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Inactivators in competition How to deal with them . . . and not!

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Abstract

A method is described to determine the values of the equilibrium (K) and rate (k_2) constants for enzyme inactivations which occur according to two-step pathways involving a first non-covalent complex and a covalent, irreversibly inactivated adduct.

The method rests on a competition between a reference compound [R] for which the k_2 and K values are already known and another inactivator [C]. During the experiments, the disappearance of the reference compound or the appearance of the ER* adduct is monitored. The analysis shows that under conditions where the k_2 and K values for the competing substrate can be determined, the measured apparent first-order rate constant for the disappearance of the reference compound is not the sum of the rate constants obtained for each inactivator in the absence of the other.

The method can be used to determine the K and k_2 constants when an adequate reference compound is available, in particular, for the interactions between β-lactam antibiotics and penicillin-binding proteins. The precautions which must be taken to avoid large errors on the estimation of the parameters of the competing inactivator are discussed.

Examples found in the literature are discussed where an erroneous simplified equation has been utilised, thus yielding incorrect values for k_2 and K. Interestingly, the correct values can be calculated on the basis of the published results which do not contain the raw experimental data. But some of the values should be considered with a lot of caution since the experiments have not been performed under optimal conditions.

Keywords: Enzyme inactivation; Competition; β-Lactams; Kinetics; *Staphylococcus aureus* BlaR; *Staphylococcus aureus* PBP2a

1. Introduction

Specific enzyme inactivators are extremely useful both as tools in fundamental research and as potential drug candidates. These compounds whose structures usually mimic those of the substrates bind to the active site and then react irreversibly with an essential amino acid side chain. In some cases, this reaction is made more efficient by the catalytic machinery of the enzyme itself so that the term ''mechanism-based inactivator'' has been coined. Although more complex pathways can prevail, many interactions are adequately described by the model of Kitz and Wilson [1]:

$$E + C \overset{K}{\rightleftharpoons} EC \overset{k_2}{\rightarrow} EC^* \qquad \text{(model 1)}$$

where E is the enzyme, C the inactivator, EC the non-covalent complex and EC* the inactivated adduct. K is the dissociation constant of EC and k_2 is the first-order rate constant characterising the formation of the adduct. To rationalize the factors which can affect the efficiency of an inactivator and open the way to the design of better molecules, it is extremely useful to determine the individual k_2 and K values.

Antibiotics of the β-lactam family inactivate their target enzymes (DD-carboxy- and trans-peptidases, also called penicillin-binding proteins or PBPs) according to model (1) where EC* is an acylenzyme formed by reaction of the β-lactam carbonyl with the active-site serine [2]. The EC* adduct is not completely stable and undergoes spontaneous hydrolysis which regenerates active enzyme but although a very small number of exceptions have been recorded, the reactivation reaction is too slow to significantly influence the physiologically relevant sensitivity of a PBP to the

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antibiotic or the rate and efficiency of the inactivation reaction. Since the values of K are generally well above the clinically useful antibiotic concentrations, this sensitivity is determined by the values of the k_2/K ratio, a second-order rate constant. However, from a fundamental point of view and for the reasons given above, the determination of the individual k_2 and K values remains useful.

In this commentary, we show that competition methods allow the measurement of the k_2, K and k_2/K values and we discuss the limitations of these methods and the major traps to be avoided. The interactions between PBPs and β-lactam antibiotics are used as examples.

2. Direct determination of k_2/K

Various methods have been proposed to determine the value of k_2/K in which the time-course of the formation of EC^* can be followed by monitoring the fluorescence or the absorbance of the mixture or the formation of a labelled covalent adduct. The first method requires that the fluorescence spectrum of the EC^* be different from that of E and EC [3], which is not the case in most interactions.

The second relies on the fact that the absorption spectrum of the antibiotic is modified upon opening of the β-lactam ring [4]. The drawbacks of the latter method are that rather high concentrations of pure PBPs must be used and that in contrast to those of cephalosporins and carbapenems, the spectra of penicillins and monobactams are modified below 230 nm and with relatively low variations of the molecular extinction coefficients.

Formation of the acylenzyme can be directly monitored with a fluorescent or radioactive β-lactam by separating the protein from the excess of reagent by gel electrophoresis and quantifying the extent of labelling by fluorography [5]. For unlabelled compounds, a counter-labelling technique can be used in which the unreacted enzyme is labelled by the radioactive or fluorescent compound [5,6]. These time-consuming, ''point-by-point'' methods cannot be utilised under conditions where the reaction is quite fast since it is difficult to take samples out of a reaction mixture at a frequency higher than a few samples per minute unless a quenched-flow apparatus is utilised.

3. Determination of k_2/K by competition

Nitrocefin [7] is a chromogenic cephalosporin whose absorbance at 480–500 nm strongly increases upon opening of the β-lactam ring ($\Delta\varepsilon = 17{,}000\ M^{-1}\ cm^{-1}$). Its interaction with PBPs is thus easy to monitor and stopped-flow techniques can be utilised if the reaction is rapid. After the k_2/K ratio of nitrocefin has been measured, that of other compounds can easily be obtained by a simple, direct competition method [8]. If the enzyme is added to a mixture of nitrocefin (R) and another β-lactam (C), the following system prevails:

$$\begin{aligned} E + R &\overset{K_R}{\Leftrightarrow} ER \overset{k_{2R}}{\rightarrow} ER^* \\ E + C &\overset{K_C}{\Leftrightarrow} EC \overset{k_{2C}}{\rightarrow} EC^* \end{aligned} \qquad \text{(model 2)}$$

Note that the degradation of EC^* is neglected in the following analysis (as mentioned above) and that (R) and (C) are significantly higher than E_0 (at least 10-fold).

The rates of accumulation of ER^* and EC^* are, respectively, k_{2R}(ER) and k_{2C}(EC). In consequence, the $(ER^*)/(EC^*)$ ratio remains constant throughout and is given by:

$$\frac{(EC^*)}{(ER^*)} = \frac{(k_{2C}/K_C)(C)}{(k_{2R}/K_R)(R)} \qquad (1)$$

The easiest procedure is to let the reaction go to completion and to measure the final $(EC^*)/(ER^*)$ ratio [8]. Clearly, this is only valid if no significant degradation of both ER^* and EC^* occurs before the reaction is completed, but this is usually not a major problem since C_0 and R_0 can easily be sufficiently and simultaneously increased to fulfill this condition. Note, however, that it is not absolutely necessary to let the reaction go to completion, since the $(EC^*)/(ER^*)$ ratio remains the same throughout, but the concentration of the residual non-acylated enzyme [i.e. (E) + (ER) + (EC)] must then be determined. This can be done by measuring the residual enzyme activity after suitable dilution or elimination of both R and C by a β-lactamase. However, the most reliable results are obtained with a complete reaction and R_0 and C_0 concentrations adjusted so that the final (ER^*) value lies between 10 and 80% of that obtained in the absence of C.

The direct monitoring of the $(EC^*)/(ER^*)$ ratio requires the utilisation of sufficient enzyme concentrations (1 μM or more) to obtain a significant absorbance variation. If only small amounts of enzyme are available, nitrocefin might in a second step be replaced by a radioactive or fluorescent compound and the following procedure can be used:

1. measure the k_2/K ratio for nitrocefin;
2. measure that of the radioactive or fluorescent inactivator by competition with nitrocefin;
3. measure those of other compounds by competition with the labelled compound;
4. An alternative is to directly measure the k_2/K ratio for the labelled compound (which then becomes the reference compound R) by a point-by-point method if conditions can be found where this reaction is sufficiently slow.

4. Individual values of k_2 and K

Obtaining the individual values of k_2 and K on the basis of competition experiments is also possible. To do so, one needs to record the time-courses of the nitrocefin reaction

in the absence and in the presence of the competing compound. Model (2) yields the following differential equations:

$$\frac{d(ER^*)}{dt} = k_{2R}(ER)$$

$$\frac{d(EC^*)}{dt} = k_{2C}(EC)$$

and since E, ER and EC are assumed to be in rapid equilibrium

$$\frac{d\alpha}{dt} = -k_{2R}(ER) - k_{2C}(EC)$$

where

$$\alpha = (E) + (EC) + (ER)$$

Integration of these equations shows that the appearance of both EC^* and ER^* as well as the disappearance of non-acylated enzyme are characterised by the same rate constant, k_a, but the three rates are different.

$$\frac{\alpha}{E_0} = e^{-}k_a t$$

$$\frac{(ER^*)}{E_0} = k_{aR}\frac{(1 - e^{-}k_a t)}{k_a}$$

and

$$\frac{(EC^*)}{E_0} = k_{aC}\frac{(1 - e^{-}k_a t)}{k_a}.$$

Note that $(EC^*)/(ER^*) = k_{aC}/k_{aR}$, in agreement with Eq. (1) and that $(ER^*) + (EC^*) = E_0 - \alpha$.

The value of k_a, a pseudo-first-order rate constant can be considered to be the sum of two other constants, as detailed by Eqs. (2)–(4)

$$k_a = k_{aC} + k_{aR} \tag{2}$$

where

$$k_{aC} = \frac{k_{2C}K_R(C)}{D} \tag{3}$$

$$k_{aR} = \frac{k_{2R}K_C(R)}{D} \tag{4}$$

and

$$D = K_C(R) + K_R K_C + K_R(C)$$

Since k_{2R} and K_R are known, Eq. (1) can also be written as:

$$k_a = \frac{1 + b(C)}{c + d(C)} \tag{5}$$

where

$$b = \frac{k_{2C}}{K_C}\frac{K_R}{k_{2R}}\frac{1}{(R)} \tag{5a}$$

$$c = \frac{K_R + (R)}{k_{2R}(R)} \tag{5b}$$

and

$$d = \frac{K_R}{K_C k_{2R}(R)} \tag{5c}$$

A plot of k_a versus (C) yields a rectangular hyperbola from which the values of b and d and thus of k_{2C} and K_C can be derived (since c only contains (R), k_{2R} and K_R).

In the absence of C,

$$k_a = \frac{k_{2R}(R)}{(K_R + (R))} = k_R \tag{6}$$

And, conversely, in the absence of nitrocefin:

$$k_a = \frac{k_{2C}(C)}{(K_C + (C))} = k_C \tag{7}$$

But it is clear that when both compounds are present together, k_a is not equal to $k_R + k_C$ since k_{aR} and k_{aC} are different from k_R and k_C, respectively (compare Eqs. (3) and (4) to Eqs. (6) and (7)).

5. Simplified equations

(a) If $(R) \ll K_R$, the k_a value simplifies to:

$$k_a = \frac{k_{2C}(C)}{(K_C + (C))} + \frac{k_{2R}K_C(R)}{(K_C K_R + K_R(C))} \tag{8}$$

$$= k_C + \frac{k_{2R}K_C(R)}{(K_C K_R + K_R(C))} \tag{8a}$$

Thus, k_a is not linear versus (C) and both k_{2C} and K_C can be obtained by measuring k_a for various values of (C). But if the first right-hand term of the equation is equal to k_C, the second is not equal to k_R and the dependence of k_a versus (C) is characterised by Eq. (5) with the only difference that

$$c = \frac{K_R}{k_{2R}(R)}$$

(b) Similarly, if $(C) \ll K_C$,

$$k_a = \frac{k_{2C}K_R(C)}{K_C K_R + K_C(R)} + \frac{k_{2R}(R)}{(K_R + (R))} \tag{9}$$

$$k_a = \frac{k_{2C}K_R(C)}{K_C K_R + K_C(R)} + k_R$$

Now, k^a is linear versus (C) but only the k^{2C}/K^C ratio can be derived and not the individual values of k^{2C} and K^C.

(c) Finally, if (C) ≪ than K_C and $(R) \ll K_R$,

$$k_a = \frac{k_{2C}(C)}{K_C} + \frac{k_{2R}(R)}{K_R} \tag{10}$$

In this case and in this case only, $k_a = k_C + k_R$ and k_a is linear versus both (C) and (R) but it is clear that, as above in case (b), the individual values of k_{2C} and K_C cannot be derived.

6. Variation of k_a versus (C)

In conclusion, if (C) $\ll K_C$ (simplified cases (b) and (c)), k_a increases linearly with (C) whatever the (R)/K_R ratio (Eqs. (9) and (10)). If all the experiments are performed at the same (R), k_{aC} can be computed as $k_a - k_R$ but the individual k_{2C} and K_C values cannot be derived whatever the R concentration. By contrast, in the general case and in simplified case (a), k_a might increase or decrease with (C) or even be independent of C. When k_a varies with (C), it does so in a hyperbolic way and k_{2C} and K_C can be deduced (but see Section 7 for practical limitations). If k_a does not vary with (C), the experiments should be repeated at another concentration of the reference inactivator (see next paragraph).

That k_a might decrease when (C) increases is not surprising: intuitively, if $k_{2C} < k_{2R}$ and the concentration of the competing compound is close to K_C (a prerequisite for measuring K_C, as stated above), some enzyme is immobilised as EC which transforms into EC* more slowly than ER does to ER* and the rate constant decreases. In a more mathematical way, the first derivative of Eq. (5) is

$$(k_a)' = \frac{bc - d}{(c + dx)^2}, \quad \text{where } x = (C)$$

Thus, $(k_a)'$ is positive and k_a increases with increasing (C) if $bc > d$ and k_a decreases with increasing (C) if $bc < d$.

On the basis of the values of b, c and d (Eqs. (5a)–(5c)), it follows that if $k_{2C} > k_{2R}(R)/(K_R + (R))$, k_a increases with increasing (C) values, while it decreases if k_{2C} is $< k_{2R}(R)/(K_R + (R))$. When $k_{2(C)} = k_{2R}(R)/(K_R + (R))$, k_a is independent of (C) and remains equal to $k_{2R}(R)/(K_R + (R))$, i.e. the k_R value. It is clear that in the latter case, a decrease of the reference inactivator concentration results in a k_a value which increases versus (C) in a hyperbolic way and that an increase of (R) results in a k_a value which decreases versus (C).

7. Practical limitations

Thus, competition experiments can theoretically be useful for determining the k_2 and K values of a series of "spectroscopically silent" compounds when the parameters are known for a reference compound for which the continuous monitoring of the accumulation of ER* is possible, but the adequate Eq. (5) should be used to calculate the parameters of the competing inactivator. However, a practical difficulty arises from the fact that the difference between the k_a value in the absence and that in the presence of a sufficient concentration of C can be rather small. To highlight this problem, we have performed a series of simulations using Eq. (5) and adding a 2.5% "noise" to simulate experimental errors. As can be seen by comparing examples 3 and 4 to examples 1, 2 and 5 in Table 1 (see also Fig. 1), very large errors are to be expected when the relative difference between k_R and k_{2C} becomes small. To avoid this difficulty, the concentration of the reference compound can be decreased, which results in a decrease of k_R (examples 6 and 7). The errors on K_C and k_{2C}/K_C are then significantly lower. However, another problem is then encountered: the amplitude of the recorded signal significantly decreases when large concentrations of C are utilised (examples 6 and 7) to increase the difference between k_R and k_{2C}. This means that to obtain good results, a rather large signal must be observed in the absence of C, a situation which requires the utilisation of rather large enzyme concentrations. One should note, however, that an independent measurement of k_{2C}/K_C performed as discussed above in Section 2 might improve the reliability of the individual k_{2C} and K_C values.

8. Examples of incorrect derivations of k_2 and K

An equation similar to Eq. (10) has been utilised by Graves-Woodward and Pratt [9] to measure the k_2/K values for a series of antibiotics using nitrocefin as the reference compound. The authors mention that all k_a plots were linear for the concentrations of β-lactams used which shows that Eq. (10) was indeed valid under the conditions they utilised.

By contrast, in two recent papers, Golemi-Kotra et al. [10] and Fuda et al. [11] have utilised an equation similar to Eq. (4) (referring to the paper by Graves-Woodward and

Table 1
Errors on the measured parameters for various values of k_R and k_{2C}

Example	k_{2C}	[R]	Range of k_a values[a]	k_a at (C) = 1000	Amplitude at (C) = 1000[b]	Error on k_{2C}/K_C (%)	Error on K_C (%)
1	1	100	0.5–1.0	0.86	17	29	33
2	2	100	0.5–2.0	1.57	9	19	26
3	0.6	100	0.5–0.6	0.57	25	106	117
4	0.4	100	0.5–0.4	0.43	33	72	64
5	0.2	100	0.5–0.2	0.28	50	16	10
6	0.4	10	0.09–0.4	0.34	4.8	13	17
7	0.6	10	0.09–0.6	0.51	3.2	12	16

The values of the other constants are $k_{2R} = 1$, $K_R = 100$, $K_C = 200$ and (C) was varied between 0 and 1000. The units are arbitrary.
[a] The lower value is equal to k_R, i.e. $1/c$ in Eq. (5) and the higher value to k_{2c}, i.e. b/d in Eq. (5).
[b] The amplitude at (C) = 0 was taken as 100.

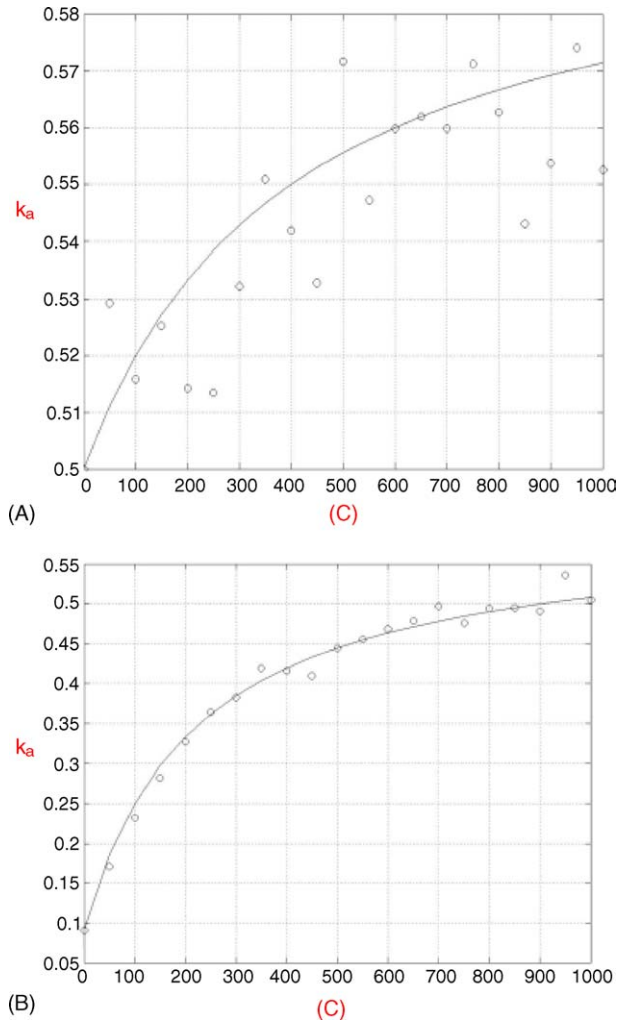

Fig. 1. Simulated k_a values (examples 3 and 7 of Table 1). The improvement of the fit is quite clear when the k_R value is decreased. The k_a values were calculated using Eq. (1). To simulate experimental errors, a random "noise" was introduced corresponding to a maximum of 2.5% of the highest calculated value. The lines were drawn by non-linear regression on the basis of Eq. (5) yielding the following parameters (arbitrary units). (A) Example 3: $b = 0.00201$; $d = 0.00323$; hence $K_c = 312$ and $k_{2c} = 0.624$ (theoretical values 200 and 0.6, respectively). (B) Example 7: $b = 0.0294$; $d = 0.0491$; hence $K_c = 204$ and $k_{2c} = 0.61$ (theoretical values 200 and 0.6, respectively). The errors on the determined K_C and k_{2C}/K_C values are those given in Table 1. The units are arbitrary.

Pratt) to compute the individual k_2 and K values for various β-lactam using the same competition method with nitrocefin as reference.

The authors have calculated k_{aC} as $k_a - k_R$. Apparently, they obtained a hyperbolic dependence of k_{aC} versus (C) and they deduced k'_{2C} and K'_C values (in the following discussion, the incorrect k_{2C} and K_C values derived by these authors will be referred to as k'_{2C} and K'_C, respectively) for a series of competitors by assuming that

$$k_{aC} = k_C = \frac{k'_{2C}}{(K'_C + (C))}.$$

Table 2
Values of k_a computed on the basis of Eq. (5) and of the parameters reported by Golemi-Kotra et al. [10]

β-Lactam	k'_{2C} (s^{-1})	K'_C (μM)	(C) (μM)	Calculated k_a (s^{-1})
Ampicillin	1.0	23	23	5.6
Cefepime	1.4	25	25	5.8
Ceftazidime	4.1	68	68	6.8
FA-penicillin[a]	1.6	13	13	5.8

The PBP is the BlaR C-terminal domain of *Staphylococcus aureus*. The reference substrate is 12 μM nitrocefin ($k_{2R} = 26\ s^{-1}$, $K_R = 24$ μM, $k_R = 8.7\ s^{-1}$). The concentration of the competing β-lactam has been chosen as equal to K_C in all cases. Note that all the k_a values are lower than k_R, which underlines the incoherence between the published values of the k_{2C} and K_C parameters and the experimental data.
[a] Furacroylpenicillin.

However, as shown above, if k_a varies with (C) in a hyperbolic way, k_{aC} is not equal to $k_a - k_R$ and k_{aC} is different from k_C.

By applying Eq. (5), the k_a values which can be calculated on the basis of the k'_{2C} and K'_C values derived by Golemi-Kotra et al. [10] are, with several competing β-lactams, lower than the k_R value for nitrocefin alone (Table 2). The situation with furacroyl penicillin is depicted in more detail in Table 3: k_a decreases versus (C) in a hyperbolic way. In consequence, the calculations highlight a complete incoherence between the published kinetic parameters and the assumption that k_{aC} was equal to $k_a - k_R$ since k_a is found to be smaller than k_R. How is this possible? In other words, should one not expect the experimental data to indicate that the wrong equations are applied? As long as k_a increases versus (C), the answer is no. Indeed, Eq. (2) shows that in this case, k_a varies in a hyperbolic way from k_R when (C) = 0 to a limiting value $[(k_a)_{lim}]$ of k_{2C} when (C) is very large. Thus, and as discussed in Section 6, if $k_{2C} > k_R$, k_a increases versus (C) and $k_a - k_R$ also increases versus (C) in a hyperbolic way. This can be easily shown by simulating a situation where $k_{2R} = k_{2C} = 1$, $K_R = 100$ and $K_C = 200$ (the units are arbitrary). By assuming (R) = 100 and varying (C) from 50 to 800, it appears that $k_a - k_R$ is indeed hyperbolic but utilisation of the incorrect equation:

$$k_a - k_R = k_C = \frac{k'_{2C}(C)}{(K'_C + (C))} \quad (11)$$

yields erroneous values of 0.5 and 400 for k'_{2C} and K'_C.

Table 3
Expected concentration dependence of k_a for furacroylpenicillin on the basis of the parameters reported by Golemi-Kotra et al. [10]

(FA-penicillin) (μM)	Calculated k_a values (s^{-1})
0	8.7
6.5	6.9
13	5.8
19.5	5.1
26	4.6
39	4.0
52	3.5
65	3.2

The reference substrate is 12 μM nitrocefin ($k_{2R} = 26\ s^{-1}$, $K_R = 24$ μM).

Table 4
Correct values of k_{2C} and K_C calculated on the basis of the incorrect k'_{2C} and K'_C of Golemi-Kotra et al. [10]

	k_{2c} (s^{-1})	K_C (μM)
Nitrocefin[a]	26	24
Ampicillin	9.7	16
Cefepime	10.1	17
Ceftazidime	12.8	45
FA-penicillin	10.3	9
Oxacillin	27	32
Imipenem	15	120

[a] Reference compound. The authors' values are taken as correct.

Is it possible to estimate the real values of k_{2C} and K_C on the basis of the published data? Although the raw experimental data of the authors are not available, Eq. (5) shows that the limit value of k_a for large values of (C) is k_{2C}. Since the authors have used the incorrect Eq. (11), it can be seen that the real limit value of k_a is $k_R + k'_{2C}$. Thus, the real values of k_{2C} are easily obtained as $k_R + k'_{2C}$. Similarly, it can be shown that the erroneous K'_C values are related to K_C by Eq. (12):

$$K'_C = K_C \frac{K_R + (R)}{K_R} \quad (12)$$

and the correct k_{2C} and K_C values are given in Table 4.

However, these values should be considered with a lot of caution, particularly for ampicillin, cefepime and FA-penicillin. Indeed, in these cases, k_{2C} is only 10–20% larger than k_R and large S.D. values are to be expected (see example 3 in Table 1). More reliable values should be obtained by repeating the experiments with lower nitrocefin concentrations, thus, decreasing the k_R values and increasing the difference between $(k_a)_{lim}$ (i.e. k_{2C}) and k_R.

When the same analysis is applied to the data of Fuda et al. [11], some k_a values computed on the basis of the incorrect K'_C and k'_{2C} values are also found to decrease versus (C) (Table 5). Here, however, the k'_{2C} values range between 50 and 270% of k_R so that the k_{2C} and K_C values calculated by us are probably more reliable (Table 6).

Table 6
Correct values of k_{2C} and K_C calculated on the basis of the k'_{2C} and K'_C values of Fuda et al. [11]

Enzyme	β-Lactam	k_{2C} (s^{-1})	K_C (μM)
Wild-type PBP2a	Nitrocefin[a]	3.7×10^{-3}	192
	Oxacillin	3×10^{-3}	112
	Ceftazidime	2.4×10^{-3}	420
	Cefepime	2.9×10^{-3}	1000
	Ampicillin	4.8×10^{-3}	420
	Imipenem	3.1×10^{-3}	370
K406A	Nitrocefin[a]	4.5×10^{-5}	200
	Oxacillin	2.9×10^{-5}	500
	Ceftazidime	2.5×10^{-5}	320
Y519F	Nitrocefin[a]	2.8×10^{-3}	230
	Oxacillin	3.6×10^{-3}	390
	Ceftazidime	2.5×10^{-3}	920
K406A/Y519F	Nitrocefin[a]	5.2×10^{-5}	200
	Oxacillin	3.2×10^{-5}	690
	Ceftazidime	3.9×10^{-5}	580

[a] Reference compound. The authors' values are taken as correct.

In summary, in both cases, the published k'_{2C} values are always smaller than the real k_{2C} and the K'_C values larger than K_C so that the k'_{2C}/K'_C ratios are sometimes one order of magnitude too low (15-fold for ampicillin and BlaR, compare Tables 2 and 4).

9. A protocol for obtaining correct parameters

To obtain correct k_{2R} and K_R values, the following protocol can be suggested. In all cases, it should be remembered that both (C) and (R) must be significantly higher than E_0 (at least 10-fold).

(a) First measure the k_{2C}/K_C ratio as described in Section 3 and with the help of Eq. (1).

Table 5
Values of k_a computed for PBP2a and its mutants on the basis of the parameters reported by Fuda et al. [11]

Enzyme	k_R (s^{-1})	β-Lactam	K'_C (μM)	k'_{2C} (s^{-1})	β-Lactam (μM)	Calculated k_a (s^{-1})
Wild-type	1.42×10^{-3}	Oxacillin	180	1.6×10^{-3}	90	1.46×10^{-3}
					180	1.49×10^{-3}
					270	1.51×10^{-3}
		Ceftazidime	670	1.0×10^{-3}	335	1.32×10^{-3}
					670	1.26×10^{-3}
					1035	1.22×10^{-3}
K406A	1.69×10^{-5}	Oxacillin	800	1.2×10^{-5}	400	1.57×10^{-5}
		Ceftazidime	510	0.8×10^{-5}	255	1.48×10^{-5}
Y519F	0.96×10^{-3}	Oxacillin	590	2.6×10^{-3}	295	1.41×10^{-3}[a]
		Ceftazidime	1400	1.5×10^{-3}	700	1.16×10^{-3}[b]
K406A/Y519F	1.95×10^{-5}	Oxacellin	1100	1.2×10^{-5}	550	1.77×10^{-5}
		Ceftazidime	930	1.9×10^{-5}	465	1.94×10^{-5}

The reference compound is nitrocefin at the concentration (120 μM) utilised by the authors. In all cases, the competing substrate concentrations are indicated. The calculated k_a values are sometimes lower than k_R or are not significantly modified.

[a] $k_R + k_C = 1.8 \times 10^{-3}\ s^{-1}$.

[b] $k_R + k_C = 1.46 \times 10^{-3}\ s^{-1}$.

(b) Measure the k_{2R} and K_R values. This is, however, not absolutely necessary. If the K_R value remains linear versus (R) up to the highest R concentration, Eq. (8a) applies and can be rewritten as:

$$k_a = k_C + \frac{k_{2R}(R)}{K_R} \frac{K_C}{K_C + (C)} \quad (8b)$$

where only the k_{2R}/K_R ratio needs to be known to determine k_{2C} and K_C.

(c) Choose conditions in which C significantly modifies the k_a values. Ideally, k_a should decrease or increase by at least 50% at the highest (C) values.

(d) The k_a should vary in a hyperbolic way versus (C). This requires the utilisation of C concentrations close to K_C in simplified case (a) (Eq. (8b)) but higher than K_C if (R) is close to or larger than K_R (general case).

The following points need special attention.

- At high (C) values, the amplitude of the signal might become quite small (Table 1). The concentrations of the enzyme and/or R should then be increased.
- If the difference between k_a and k_R is not sufficient, the simplest solution is to decrease (R) but this will not always be possible due to the amplitude problem (see preceding point).
- If k_a decreases with increasing (C) values, rather high concentrations of R improve both the amplitude of the signal and the difference between k_R and k_a.

Finally, numerical simulation and fitting methods are now available, which facilitate the analysis of the results. These methods have the same dangers inherent to their application as does the method described here: poor results due to small differences between k_a and k_R or to low amplitudes of the signal yield inaccurate parameters even when analysed by a sophisticated fitting method.

10. Conclusion

In this commentary, we have described a competition method which can be used for studying the inactivation of PBPs and of other enzymes for which a reference inactivator is available whose reaction with the target enzyme can be continuously and directly monitored. We have derived the characteristic equation and shown how the incorrect utilisation of a simplified equation can lead to erroneous values of the competing inactivator's kinetic parameters. We have also attempted to estimate the correct parameters on the basis of some published incorrect values.

Finally, we have discussed the limitations of the method by underlining that, in some cases, increasing the range of k_a measurements results in a simultaneous decrease of the amplitude of the measured signal.

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