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Broad Ligand Specificity of the Transcriptional Regulator of the *Bacillus subtilis* Multidrug Transporter Bmr

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The expression of the *Bacillus subtilus* multidrug-efflux transporter Bmr can be induced by two of its structurally dissimilar substrates, rhodamine 6G and tetraphenylphosphonium, through their direct interaction with the transcriptional regulator BmrR (Ahmed et al., J. Biol. Chem. 269, 28506). Here, by screening a chemical library, we identified four additional ligands of BmrR inducing Bmr expression at micromolar concentrations. BmrR ligands, although sharing a positive charge and moderate hydrophobicity, are structurally very diverse. At the same time, not all hydrophobic positively charged compounds, including many structural analogs of the inducers, induce Bmr expression, thus suggesting that local chemical interactions and not merely physical properties of the ligands are important for their recognition by BmrR. These results confirm that this soluble protein, like the membrane transporter it regulates, has a uniquely broad substrate specificity. © 1997 Academic Press

The expression of multidrug efflux transporters in bacteria and eukaryotes is a well recognized cause of resistance to multiple antibacterial and chemotherapeutic agents (reviewed in refs. 1, 2). For example, the multidrug transporter Bmr of *Bacillus subtilis* confers resistance to a wide range of structurally unrelated toxic compounds including fluoroquinolone antibiotics, ethidium bromide, rhodamine 6G, acridine dyes, puromycin and tetraphenylphosphonium (TPP) by means of their active extrusion from the cell (3, 4). The mechanism underlying the extraordinarily broad substrate specificity of Bmr, or, for that matter, any other multidrug transporter, remains unknown.

We have shown previously that the expression of Bmr is regulated by the MerR-type transcriptional activator BmrR, encoded immediately downstream of the transporter gene (5). Interestingly, two of the substrates of Bmr, namely rhodamine 6G and TPP, interact directly with BmrR and augment multidrug resistance by inducing expression of the transporter (5). This finding suggested that Bmr and BmrR together constitute an inducible nonspecific detoxification system, although the extent to which BmrR could recognize dissimilar toxins was unclear. In particular the much higher affinity of rhodamine for BmrR (K_D 1 - 2 μM) than that of TPP (K_D 0.1 - 1 mM) suggested that BmrR may actually exhibit significant ligand specificity.

In this study, in order to identify additional inducers of BmrR, we screened approximately twenty one hundred compounds from a library of diverse organic molecules with molecular masses of 200-700 (DiverSet96, Chembridge Corp., Glenview, IL). These compounds were tested for the ability to induce resistance in the wild type *B. subtilis* strain BD170 (trpC2, thr-5) to the Bmr substrate ethidium bromide. Logarithmically growing cells, diluted in LB medium to an OD_{600} of 0.002, were preincubated for 45 min in 96 well plates with potential inducers (2 μg/ml), or rhodamine (0.06 μg/ml). Subsequently, ethidium bromide was added to a final concentration of 4 μg/ml, plates were incubated in a humidified chamber at 37 °C for 5 hours and wells examined for bacterial growth. Fifteen potential inducers were identified which, like rhodamine, stimulated growth of wild type cells in this otherwise bacteriostatic concentration of ethidium.

Figure 1 shows the structures of rhodamine, TPP and the four most potent newly identified inducers, which were active in the assay described above at concentrations of less than 1 μg/ml. Checkerboard titrations were performed with twofold serial dilutions of inducer from 10 μg/ml to 0.002 μg/ml versus a linear gradient of ethidium bromide. As shown in Figure 2, incubation of cells with each of the inducers at concentrations greater than 0.3 μg/ml resulted in at least a two-fold increase in the mini-

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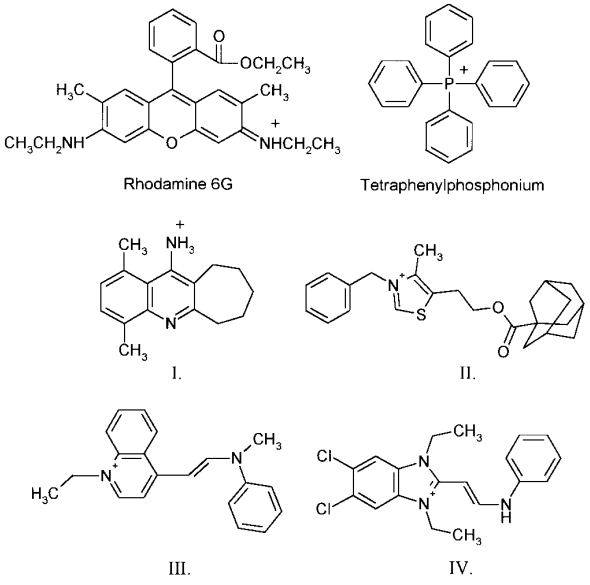

FIG. 1. Chemical structures of the inducers of Bmr expression. Compounds I - IV were identified in this study.

mal inhibitory concentration (MIC) of ethidium bromide. Compound III and IV exhibited some toxicity at 10 μg/ml which was reflected in a decrease in bacterial growth at this concentration. Under the same conditions, rhodamine 6G displayed its maximal induction of ethidium resistance between 0.03 and 0.12 μg/ml but displayed toxicity at higher concentrations.

The observed stimulation of ethidium resistance was due to increased expression of the Bmr transporter: none of the compounds increased the MIC of ethidium for the *B. subtilis* strain BD170/*bmr::cat* (5), in which the *bmr* gene is disrupted (data not shown). In fact, these inducers were shown to increase transcription from the *bmr* promoter as evaluated in the reporter strain BP 3.1 in which the *bmr* promoter controls the expression of *lacZ* (5). Logarithmically growing BP 3.1 or BD170 cells (OD_{600} 0.7) were incubated in the presence or absence of 1 μg/ml of each compound for 45 min and β-galactosidase activity was determined using 4-methyl umbelliferyl β-D-galactoside as a substrate (6). Whereas none of the compounds affected the level of β-galactosidase activity in the control cells, each inducer resulted in increased expression of this enzyme in the strain BP 3.1 (Fig. 3) demonstrating that these compounds increase transcription of *bmr*.

Similar to the inducers rhodamine and TPP (5), the stimulation of Bmr expression by the newly identified inducers was mediated by the transcriptional regulator BmrR. No induction of ethidium resistance was observed in the strain BD170/*bmrR::cat* (5), in which the *bmrR* gene is disrupted (data not shown). It was likely, therefore, that these inducers interacted with BmrR directly, a notion that was confirmed for compound I (Fig. 1) by biochemical analysis. We have previously shown that the interaction of BmrR with rhodamine and TPP is confined to its C-terminal 125 amino acids. This portion of BmrR, expressed as an individual protein (BRC), effectively bound rhodamine in an equilibrium dialysis ligand-binding assay, with TPP competing with rhodamine for binding (7). When compound I was tested in a similar assay, it was also shown to inhibit, at micromolar concentrations, the binding of rhodamine to BRC (Fig. 4), indicating that this compound interacts directly with the inducer-binding domain of BmrR.

Since the previously known ligands of BmrR, rhodamine and TPP, are substrates of the Bmr transporter, it was interesting to determine whether the newly identified BmrR ligands are also Bmr substrates. The MICs of compounds III and IV for *B. subtilis* could be reduced two-fold by reserpine, the inhibitor of Bmr (3), or by the genetic disruption of the Bmr transporter gene (data not shown), suggesting that these two compounds are indeed Bmr substrates. Compounds I and II were not toxic to *B. subtilis* at concentrations as high as 50 μg/ml and, therefore, could not be evaluated by the same approach.

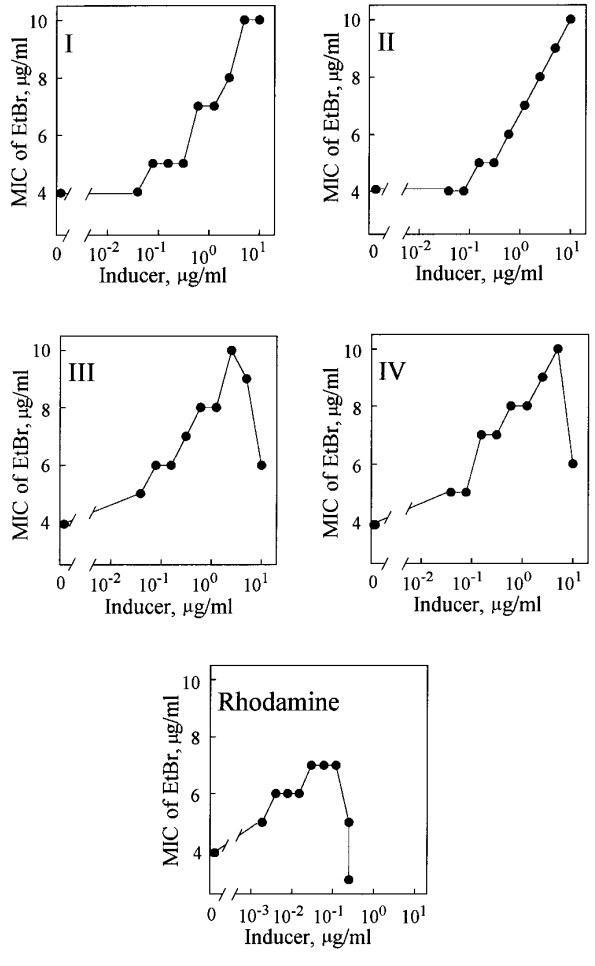

FIG. 2. Effect of Bmr inducers on the minimal inhibitory concentration (MIC) of ethidium bromide for *B. subtilis* (strain BD170).

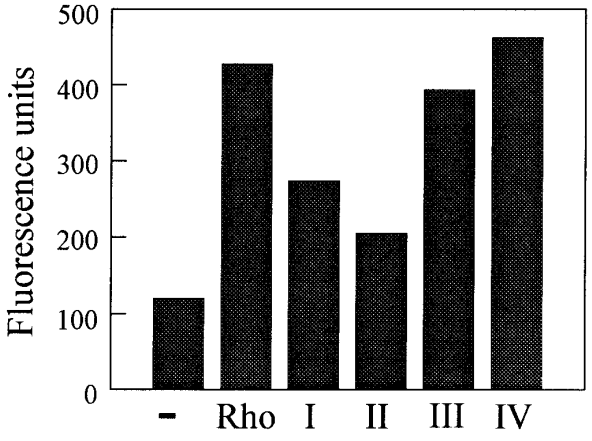

FIG. 3. β-galactosidase activity in the *bmr* reporter strain BP 3.1 incubated with Bmr inducers (1 μg/ml for compounds I-IV; 0.06 μg/ml for rhodamine). Background β-galactosidase activity in the parental strain BD170 incubated with the same drugs (75 $\pm$ 3 fluorescence units) was subtracted from each value.

The newly identified Bmr inducers share little structural similarity with either rhodamine 6G, TPP, or each other, the only commonality being the presence of a positive charge and a moderate hydrophobicity (Fig. 1). It should be noted that only a subset of positively charged hydrophobic compounds can induce Bmr expression. For example, neither the Bmr substrates ethidium and acridine, nor most of the positively charged hydrophobic compounds of the library screened in this work, are active in this respect. Furthermore, many of the relatively close structural analogs of the identified inducers were found to lack an inducer activity.

In these experiments structural homologs of inducers I and IV (approx. 20 for each) were identified in the DiverSet library using the ISIS Base software (MDL Information Systems, San Leandro, CA) and screened, at 1 and 0.2 μg/ml, for the ability to induce ethidium resistance in *B. subtilis* cells as described above. Whereas some analogs, shown in Fig. 5, demonstrated an inducing activity similar to that of the original inducers and some demonstrated weaker activity (not shown), others, shown in Fig. 5, were completely inactive in promoting ethidium resistance. No obvious structure-activity correlations could be derived from these results. Nevertheless, these experiments demonstrated that ligand recognition by BmrR is not based solely on the physical characteristics of ligands, such as charge and hydrophobicity, but rather involves local chemical interactions.

In conclusion, we have identified several additional ligands of the transcriptional regulator BmrR, of which at least two appear to be substrates of Bmr. Thus, BmrR, like the transporter whose expression it regulates, is capable of interacting with a broad range of structurally diverse molecules. Since the broad specificity of BmrR is contained within a region comprised of only 125 amino acids, it seems likely that this protein possesses a single multi-ligand recognition site rather than multiple independent binding sites for different inducers. The ongoing structural analysis of BRC (8) should reveal the molecu-

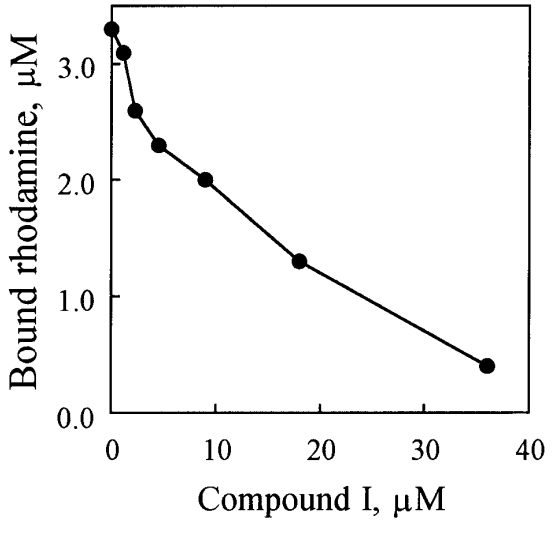

FIG. 4. Inhibition of rhodamine binding to BRC by compound I. Shown is the molar concentration of rhodamine bound to BRC (5 μM) in the presence of increasing concentrations of Compound I. The concentration of free rhodamine was 2.3 μM.

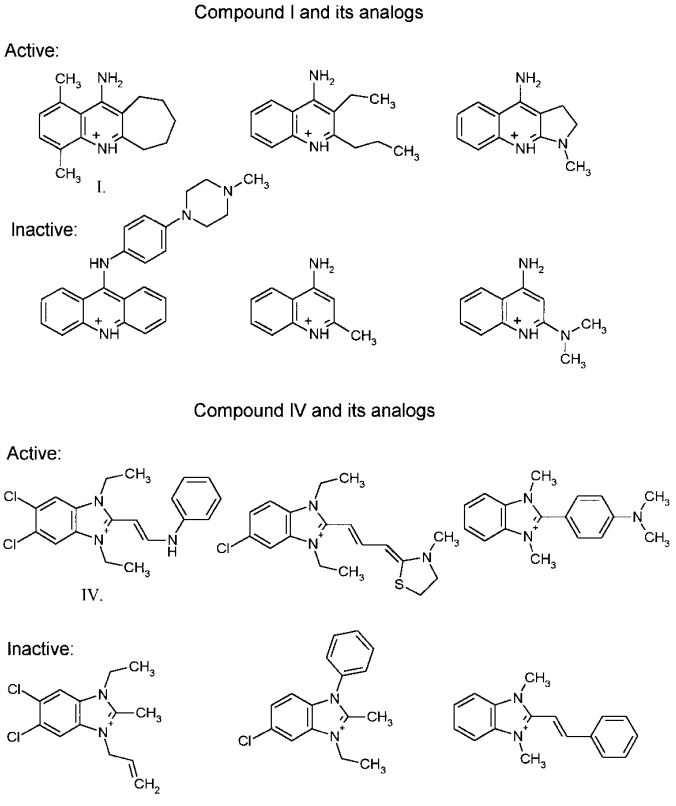

FIG. 5. Structures of compounds similar to inducers I, or IV, which either induce, or do not induce ethidium resistance in *B. subtilis*. Structures of only some compounds of each class are shown.

lar interactions involved in ligand binding, and shed light on the molecular mechanism underlying the presently obscure phenomenon of multidrug recognition.

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