Supplementary Material for: A Microtiter Plate-Based β-Lactam Binding Assay for Inhibitors of the High Molecular Mass Penicillin-Binding Proteins

Miglena Stefanova, Sudheer Bobba, and William G. Gutheil

DETAILED MATERIALS and METHODS

Preparation of BIO-AMP: Biotin ampicillin conjugate (BIO-AMP) was prepared by a modification of the method of Dargis and Malouin [1]. Ampicillin (sodium salt, Sigma # A-9518) (100 μL of a 50 mM solution, 5 μmol) in 0.1 M phosphate buffer/150 mM NaCl pH 8.0 (PBS) was treated with of EZ-Link-Sulfo-NHS-LC-Biotin (12.5 mg, 20 μmol) (Pierce) for 30 min. The primary amino group of ampicillin was completely acylated as determined using a ninhydrin spot test for free primary amino groups. Glycine (6 mg, 80 μmol) was then added to consume unreacted biotin conjugating reagent, and after 30 min the mixture was diluted to 200 μL with water to make a 20 mM stock solution of the BIO-AMP labeling reagent, which was stored frozen at -20 °C.

Figure S1

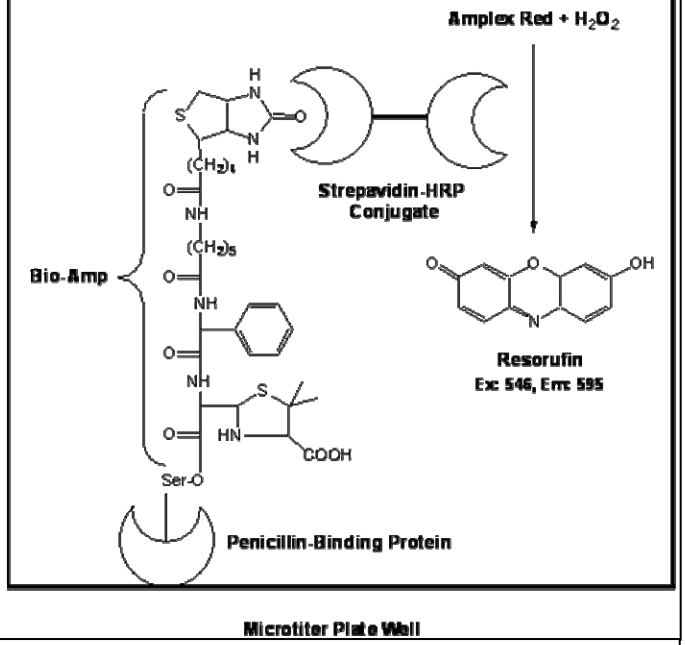

Figure S1: Diagram of the final complex formed in the assay and of the reaction catalyzed by HRP to provide a detectable fluorescent product.

PBPs: EC PBP1B [2, 3], NG PBP 1 [4], NG PBP 2 [5], NG PBP3 [6], and EC PBP 5 [7, 8] were generous gifts from Professor Robert Nicholas (University of North Carolina).

Data analysis: Data were fit to the appropriate expressions given below using the non-linear regression function in SPSS for Windows (Chicago,IL).

PBP loading onto microtiter plates, and labeling and detection (Fig. S1): As described in the main article text.

Determination of BIO-AMP K_m for binding vs various PBPs: PBPs turnover β–lactams (albeit very slowly) following the simplified general Scheme

$$E + I \xrightarrow{k_1} E\text{-}I \xrightarrow{k_2} E + P \qquad \text{Eqn. S1}$$

The steady-state equation for product formation is

$$v = \frac{[E]_T * k_{cat} * [I]}{K_m + [I]} \qquad \text{Eqn. S2}$$

Where

$$k_{cat} = k_2 \qquad \text{Eqn. S3}$$

and

$$K_m = \frac{k_2}{k_1} \qquad \text{Eqn. S4}$$

In the present case we are not measuring product formation, but the fraction of enzyme (PBP) with bound BIO-AMP. The apparent binding isotherm for this system will be

$$[E\text{-}I] = \frac{[I] * [E]_T}{K_m + [I]} \qquad \text{Eqn. S5}$$

The expression for the observed fluorescence (relative fluorescence units, RFU) as a function of added BIO-AMP will be

$$RFU = \frac{RFU_{max} * [I]}{K_m + [I]} \qquad \text{Eqn. S6}$$

To take into account the background (blank) fluorescence, this equation was expanded to

$$RFU = RFU_0 + \frac{RFU_{max} * [I]}{K_m + [I]} \qquad \text{Eqn. S7}$$

To assess BIO-AMP binding to a given PBP, the microtiter plate bound PBP was treated with serially diluted (steps of 5) concentrations of BIO-AMP (each concentration tested in quadruplicate), and the remaining steps of the assay performed as described above. Signals were plotted, and the set of 5 data points bracketing the midpoint of the saturation curve were analyzed for the K_m of binding by fitting the data to Eqn. S7.

Application to HMM PBP-inhibitor screening and characterization: For inhibitor screening and characterization the concentration of BIO-AMP was used at a fixed concentration equal to the determined K_m for a PBP. This was selected since it was high enough give 1/2 of the maximum possible signal and low enough to still allow inhibition to be readily detected. To demonstrate this capability, NG PBP2 was characterized for inhibition by ampicillin. NG PBP2 was first attached to the wells of a microtiter plate as described above. Serially (steps of 5) diluted solutions of ampicillin in 50 μL PBS were added to the wells (each concentration tested

in quadruplicate). After 15 minutes 5 μL of a 28 μM solution of BIO-AMP was added (final concentration of 1.1 μM BIO-AMP in assay mixture). After 15 minutes the binding reactions were stopped by heat denaturation and the plates developed as described above. The general expression for a competitive inhibitor in this assay is

$$RFU = \frac{RFU_{max} * [BIO\text{-}AMP]}{K_m * (1 + [I]/K_I) + [BIO\text{-}AMP]} \qquad \text{Eqn. S8}$$

Where K_m is the K_m for BIO-AMP vs the particular PBP, [BIO-AMP] is the concentration of BIO-AMP, K_I is the apparent binding constant for the alternative inhibitors, and [I] is the concentration of the alternative inhibitor. With [BIO-AMP] = K_m this expression reduces to

$$RFU = \frac{RFU_{max}}{[I]/K_I + 2} \qquad \text{Eqn. S9}$$

To take into account the background (blank) fluorescence, this equation was expanded to

$$RFU = RFU_0 + \frac{RFU_{max}}{[I]/K_I + 2} \qquad \text{Eqn. S10}$$

Inhibitor binding data were plotted, and the set of 5 data points bracketing the midpoint of the saturation curve were analyzed for the K_I of binding by fitting the data to Eqn. S10.

Supplementary Material References

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