Biochimica et Biophysica Acta, 611 (1980) 351—357

BBA 68897

INHIBITION KINETICS OF THREE R-FACTOR-MEDIATED β-LACTAMASES BY A NEW β-LACTAM SULFONE (CP 45899)

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(Received June 28th, 1979)

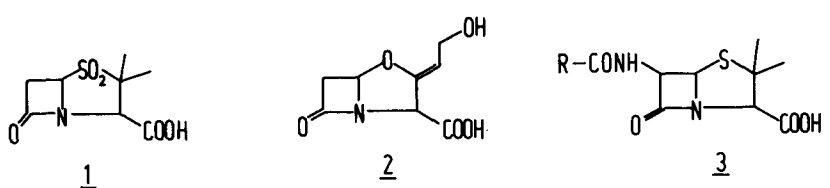
Key words: R-Factor; β-Lactamase; Sulfone CP 45899; Inhibition kinetics

Summary

A new β-lactam sulfone, CP 45899, has been proved to be a time-dependent irreversible inhibitor of three R-factor-mediated β-lactamases (penicillin amido-β-lactamhydrolase, EC 3.5.2.6): TEM-1 (pI = 5.4), TEM-2 (pI = 5.6) and Pitton's type 2 (pI = 7.7). This inhibition occurs in two principal steps: (1) formation of a reversible enzyme-inhibitor complex (characterized by a K_i); (2) evolution of this complex into one, or more, inactive protein(s) (k_{inact}). With the three β-lactamases CP 45899 shows, respectively, K_i of 0.9, 0.8 and 1.8 μM and k_{inact} of $1.2 \cdot 10^{-3}$, $0.8 \cdot 10^{-3}$ and $1 \cdot 10^{-3}$ s^{-1}; the turnover numbers are: 525, 2280 and 1220. These results are compared to those previously obtained with clavulanic acid.

Introduction

Sulfone CP 45899 (penicillanic acid sulfone) is a new semi-synthetic β-lactam [1]. The chemical structure of this compound may be considered as an



1 2 3

Fig. 1. Chemical structures of sulfone CP 45899 (1) clavulanic acid (2) and penicillins (3).

intermediate between clavulanic acid [2] and the traditional penicillins (Fig. 1). It is noticeable that sulfone CP 45899, numbered as a clavulanic acid, has no substitution on the classic 6 position of the penicillin nucleus. Reports dealing with sulfone CP 45899 [1,3,4] tend to show that an important characteristic of this molecule is to act as a β-lactamase (penicillin amido-β-lactamhydrolase, EC 3.5.2.6) inhibitor. This phenomenon is also found in bacteriology: the molecule extends or potentiates the antibacterial activity of the classical β-lactam antibiotics, such as ampicillin.

In the present paper, we wish to describe the inhibitor properties of sulfone CP 45899 towards three R-factor-mediated β-lactamases, which are now quite well known in this field [5].

Materials and Methods

β-Lactam compounds. Sulfone CP 45899 sodium salt (lot No 9807—233 IF) was a gift from Pfizer Inc. (Groton, CT), clavulanic acid was a gift from Beecham, and penicillin G a gift from Rhône-Poulenc.

Bacterial strains and β-lactamases. TEM-1 β-lactamase (pI = 5.4, M_r = 23 000) was produced by *Escherichia coli* P111, TEM-2 (pI = 5.6, M_r = 24 000) by *E. coli* RP4 and Pitton's type 2 (pI = 7.7, M_r = 21 500) by *E. coli* P453 [5,7]. After high purification, these enzymes had, respectively, specific activities of 1200, 3000 and 750 U/mg (some of these values were considerably higher that those previously described [5]). Thus, from $V = k_3 (E_0)$, the rate constants k_3 were respectively 465, 1200 and 270 s^{-1} for the three enzymes. The K_m values, for penicillin G, were respectively 21.7, 15 and 10.6 μM values which were used in the following calculations.

Kinetic and inhibition constants. The kinetic constants were monitored by on-line computerized microacidimetry, at pH 7 and 37°C [8].

Inhibition kinetics were also measured by computerized microacidimetry as described previously in the case of clavulanic acid [6]. With sulfone CP 45899, the dissociation constants for enzyme-inhibitor complex were determined by means of Dixon plots, i.e. by measuring the initial rate of hydrolysis (V_i) of the substrate (penicillin G) at various concentrations of substrate and inhibitor, with the computer's assistance. The time-dependent phase of irreversible inhibition was monitored by following the percentage of inhibition obtained with respect to the preincubation time.

Turnover numbers. The turnover numbers are defined as the number of molecules of inhibitor destroyed for each molecule of enzyme that is inactivated. Since sulfone CP 45899 hydrolysis could be monitored directly in the classical way, its turnover numbers were obtained by the ratio of the hydrolysis constants versus the inactivating constants (k_{inact}). In the case of clavulanic acid, it was easier to measure the stoichiometry of the inactivation; these values were not given in Ref. 6.

Results

Hydrolysis of sulfone CP 45899

The rate of sulfone CP 45899 hydrolysis was very low. However, with large

TABLE I

KINETIC DATA OBTAINED WITH SULFONE CP 45899

V' is given as a relative value with penicillin G(V = 100).

	K_i (μM)	V'	k_6(s^{-1})	k_{inact} (s^{-1})	Turnover numbers
TEM-1	0.9	0.12	0.63	0.0012	525
TEM-2	0.8	0.15	1.82	0.0008	2280
Type 2	1.8	0.45	1.22	0.001	1220

amounts of enzyme (about 20 units), an initial maximum rate of hydrolysis V' was measurable. It is noticeable that all of the compound was not hydrolyzed during the reaction, the enzymic activity being progessively destroyed as the compound acted as a 'suicide substrate'.

Using to the maximal specific activities reached for the enzymes and their apparent molecular weights, k_3, the rate constant of penicillin G hydrolysis, could be determined and consequently k_6, which is defined below as the rate constant of sulfone CP 45899 hydrolysis. The results are given in Table I.

Effect of sulfone CP 45899 concentration on β-lactamase inhibition

Since the phase of β-lactamase inhibition by sulfone CP 45899 appears to be much slower than which occurs in the case of the clavulanic acid, we could not use the same experimental procedure that was previously reported. Thus, we used Dixon plots with no preincubation of the enzyme with inhibitor. As an example with Pitton's type 2 β-lactamase, Fig. 2 shows that the inhibition was concentration dependent. Moreover, it demonstrated that the inhibition was competitive and allowed K_i determination to be made. The K_i values obtained are, respectively, 0.9, 0.8 and 1.8 μM for the TEM-1, TEM-2 and Pitton's type 2 enzymes.

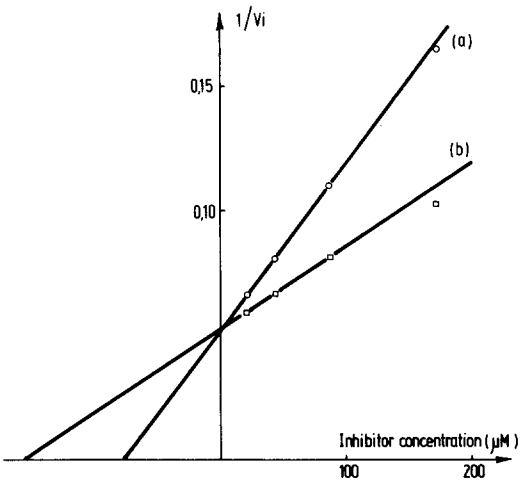


Fig. 2. Kinetic plot of inhibition of type 2 β-lactamase. The substrate is penicillin G at (a) 466 μM, and (b) 932 μM.

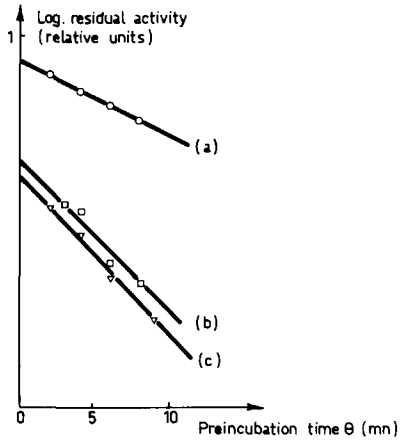


Fig. 3. Effect of preincubation time on residual β-lactamase (type 2) activity with sulfone CP 45899 at concentrations of (a) 1.8 μM; (b) 21.5 μM, and (c) 43 μM. The substrate used is penicillin G at 625 μM.

Effect of preincubation time

The inhibition of penicillin G hydrolysis was enhanced by preincubating the enzyme with sulfone CP 45899 as shown in Fig. 3. Within the range of incubation time (30 min) no recovery of activity could be detected. The inhibition appears to be irreversible since, after extensive dialysis, no more enzymic activity was obtained.

Plots of log V_i against incubation time θ, at various inhibitor concentrations were linear. This suggested that the inactivation kinetic process was pseudo first order. For each inhibitor concentration a slope k could be determined, and when sulfone CP 45899 concentration was high enough (i.e. when the active site of the enzyme was saturated with the inhibitor) k then remained constant; this value was thus called k_{inact}.

Turnover numbers

With sulfone CP 45899, the turnover numbers obtained by the ratio of k_6/k_{inact} were 525, 2280 and 1220 for, respectively, TEM-1, TEM-2 and Pitton's type 2. With clavulanic acid the stoichiometry of the reaction gave, respectively, the values of 160, 340 and 20.

Discussion

As it was previously shown for clavulanic acid [6], sulfone CP 45899 acts as a competitive and time-dependent inhibitor of the R-factor-mediated β-lactamases TEM-1, TEM-2 and Pitton's type 2 [5,7]. These compounds belong to the now rather well-known class of inhibitors named 'suicide substrates' or 'suicide reagents' [9].

Thus, the inhibition kinetics follow a particular scheme [6]:

$$E + I \underset{k_5}{\overset{k_4}{\rightleftharpoons}} (E, I) \overset{k_6}{\rightarrow} (E, I^*) \begin{matrix} \overset{k_7}{\nearrow} E - I \\ \underset{k_8}{\searrow} E + P^* \end{matrix}$$

where k_6 is the kinetic constant of the rate-determining step.

Thus, three important parameters can be described: (a) $K_i = (k_5 + k_6)/k_4$, the equilibrium constant of the rapid and reversible formation of a Michaelian enzyme-inhibitor complex; (b) $k_{inact} = k_6 \cdot k_7/k_8$, the kinetic constant of the irreversibly inhibited enzyme formation, and (c) the turnover number which represents the number of inhibitor molecules destroyed for each inactivating event; this turnover number is k_8/k_7. In the previous paper [6], this last parameter was not determined, thus some minor changes in the notations appear necessary, as the mathematical analysis is a little more complex.

These parameters are determined by the following mathematical analysis:

(a) Rate of inhibitor hydrolysis. If $[I_0] >> K_i$, and if [I] might be considered as a constant during the reaction, the rate of inhibitor hydrolysis $V'(t)$ is:

$$V'(t) = k_8[E, I^+] = k_6 \frac{k_8}{k_7 + k_8} [E, I]$$

but, as $k_8 >> k_7$ (which is verified later):

$$V'(t) = k_6[E, I]$$

During the same time, irreversible inhibition of the enzyme occurs. If $[E_0]$ is the initial amount of enzyme, at any time $[E_0] = [E, I] + [E—I]$, thus:

$$\frac{d[E - I]}{dt} = -\frac{d[E, I]}{dt} = k_7[E, I^+] = k_6 \cdot \frac{k_7}{k_8} [E, I]$$

after integration

$$[E, I] = [E_0] \exp(-k_{inact} \cdot t)$$

which leads to the expression of the rate of the inhibitior hydrolysis:

$$V'(t) = k_6[E_0] \exp(-k_{inact} \cdot t)$$

This allows the determination of k_6.

(b) The enzyme is incubated with both penicillin G and sulfone CP 45899. If we consider that the inhibitor (I) and substrate (S) concentrations remain rather constant during the reaction, the rate of penicillin G hydrolysis can be determined, as described in Ref. 6, it is:

$$v(t) = \frac{V \cdot [S]}{[S] + K_m(1 + [I]/K_i)} \exp\left(-t \cdot k_{inact} / \left(1 + \frac{[S]K_i}{[I]K_m}\right)\right)$$

During the same time, the inhibitor is also partially destroyed but at a smaller rate: its maximum rate of hydrolysis is about a thousand times smaller than those of penicillin G, thus the hydrolysis observed is mainly that of the substrate. In these conditions, Dixon plot analysis of the initial rates of penicillin G hydrolysis (V_i), in presence of inhibitor, gives the K_i by the equation:

$$\frac{1}{V_i} = \frac{1}{V}\left(1 + \frac{[I_0]K_m}{[S_0]K_i}\right)$$

(c) Effects of preincubation time of the enzyme and inhibitor. If the enzyme is preincubated with the inhibitor during a time θ, its residual activity decreases related to $[\exp(-\theta \cdot k_{inact}/(1 + K_i/[I])]$. If one adds penicillin G at that time (θ), the residual activity is given by:

$$\log \frac{V_i(\theta)}{V} = -\frac{\theta \cdot k_{inact}}{I + K_i/[I_0]} - \log\left[1 + \frac{K_m}{[S_0]} \cdot \left(1 + \frac{[I_0]}{K_i}\right)\right]$$

which allows k_{inact} determinations. After what, the turnover is obtained by the ratio: $k_6/k_{inact} = k_8/k_7$.

From the previous results [6], (Table II), it is interesting to note that, during the first step of the inhibitory pathway, sulfone CP 45899 and clavulanic acid are practically equivalent: they have similar K_i values. This emphasizes the importance of the substitution, or here the absence of a side chain, at the C-6 position of the penicillin nucleus.

Surprisingly, some observations of Fischer et al. [10] and Charnas et al. [11] with penicillanic acid, a compound without a substituent at the same position, show that it is not an inhibitor of β-lactamase, and has only a very bad affinity (K_m = 350 μM) for the TEM-like β-lactamase; the compound is also a poor substrate.

From the available kinetic data, we can see that the K_i usually obtained with the 'suicide reagents' are considerably higher that those generally obtained with reversible inhibitors of cephalosporinases. For example, with cloxacillin and a *Pseudomonas aeruginosa* cephalosporinase we found a K_i of $4.5 \cdot 10^{-3}$ μM [12], which corresponds to an affinity approximately a hundred times better. This is probably the reason why, with 'suicide reagents', the time-dependent phase is so important.

The second phase of the inhibitor process is the β-lactam opening by the enzyme. This step is also called the catalytic phase, as the product of enzymic conversion might be a molecule highly reactive towards the active site of the β-lactamase, while the parent molecule is not. With our notations, this step is characterized by k_6. With TEM-1 and TEM-2, sulfone CP 45899 appears about six times less active than clavulanic acid, but with Pitton's type 2 the reverse situation is observed.

On the other hand, the turnover numbers are only slightly higher for sulfone CP 45899 with TEM-1 and 2 (3.3 and 6.7 times, respectively) but considerably more with Pitton's type 2 (61 times).

TABLE II

KINETIC DATA OBTAINED WITH CLAVULANIC ACID

Some of these values are from [6]. V' is given as a value relative to penicillin G hydrolysis (V = 100).

	K_i (μM)	V'	k_6 (s^{-1})	k_{inact} (s^{-1})	Turnover numbers
TEM-1	0.8	0.92	4.3	0.027	160
TEM-2	0.7	0.85	10.2	0.030	340
Type 2	0.6	0.34	0.92	0.046	20

Thus, the k_{inact} values are globally 20—50 times lower for sulfone CP 45899 than for clavulanic acid. Nevertheless, sulfone CP 45899 acts synergistically with β-lactam antibiotics against a large number of bacterial strains.

Recently, Fu and Neu [4] described few inhibitory properties of this molecule, but did not take into account the time-dependent phase of the inhibition. Now, we think it is possible that (from the molecular point of view) the pathway of sulfone 45899 and clavulanic acid inhibition might be different. On the other hand, we cannot exclude the fact that the synergistic effect observed with sulfone CP 45899 and other β-lactam antibiotics might involve another mechanism such as that observed with mecillinam [13]. This aspect should be enhanced by the observations of Curtis [14] who showed that sulfone CP 45899 has some affinity for the PBP-2 of *E. coli*.

Very recently, papers on two new competitive and time-dependent inhibitors of β-lactamase have been published. One of them is the 6-α-chloropenicillanic acid sulfone [15], and the other is 6-β-bromopenicillanic acid [16—18]. Unfortunately, only a few kinetic data were presented. A recent work from Knott-Hunziker et al. [19] shows that the latter compound labels serine 44 of the *Bacillus cereus* β-lactamase.

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