

## NOTES

# Improved Sensitivity in Assays for Binding of Novel $\beta$ -Lactam Antibiotics to Penicillin-Binding Proteins of *Escherichia coli*

KAREN BUSH,\* SANDRA A. SMITH, SHARI OHRINGER, S. K. TANAKA, AND DANIEL P. BONNER

*The Squibb Institute for Medical Research, Princeton, New Jersey 08540*

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**Tigemonam and temocillin, but not aztreonam, bound to penicillin-binding proteins (PBPs) 1a and 3 of *Escherichia coli* with apparent improved affinity when challenged with benzylpenicillin at lowered temperatures. Half times for deacylation of the tigemonam-PBP complexes were shorter than were those of the corresponding aztreonam-PBP complexes. The implications of the routine testing of PBP affinities are discussed.**

Interactions of  $\beta$ -lactam antibiotics with specific receptor proteins in bacteria are examined critically in many antibiotic development programs. Early studies by Spratt (11) were instrumental in establishing assay procedures by which characteristics of these penicillin-binding proteins (PBPs) could be defined, especially with respect to their affinities for penicillins and cephalosporins. However, the recent appearance of structurally novel  $\beta$ -lactam antibiotics has required a reexamination of the nature of these interactions and a reevaluation of assay procedures used routinely for the study of these interactions.

(This work was presented in part previously [K. Bush, S. A. Smith, and D. P. Bonner, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 855, 1986].)

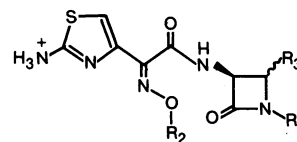
Tigemonam (Fig. 1), a new oral monobactam, exhibits excellent activity against aerobic gram-negative bacteria (3, 13) and is very similar to aztreonam in its microbiological properties. As with typical  $\beta$ -lactam antibiotics, the mode of action for killing appears to be inhibition of cell wall synthesis by binding to PBPs. In members of the family *Enterobacteriaceae*, filamentation is the primary morphological response to monobactams such as aztreonam, tigemonam, and carumonam when tested at the MIC, consistent with the observation that PBP 3 has been inhibited (5, 7, 11). Although MICs are identical for the three monobactams, aztreonam and carumonam bind to PBP 3 of *Escherichia coli* at concentrations lower than 0.1  $\mu$ g/ml (2, 7), whereas significant binding of tigemonam to PBP 3 was observed at a concentration at least 10-fold higher. Similar observations have been reported for temocillin and SQ 81,377 (Fig. 1), for which binding to PBP 3 also was not consistent with the observed biological activity (5, 8).

Because of these anomalies, we investigated the possibility that the standard conditions used to test the binding of  $\beta$ -lactams to PBPs as described by Spratt (11) may result in an underestimation of actual affinities for PBPs. Based on the studies with temocillin by Labia et al. (8), assay conditions were developed in this laboratory that gave results

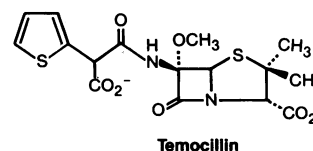
more consistent with the observed biological activity of certain antibiotics.

Solubilized membranes were prepared from *E. coli* SC8294, a strain that produces no detectable  $\beta$ -lactamase activity. Binding of  $\beta$ -lactam antibiotics was determined by procedures described previously (4, 11). Modifications of incubation conditions were developed from the studies of Labia et al. (8). Under standard assay conditions (12), membranes were incubated with monobactam for 10 min, followed by a 10-min incubation with 10 nmol (0.2 mM) of [ $^{14}$ C]benzylpenicillin, all at 30°C. When altered conditions were used, the second incubation temperature was lowered to 0°C. Reaction mixtures were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9), followed by fluorography (1, 10). X-ray films were analyzed with a laser densitometer (model 2212; LKB Instruments, Inc.).

For determination of deacylation rates, incubation times with [ $^{14}$ C]benzylpenicillin were varied from 2 to 30 min.



Compound	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
SQ 81,377	—SO <sub>3</sub> <sup>-</sup>	—CH <sub>3</sub>	H, H
SQ 81,402	—SO <sub>3</sub> <sup>-</sup>	—C(CH <sub>3</sub> ) <sub>2</sub> CO <sub>2</sub> <sup>-</sup>	H, H
Aztreonam	—SO <sub>3</sub> <sup>-</sup>	—C(CH <sub>3</sub> ) <sub>2</sub> CO <sub>2</sub> <sup>-</sup>	$\alpha$ -CH <sub>3</sub> , $\beta$ -H
Tigemonam	—OSO <sub>3</sub> <sup>-</sup>	—CH <sub>2</sub> CO <sub>2</sub> <sup>-</sup>	$\alpha$ -CH <sub>3</sub> , $\beta$ -CH <sub>3</sub>



Temocillin

FIG. 1. Structures of  $\beta$ -lactam antibiotics included in this study.

\* Corresponding author.

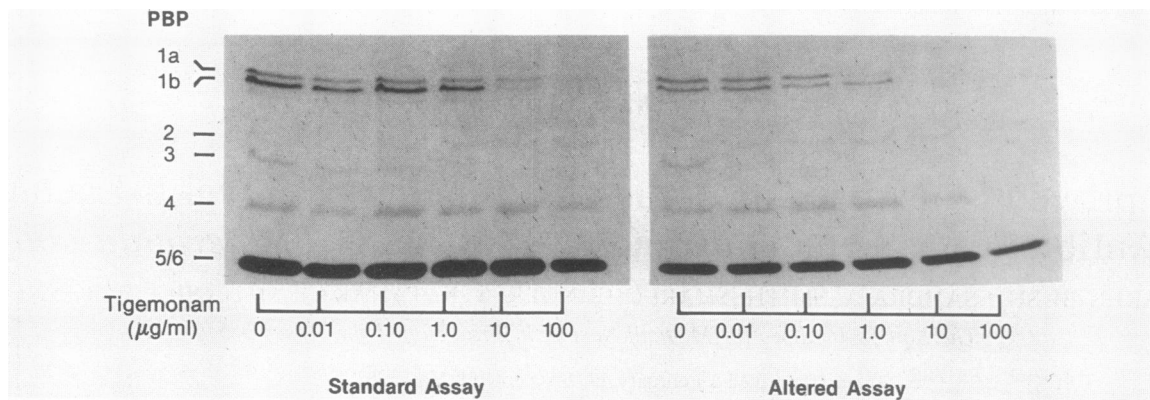


FIG. 2. Binding of tigemonam to PBPs of *E. coli* followed by challenge with [ $^{14}$ C]benzylpenicillin. Under standard conditions, all incubations were performed at 30°C. When altered conditions were used, incubation of tigemonam with solubilized membranes proceeded at 30°C, followed by incubation with [ $^{14}$ C]benzylpenicillin at 0°C.

Acylation rates were studied by incubating monobactam with membranes for fixed times ranging from 30 s to 10 min before challenge with [ $^{14}$ C]benzylpenicillin. Temperatures were maintained at 30°C throughout the kinetic studies.

Antibiotic activity was determined by twofold serial dilutions in yeast-beef agar (Difco Laboratories) with a bacterial inoculum of  $10^4$  CFU. All monobactams were prepared at The Squibb Institute for Medical Research. Temocillin was obtained from Beecham Laboratories, and cefotaxime was obtained from Hoechst-Roussel Pharmaceuticals Inc.

Binding of  $\beta$ -lactam antibiotics to PBPs is determined after an antibiotic is equilibrated with solubilized membranes and then challenged with saturating levels of [ $^{14}$ C]benzylpenicillin. Because this method is based on the competition between an antibiotic and benzylpenicillin for a PBP, transiently stable PBP-antibiotic complexes may not be detected; these complexes could dissociate within the time of the assay and reform as the [ $^{14}$ C]benzylpenicillin-PBP complex. Therefore, it is necessary to select assay conditions that favor the antibiotic-PBP complex over the benzylpenicillin-PBP complex. In our altered assay conditions, a lower incubation temperature was used for the challenge with [ $^{14}$ C]benzylpenicillin, thus allowing fewer kinetic events to occur with respect to complex degradation or formation.

Tigemonam appeared to bind much more tightly to PBPs 1a, 3, and 4 when lower incubation temperatures were used for the benzylpenicillin challenge (Fig. 2). The shifts in concentrations for 50% inhibition of binding of tigemonam and SQ 81,377 to PBPs (Table 1) were similar to observations reported previously for temocillin (8), which was included in our study as a reference. However, binding of aztreonam and the related monobactam, SQ 81,402, was not significantly affected by alterations in assay conditions, indicating differences in behavior within classes of  $\beta$ -lactam antibiotics.

Because the effect of lowered temperature in the [ $^{14}$ C]benzylpenicillin challenge was quite marked for tigemonam, temocillin, and SQ 81,377, kinetic interactions between these antibiotics and various PBPs must have been affected. Deacylation rates of tigemonam were then studied with PBPs 1a and 3, as shown in Fig. 3. The half times for deacylation ( $\pm$  standard deviation) were  $4.7 \pm 0.62$  min for PBP 1a ( $n = 5$ ) and  $6.6 \pm 2.4$  min for PBP 3 ( $n = 10$ ) at 30°C. In contrast, deacylation half times for aztreonam were  $>30$  min for each of these PBPs. Acylation of PBP 3 occurred rapidly for both antibiotics, with completely acylated PBP 3 complexes detected after a 30-s incubation of monobactam with membranes. Therefore, the improved binding observed

TABLE 1. Binding of  $\beta$ -lactam antibiotics to PBPs under varied assay conditions

Antibiotic	Incubation temp (°C)	$I_{50}$ ( $\mu$ g/ml) <sup>a</sup> for binding of antibiotic to PBP:						MIC <sup>b</sup> ( $\mu$ g/ml)
		1a	1b	2	3	4	5/6	
Tigemonam	30	6.0	2.7	>200	0.72	>200	>200	0.40
	0	0.46	2.4	>200	0.015	39	130	
SQ 81,377	30	1.25	64	>200	12	25	>200	3.1
	0	0.65	2.4	98	0.052	2.3	72	
Temocillin	30	18	16	>200	>200	>200	>200	25
	0	2.0	7.5	>200	1.3	6.5	43	
Aztreonam	30	1.6	65	>200	0.008	30	>200	0.40
	0	1.3	50	>200	0.005	46	>200	
SQ 81,402	30	19	>100	>100	0.016	50	>100	0.40
	0	13	>100	>100	0.039	60	>100	

<sup>a</sup> Antibiotic concentration that inhibited binding of [ $^{14}$ C]benzylpenicillin by 50%.

<sup>b</sup> Determined for *E. coli* SC8294 at  $10^4$  CFU.

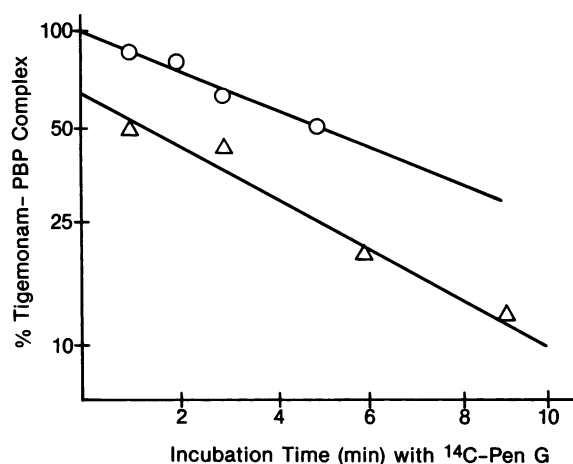


FIG. 3. Deacylation of tigemonam-PBP complexes. Membrane preparations were incubated for 10 min with tigemonam at 30°C before the addition of [<sup>14</sup>C]benzylpenicillin (Pen G). At various times, the reaction was stopped as described previously (4). Percent tigemonam-PBP complex was determined with respect to a control incubated for the same time with water instead of tigemonam. ○, PBP 3 after incubation with tigemonam at 0.10 μg/ml; △, PBP 1a after incubation with tigemonam at 0.50 μg/ml.

at lower assay temperatures may be explained at least in part by the differential in deacylation rates, a possibility initially recognized by Spratt (12).

Although deacylation occurred much more rapidly for tigemonam than for aztreonam, the overall microbiological activities are identical. Therefore, in the growing bacterium, a range of deacylation rates for the PBP-antibiotic complex is apparently possible for an effective antimicrobial agent, as long as the following criteria are met for at least one essential PBP. First, the antibiotic must have a greater affinity for the PBP than for the natural substrate. Second, the antibiotic must be present in saturating amounts so that any free PBP formed (after deacylation) will preferentially bind another molecule of antibiotic.

As discussed previously by several investigators (6, 12), meaningful data for the study of PBP interactions with antibiotics can be difficult to attain, due to complex kinetic interrelationships with multiple enzymes in the membrane preparations. Our results have provided examples of β-lactam antibiotics whose activities have been underesti-

mated with respect to PBP binding. If an inspection of PBP binding data suggests that an antibiotic does not bind to essential PBPs as tightly as predicted based on MIC data, it is recommended that less stringent conditions for [<sup>14</sup>C]benzylpenicillin binding be used to reevaluate the interactions of that molecule with PBPs.

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