inhibition constant $K$ is even slightly larger than the enzyme concentration $E$, it becomes impossible to determine both $K$ and $E$ from the same dataset. The only two model parameters that can be determined from a dose–response curve are $V$ and $K$, and $E$ must be treated as a fixed constant in Eq. (4). In that case, the determinant of the Box–Lucas sensitivity matrix is defined by Eq. (5), where $I_1$ and $I_2$ are inhibitor concentrations comprising the two-point experimental design:

$$D(I_1, I_2) = \begin{vmatrix} \frac{\partial v(I_1)}{\partial V} & \frac{\partial v(I_1)}{\partial K} \\ \frac{\partial v(I_2)}{\partial V} & \frac{\partial v(I_2)}{\partial K} \end{vmatrix}. \quad (5)$$

One of the two adjustable model parameters is the "control" reaction velocity, $V$, observed in the absence of inhibitors. It follows that one of the two design points must, in fact, correspond to zero inhibitor concentration, $I_1 = 0$. Thus, the determinant simplifies to Eq. (6):

$$D(0, I_2) = \begin{vmatrix} 1 & 0 \\ \frac{\partial v(I_2)}{\partial V} & \frac{\partial v(I_2)}{\partial K} \end{vmatrix} = \left|\frac{\partial v(I_2)}{\partial K}\right|. \quad (6)$$

From solving the equation $\partial|D(0,I_2)|/\partial I_2 = 0$ for $I_2$, it follows that the maximum of $|D(0,I_2)|$ occurs at $I_2 = E + K$. This result is very similar to what other authors [5] have found for certain classes of ligand-binding studies. In summary, if the enzyme concentration must be treated as a fixed constant, the two-point optimal design includes inhibitor concentrations defined by Eqs. (7) and (8).

Two-point design for fixed $E$:

$$I_1 = 0 \quad (7)$$

$$I_2 = K + E \quad (8)$$

*Optimal design for simultaneous fit of E and K*

We previously found [8] that under experimental conditions where $0.01 < E/K < 1$, both $E$ and $K$ can be determined from the same dose–response curve. This amounts to performing an active site titration while simultaneously measuring the inhibition constant of the particular tight-binding inhibitor. Under such circumstances, the determinant of the Box–Lucas sensitivity matrix is defined by Eq. (9), where $I_1$, $I_2$, and $I_3$ are inhibitor concentrations comprising the three-point experimental design:

$$D(I_1, I_2, I_3) = \begin{vmatrix} \frac{\partial v(I_1)}{\partial V} & \frac{\partial v(I_1)}{\partial K} & \frac{\partial v(I_1)}{\partial E} \\ \frac{\partial v(I_2)}{\partial V} & \frac{\partial v(I_2)}{\partial K} & \frac{\partial v(I_2)}{\partial E} \\ \frac{\partial v(I_3)}{\partial V} & \frac{\partial v(I_3)}{\partial K} & \frac{\partial v(I_3)}{\partial E} \end{vmatrix}. \quad (9)$$

As before, one of the optimally chosen inhibitor concentrations must be zero, $I_1 = 0$, because one of the adjustable model parameters is the control reaction rate $V$. At zero inhibitor concentration the partial derivatives with respect to $K$ and $E$ both are zero and $\partial v/\partial V = 1$. Therefore, Eq. (9) simplifies to Eq. (10)

$$|D(0, I_2, I_3)| = \left|\frac{\partial v(I_2)}{\partial K}\frac{\partial v(I_3)}{\partial E} - \frac{\partial v(I_2)}{\partial E}\frac{\partial v(I_3)}{\partial K}\right|. \quad (10)$$

According to the Box–Lucas method, finding the optimally chosen inhibitor concentrations $I_2$ and $I_3$ (in addition to $I_1 = 0$) amounts to finding those two particular inhibitor concentrations at which the absolute value $|D(0, I_2, I_3)|$ is at a maximum. In this instance we no longer can find an analytic (algebraic) solution to the maximization problem and must resort to numerical iterative methods. In this work, the maximization of $|D(0, I_2, I_3)|$ in Eq. (10) was performed by Powell's method [9].

## Results

*Empirical design for simultaneous fit of K and E*

According to Eq. (10), the optimal inhibitor concentrations of a three-point experimental design are found by maximizing the absolute value of the determinant $D$. Fig. 1 shows the numerical values of the determinant at one particular value of the inhibition constant ($K$ = 0.1 nM) and one particular value of the enzyme concentration ($E$ = 1 nM). The open circle in Fig. 1 represents the corresponding optimal design, namely, $I_1 = 0, I_2 = 1.3$ nM, and $I_3 = 0.6$ nM.

The reason why there are two "hot spots" in Fig. 1 is that the two inhibitor concentrations we are concerned with are interchangeable. Indeed, labeling one of them as $I_1$ (the "first" inhibitor concentration) and the other as $I_2$ (the "second" concentration) is purely arbitrary. For this reason, the contour plot in Fig. 1 is by definition diagonally symmetrical.

Numerical maximization of the Box–Lucas determinant value defined by Eq. (10) was performed across a wide range of inhibition constants $K$. The results are summarized in Fig. 2.

Fig. 2 shows that the dependence of the optimally chosen inhibitor concentrations $I_2$ and $I_3$ on the "true" value of the inhibition constant $K$ is slightly nonlinear. One of the two inhibitor concentrations ($I_2$) stays approximately constant, varying only slightly between $I_2 = 0.6$ nM and $I_2 = 0.8$ nM. The other inhibitor concentration ($I_3$) increases approximately linearly with the slope

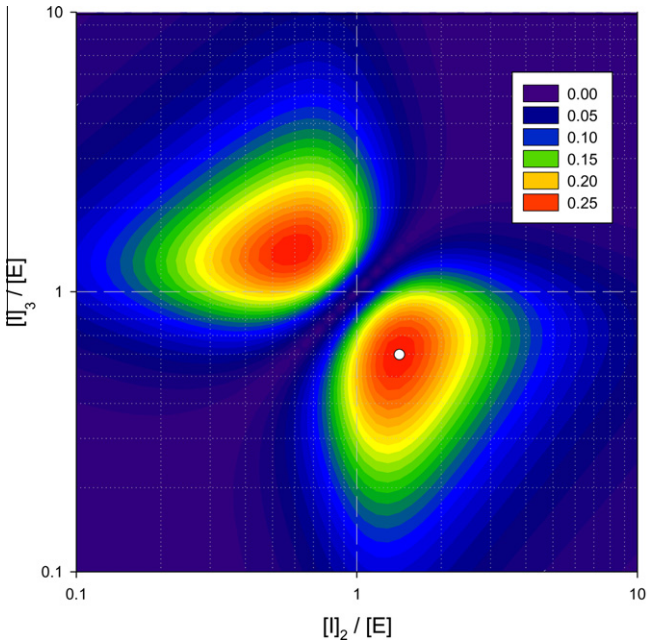


**Fig.1.** Representative plot of the Box–Lucas determinant $|D(0, I_2, I_3)|$ according to Eq. (10) against the inhibitor concentrations $I_2$ and $I_3$, assuming $E = 1$ nM and $K = 0.1$ nM. The maximum value of $|D|$ (white circle) occurs at $I_2 = 1.3$ nM and $I_3 = 0.6$ nM.

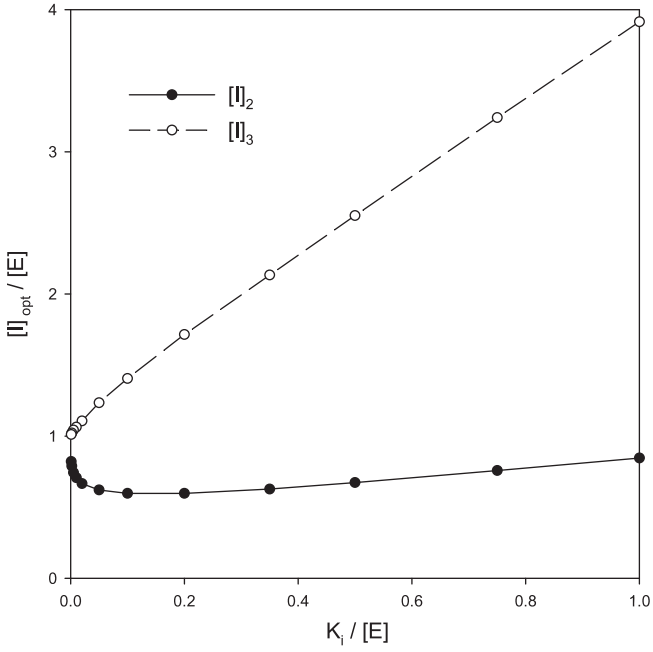


**Fig.2.** Plot of the optimal inhibitor concentrations $I_2$ and $I_3$ against the inhibition constant $K$, assuming $E = 1$ nM. Each point corresponds to a particular maximum value of $|D(0, I_2, I_3)|$ according to Eq. (10), as illustrated in Fig. 1 for the particular case of $K = 0.1$ nM.

of 3 and intercept equal to 1. These trends are summarized in the empirical Eqs. (11) to (13).

Three-point design for optimized $E$:

$$I_1 \approx 0 \tag{11}$$

$$I_2 \approx 0.7E \tag{12}$$

$$I_3 \approx 3K + E. \tag{13}$$

**Table 1**
An eight-point rational design for inhibitor screening at $E = 1$ nM.

| $K$ (nM) | $I_1$ (nM) | $I_2$ (nM) | $I_3$ (nM) | Point | $I_{opt}$ (nM) |
|---|---|---|---|---|---|
| 1000 | 0 | 1001 | – | 1 | 1001 |
| 100 | 0 | 101 | – | 2 | 101 |
| 10 | 0 | 11 | – | 3 | 11 |
| 1 | 0 | 4 | 0.7 | 4 | 4 |
| 0.1 | 0 | 1.3 | 0.7 | 5 | 1.3 |
| 0.01 | 0 | 1.03 | 0.7 | 6 | 1.03 |
| | | | | 7 | 0.7 |
| | | | | 8 | 0 |

In creating Figs. 1 and 2, we used a scaled variant of the rate Eq. (4), where the enzyme concentration was eliminated by introducing the scaled inhibitor concentration $I^* = I/E$ and the scaled inhibition constant $K^* = K/E$. The resulting Eq. (14) is exactly equivalent to Eq. (1)

$$v(I) = \frac{V}{2}\left(1 - I^* - K^* + \sqrt{(1 - I^* - K^*)^2 + 4K^*}\right) = \frac{V}{2}\left(1 - \frac{I}{E} - \frac{K}{E} + \sqrt{\left(1 - \frac{I}{E} - \frac{K}{E}\right)^2 + 4\frac{K}{E}}\right). \tag{14}$$

*Rational design for inhibitor screening*

For illustration, let us assume that, in a given inhibitor screening project, we are interested in capturing inhibitory activities spanning five orders of magnitude from $K = 0.01$ nM (10 picomolar) to $K = 1000$ nM (1 micromolar). Let us also assume that, for various logistical reasons, the enzyme concentration in the screening study is $E = 1$ nM. Table 1 illustrates how we can arrive at an optimal choice of inhibitor concentrations to construct dose–response curves.

The first column shows the appropriate order of magnitude of the apparent inhibition constant, $K$. For $K$ values larger than $E$, we choose a two-point optimal design according to Eqs. (7) and (8), because under those particular experimental conditions it is not possible to determine $K$ and $E$ simultaneously [8]. Conversely, for lower values of the inhibition constant, $K \leqslant E$, we choose the three-point design according to Eqs. (11) to (13). Finally, scanning the optimal values of inhibitor concentrations listed in the rightmost column of Table 1, the consolidated eight-point screening design emerges as $I = 1001, 101, 11, 4, 1.3, 1.03, 0.7,$ and 0 nM.

Let us illustrate the efficiency of this optimal design by assuming that a given tight-binding enzyme inhibitor is characterized by the inhibition constant $K = 50$ pM. Fig. 3 shows a simulated dose–response curve for such a hypothetical inhibitor. The filled rectangles in Fig. 3 (pseudo-experimental data points) were simulated using Eq. (4) with 3% Normally distributed pseudo-random error. The smooth curve represents the best least-squares fit of the data points to Eq. (4). The best-fit values of adjustable model parameters are $E = (1.07 \pm 0.05)$ nM and $K = (42 \pm 11)$ pM. This is very close to the simulated or "true" value of $K = 50$ pM.

*Monte Carlo study: scan of inhibition constants*

The purpose of the following heuristic simulation study was to evaluate the efficiency of the $D$-optimal design not just for one particular value of the inhibition constant, illustrated in Fig. 3 ($K = 50$ pM), but for a wide range of simulated (i.e., "true") inhibition constants spanning from 10 pM to 1 μM. To this end, 1000 dose–response curves were simulated at randomly chosen values of the inhibition constant $K$. As before, each curve was simulated using Eq. (4) as the underlying theoretical model. The

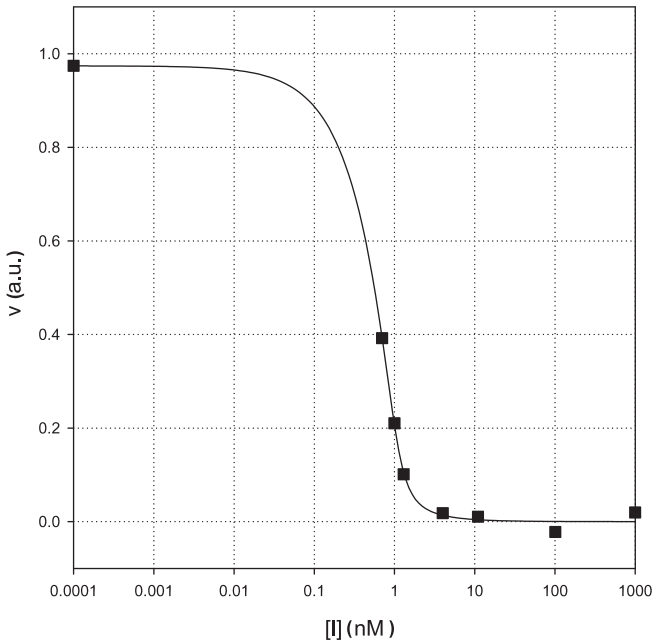


**Fig.3.** Example of an optimal design for screening. The simulated data points correspond to $K = 50$ pM. The smooth curve corresponds to the best least-squares fit to Eq. (1), yielding $K = (42 \pm 11)$ pM. For further details, see text.

pseudo-random error superimposed on each simulated data point was 3%. The simulated dose–response curves were subjected to nonlinear regression to determine best-fit value of $K$. The results are summarized in Fig. 4.

Fig. 4 shows that the recovery of the "true" inhibition constants from the simulated datasets is very satisfactory at $K/E > 1$. More important, even for extremely tight-binding inhibitors ($0.01 < K/E < 0.1$), the best-fit values of inhibition constants agree with the simulated or "true" values within an order of magnitude.

*Comparison with a serial dilution design*

The advantages of the optimal experimental design, derived from the Box–Lucas $D$-optimality criterion, are best illustrated in a comparison with the classic serial dilution design that is commonly used in inhibitor screening studies. With target inhibition constants spanning from 10 pM to 1 μM, a typical serial dilution design might involve inhibitor concentrations equal to 1000, 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 nM. The maximum inhibitor concentration is equal to the largest relevant value of the inhibition constant. The remaining concentrations are derived by 10-fold serial dilution. Let us assume that a particular tight-binding inhibitor is characterized by the apparent inhibition constant $K = 50$ pM. Fig. 5 shows a simulated serial dilution dose–response curve for such a hypothetical inhibitor.

As before, the filled rectangles in Fig. 5 (pseudo-experimental data points) were simulated using Eq. (1) with 3% normally distributed pseudo-random error. The smooth curve represents the best least-squares fit of the data points to Eq. (1). The best-fit values of adjustable model parameters are $E = (1.1 \pm 0.2)$ nM and $K = (26 \pm 38)$ pM.

These results illustrate the inferiority of the traditional fixed ratio serial dilution series in two different ways. First, the "best-fit" value of the inhibition constant ($K^{(\mathrm{fit})} = 26$ pM) is significantly different from the simulated or "true" value ($K^{(\mathrm{true})} = 50$ pM). More important, the formal standard error of the inhibition constant (±38 pM) is so large that it is physically meaningless. We must conclude that no meaningful value for $K$ could be obtained from the synthetic dataset pictured in Fig. 5.

A striking illustration of how poorly the serial dilution design performs is shown in Fig. 6. A Monte Carlo confidence interval study was performed according to the computational technique previously described by Straume and Johnson [10]. Here 1000 dose–response curves were simulated for a hypothetical enzyme inhibitor characterized by $K = 50$ pM using either the classic serial dilution design, $I = 1000, 100, 10, 1, 0.1, 0.01, 0.001$, and 0.0001 nM, or the optimized design, $I = 1001, 101, 11, 4, 1.3, 1, 0.7$, and 0.0001 nM. In both cases, the dose–response curves contained

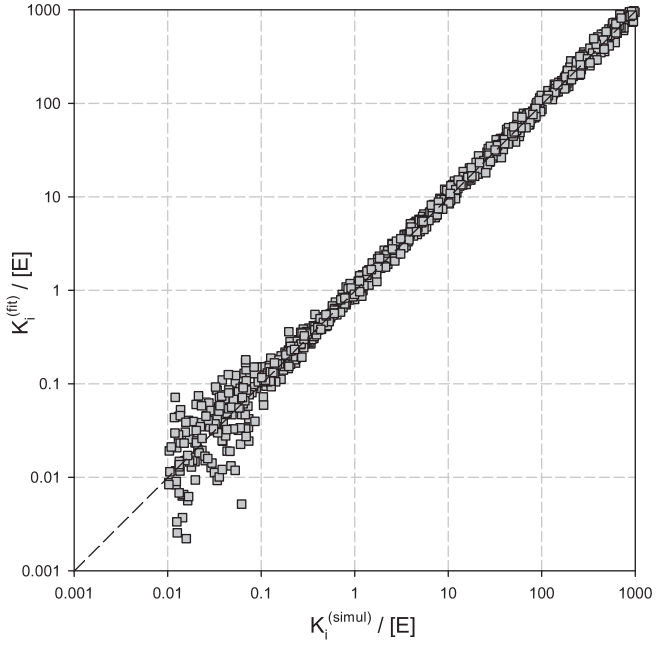


**Fig.4.** Correlation of simulated ("true") versus fitted values of inhibition constants using the optimized design $I = 1001$, 101, 11, 4, 1.3, 1, 0.7, and 0 nM and assuming $E = 1$ nM.

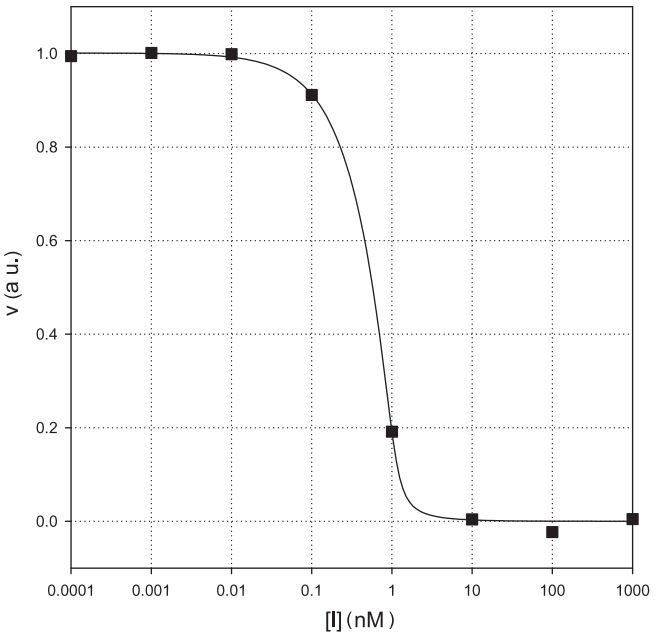


**Fig.5.** Example of a serial dilution experimental design. The simulated data points correspond to $K = 50$ pM. The smooth curve corresponds to the best least-squares fit to Eq. (1), yielding $K = (26 \pm 38)$ pM. For further details, see text.

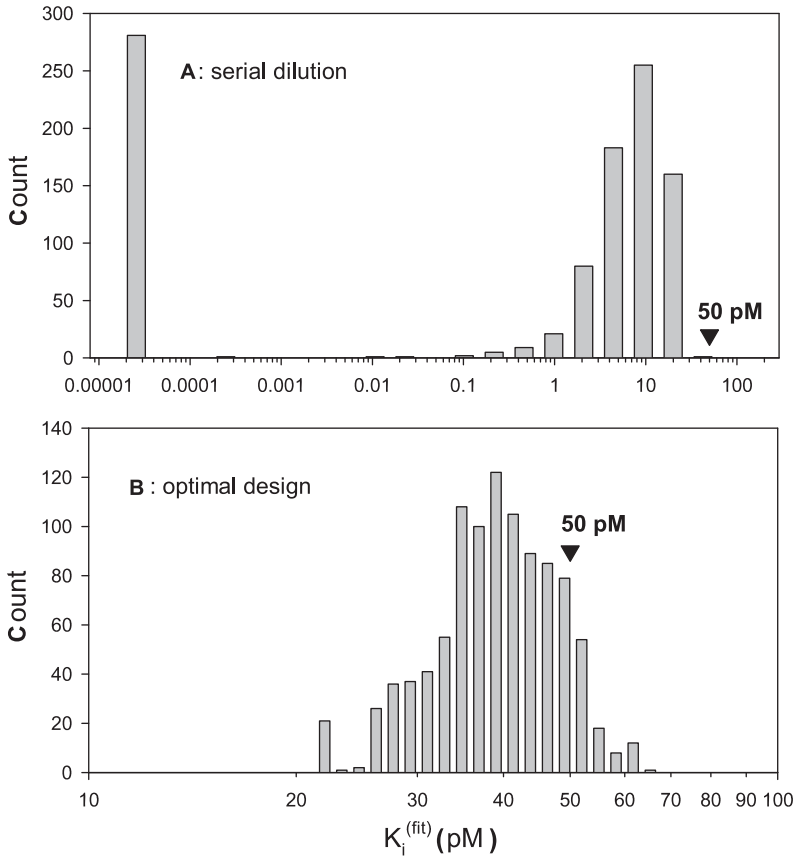


**Fig.6.** Monte Carlo confidence intervals for the inhibition constant $K$ using either the conventional serial dilution experiment design $I$ = 1000, 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 nM (A) illustrated in Fig. 5 or the optimized design $I$ = 1001, 101, 11, 4, 1.3, 1, 0.7, and 0.0001 nM (B) illustrated in Fig. 3. The "true" value $K$ = 50 pM is shown by the black triangle.

eight data points. The pseudo-random experimental error was 3%. Each simulated curve was fit to the underlying theoretical model given by Eq. (4). For each particular experimental design, a histogram of distribution for the 1000 best-fit values of $K$ is shown in Fig. 6.

Fig. 6 clearly shows that the recovery of the "true" (i.e., simulated) inhibition constant $K$ = 50 pM is very satisfactory for the optimized design (Fig. 6B). The most frequently represented best-fit value is $K \approx 40$ pM; nearly all best-fit values are spread over a relatively narrow interval, spanning from $K$ = 20 pM to $K$ = 60 pM.

In contrast, the classic serial dilution experimental design produced the most frequently represented best-fit value of $K$ that is practically indistinguishable from zero, as is shown by the tall histogram bar at the left Fig. 6A. The rest of the best-fit values seem to be centered around $K$ = 10 pM, which is significantly different from the "true" value of $K$ = 50 pM.

## Discussion

Many experiments in biochemical laboratories are performed by using ad hoc experimental designs, usually handed down by local tradition. For example, an informal survey of the pharmaceutical industry revealed that one laboratory always uses a 12-point 1:3 serial dilution design starting from 10 μM and ending with 56 pM ($I$ = 10000, 3333.33, 1111.11, . . ., 0.17, 0.056 nM). Another laboratory always uses an 8-point 1:4 serial dilution ranging from 1 μM down to 61 pM ($I$ = 1000, 250, 62.5, . . ., 0.244, 0.061 nM), and so on.

What are the relative merits of these various experimental designs? Is it possible to come up with a rationally designed dilution series for inhibitor screening, perhaps one that is not strictly regular but one that would allow the most precise and accurate measurements possible? These are the questions addressed by the theory of optimal experimental design [1,2]. Many important theoretical ideas, such as the Box–Lucas approach to optimal design [3], did eventually filter into enzymological studies [4,5]. This article is an extension of the Box–Lucas design ideas for dose–response screening.

The main findings of the current study, summarized in the form of a "cookbook" recipe, are as follows.

First, choose an enzyme concentration, $E$, that is as low as possible while still maintaining sufficient sensitivity and a reasonably short overall duration of the enzyme assay. This particular choice of the enzyme concentration determines the lower limit of the apparent inhibition constant that can be reliably determined, $K_{min}$. Based on a previous theoretical study [8], it is practically possible to reliably determine $K$ values that are approximately 20 to 50 times lower than $E$, but only if optimally chosen inhibitor concentrations are used.

Next, choose the maximum value of inhibition constant that is still of interest in a particular screening project (a typical example in the pharmaceutical industry is $K_{max} = 10$ μM). Any inhibitor with $K > K_{max}$ can be declared "inactive." Now compose a "design table" similar to Table 1. In the left-most columns, list the values of inhibition constants ranging from $K_{max}$ to $K_{min}$ stepping by an order of magnitude or less. For each particular $K$ value of interest, either use Eqs. (7) and (8) if $K > E$, or use Eqs. (11) to (13) if $K \leqslant E$.

Finally, consolidate all concentrations generated in the "design table," such that all possible duplicates are removed and concentrations that are sufficiently similar (e.g., within two significant

**Table 2**
A 12-point rational design for inhibitor screening at $E = 0.1$ nM.

| $K$ (nM) | $I_1$ (nM) | $I_2$ (nM) | $I_3$ (nM) | Point | $I_{opt}$ (nM) |
|---|---|---|---|---|---|
| 10,000 | 0 | 10000.1 | – | 1 | 10,000 |
| 2000 | 0 | 2000.1 | – | 2 | 2000 |
| 400 | 0 | 400.1 | – | 3 | 400 |
| 80 | 0 | 80.1 | – | 4 | 80 |
| 16 | 0 | 16.1 | – | 5 | 16 |
| 3.2 | 0 | 3.3 | – | 6 | 3.3 |
| 0.64 | 0 | 0.74 | – | 7 | 0.74 |
| 0.128 | 0 | 0.228 | – | 8 | 0.23 |
| 0.0256 | 0 | 0.07 | 0.177 | 9 | 0.18 |
| 0.00512 | 0 | 0.07 | 0.115 | 10 | 0.11 |
| 0.001024 | 0 | 0.07 | 0.103 | 11 | 0.07 |
| | | | | 12 | 0 |

digits) are merged into a single value. The result is the optimal design for screening according to the Box–Lucas method.

At least one additional example might further illustrate the newly proposed experimental design. Let us assume that we plan to lay out a 12-point dilution series in an inhibitor screening project, where the goal is to capture inhibition constants spanning from 10 μM down to 1 pM (seven orders of magnitude). The various values of the inhibition constants of interest are spaced by a factor of five rather than by an order of magnitude. Let us also assume that the screening concentration of the enzyme is $E = 0.1$ nM.

The "design table" for this situation is shown in Table 2, where the optimally chosen inhibitor concentrations are shown in the right-most column. For those rows where no $I_3$ value is given, we used Eqs. (7) and (8) for the two-point optimal design; otherwise, the three-point empirical formulas (Eqs. (11) to (13)) were used. Here we have consolidated the closely spaced values $I_3 = 0.115$ nM and $I_3 = 0.103$ nM into a single value $I_{opt} = 0.11$ nM. Supporting Monte Carlo simulations (results not shown) confirmed that the optimal experimental design listed in Table 2 indeed is suitable for precisely determining inhibition constants spanning seven orders of magnitude.

In summary, we have described a rational approach to designing inhibitor dose–response screening experiments in the sense of optimally choosing inhibitor concentrations. The optimality criterion is based on the Box–Lucas statistical theory of $D$-optimal designs [3]. The resulting optimized dilution series are irregular in the low-concentration region. The low-concentration region of each dilution series is closely linked to the (fixed) enzyme concentration. In creating the optimal dilution series, the enzyme concentration is optionally treated as an adjustable model parameter rather than a fixed constant, according to our previously published theoretical analysis [8]. Unlike the conventional fixed ratio dilution series, the optimal dilution series are characterized by very high efficiency in the precise and accurate determination of apparent inhibition constants. A free online "calculator" to create optimized dilution series is available at http://www.biokin.com/design/.

## References

[1] V.V. Fedorov, Theory of Optimal Experiments, Academic Press, New York, 1972.
[2] A.C. Atkinson, A.N. Donev, Optimum Experiment Designs, Oxford University Press, Oxford, UK, 1992.
[3] G.E.P. Box, H.L. Lucas, Design of experiments in non-linear situations, Biometrika 46 (1959) 77–90.
[4] R.G. Duggleby, Experimental designs for estimating the kinetic parameters for enzyme-catalysed reactions, J. Theor. Biol. 81 (1979) 671–684.
[5] L. Endrenyi, F.Y. Chan, Optimal design of experiments for the estimation of precise hyperbolic kinetics and binding experiments, J. Theor. Biol. 90 (1981) 241–263.
[6] S. Cha, Tight-binding inhibitors: I. Kinetic behavior, Biochem. Pharmacol. 24 (1975) 2177–2185.
[7] J.W. Williams, J.F. Morrison, The kinetics of reversible tight-binding inhibition, Methods Enzymol. 63 (1979) 437–467.
[8] P. Kuzmič, K.C. Elrod, L.M. Cregar, S. Sideris, R. Rai, J.W. Janc, High-throughput screening of enzyme inhibitors: simultaneous determination of tight-binding inhibition constants and enzyme concentration, Anal. Biochem. 286 (2000) 45–50.
[9] W.H. Press, S.A. Teukolsky, W.T. Vetterling, B.P. Flannery, Numerical Recipes in C, Cambridge University Press, Cambridge, UK, 1992.
[10] M. Straume, M.L. Johnson, Monte Carlo method for determining complete confidence probability distributions of estimated model parameters, Methods Enzymol. 210 (1992) 117–129.