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Association of a Zn^{2+} containing metallo β-lactamase with the anticancer antibiotic mithramycin

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ABSTRACT

Pathogenic bacteria that are resistant to β-lactam antibiotics mostly utilize serine β-lactamases to degrade the antibiotics. Current studies have shown that different subclasses of metallo β-lactamases ($E_{[MBL]}$) are involved in the defense mechanism of drug resistant bacteria. Here we report that the Zn^{2+} containing subclass B1 $E_{[MBL]}$ from *Bacillus cereus* binds to a naturally occurring anti-cancer drug mithramycin (MTR). Spectroscopic (CD and fluorescence) and isothermal titration calorimetry studies show that MTR forms a high affinity complex with the Zn^{2+} ion containing $E_{[MBL]}$. Abolished interaction of MTR with apo $E_{[MBL]}$ suggests that the formation of this high affinity complex occurs due to the potential of MTR to bind bivalent metal ions like Zn^{2+}. Furthermore, CD spectroscopy, dynamic light scattering and differential scanning calorimetry studies indicate that the strong association with sub-micromolar dissociation constant leads to an alteration in the enzyme conformation at both secondary and tertiary structural levels. The enzyme activity decreases as a consequence to this conformational disruption arising from the formation of a ternary complex involving MTR, catalytic Zn^{2+} and the enzyme. Our results suggest that the naturally occurring antibiotic MTR, a generic drug, has the potential as an $E_{[MBL]}$ inhibitor.

1. Introduction

Mithramycin (MTR, Fig. 1) is a naturally occurring antibiotic obtained from *Streptomyces plicatus* [1], which inhibits both *in vivo* and *in vitro* transcription via reversible interaction with the DNA minor groove, in the presence of bivalent metal ions like Mg^{2+} [1–5]. MTR was extensively used in the treatment of various neoplastic diseases like chronic myelogenous leukemia, testicular carcinoma, and Paget's disease of bones [1]. Presently, MTR has limited clinical use because of its systemic toxicity. Nonetheless, resurgence in application of MTR in a host of disorders besides cancer is observed as new uses and activities are ascribed to this generic drug [6–12]. High-throughput screening studies show that MTR, in spite of its small therapeutic window, is worth considering as potential lead compound for clinical application [13]. Furthermore, MTR derivatives produced by combinatorial biosynthesis show lower toxicity and better pharmacological properties, thereby enhancing the potential of this family of compounds as therapeutic agents [14–17].

Earlier, we have demonstrated the molecular basis of the mechanism of action of this antibiotic, where it is observed that the anionic antibiotic ($pK_{a[MTR]} = 5.0$) alone binds to bivalent metal ions and it is the [antibiotic:metal ion] complexes that actually act as DNA-binding ligands at and above neutral pH [18–20]. In our laboratory we have been studying the bivalent metal ion (Zn^{2+}) binding ability of the antibiotic to inhibit metalloenzymes [18]. The broad objective is to evaluate the potential of MTR for the treatment of diseases involving metalloenzymes. Zn^{2+} containing metallo β-lactamases ($E_{[MBL]}$) could, therefore, be a potential target of the drug. It is well established that a class of penicillin and penicillin derivative(s) resistant bacterial strains employ these enzymes to inhibit the bactericidal ability of commonly used β-lactam antibiotics [21]. Among different types of β-lactamases, class B β-lactamases make a unique group consisting exclusively of $E_{[MBL]}$s which utilize one or two zinc ions as co-factors [22]. Broad substrate profiles, varying metal stoichiometries and resistance to a wide range of β-lactam antibiotics have made the design of clinically appropriate inhibitors for this class of enzymes very difficult.

Here, we have shown by means of spectroscopy and calorimetry based studies that MTR binds to $E_{[MBL]}$ from *Bacillus cereus*. The association leads to MTR-induced structural alterations in the enzyme that culminates in the inhibition of its activity via a reversible mixed inhibition type of mechanism. Finally, we have proposed a binding model of MTR at the Zn^{2+} containing active site of the protein. The

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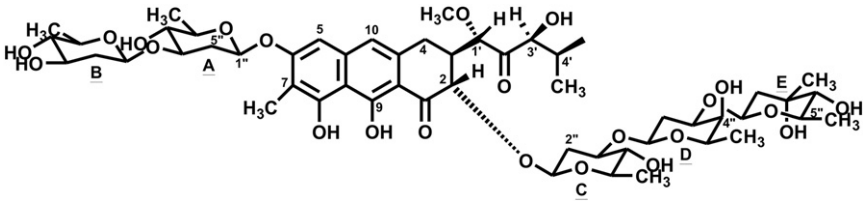

Fig. 1. Chemical structure of mithramycin (MTR).

experimental results suggest that the metal ion binding ability and energetically conducive interaction of the antibiotic with the enzyme, could lead to its antibacterial potential via inhibition of an important metalloenzyme, $E_{[MBL]}$.

2. Materials and methods

2.1. Materials

Purified and lyophilized metallo β-lactamase (from *B. cereus* 569/H/9) and benzylpenicillin were purchased from MP Biomedicals, France and used without further purification. K_2HPO_4, KH_2PO_4, Trizma base, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) Na-salt, 4-(2-pyridylazo) resorcinol (PAR), SDS, sodium chloride (NaCl), EDTA, guanidine hydrochloride and hydrochloric acid (HCl) were purchased from Sigma Chemical Co., U.S.A. MTR was purchased from MP Biomedicals, France and Sigma Chemical Co., U.S.A. according to availability of the drug stocks. MTR from each supplier and each batch was spectroscopically checked for quality (from the ratio of absorbance values at 400 nm and 440 nm) prior to each experiment. MTR used in all the experiments had A_{400}/A_{440} value of ≥3.7. All reagents used were of ACS grade, and the solutions were prepared in MilliQ (Synergy, Millipore, U.S.A.) water. Solutions were filtered through a 0.1 μm filter (Millipore, U.S.A.) prior to the experiments.

2.2. Buffers

All experiments were performed in 100 mM K-phosphate (pH 7.0) unless otherwise mentioned.

2.3. Preparation of MTR and $E_{[MBL]}$

Solution of MTR was prepared in 100 mM K-phosphate (pH 7.0) and the concentration was checked from absorbance measurements at 400 nm. The molar concentration of the antibiotic was estimated from the molar absorption coefficient, $\varepsilon = 10{,}000\ M^{-1}\ cm^{-1}$ at pH 8.0 [23].

The protein was extensively dialyzed against the experimental buffers, prior to each experiment and concentration was measured spectrophotometrically at 280 nm using molar absorption coefficient, $\varepsilon = 30{,}500\ M^{-1}\ cm^{-1}$ [24]. The purity of the commercial sample was checked in a 10% SDS-PAGE (Fig. S1). Apo $E_{[MBL]}$ was prepared as described earlier [25]. Apo $E_{[MBL]}$ was checked for its Zn^{2+} content using PAR under denaturing conditions as reported earlier [26]. The results of the experiment with PAR are provided in the Supplementary information (Fig. S2). Since the experimental samples involved incubation of the enzyme in experimental buffers for 60 min at 30°C, $E_{[MBL]}$ was incubated separately in 100 mM K-phosphate buffer, pH 7.0; 50 mM Tris–HCl, pH 7.0; 50 mM HEPES, pH 7.0 and sterile MilliQ water at 30°C for 90 min to check the potential Zn^{2+} ion depletion due to the buffers used in the current report. Zn^{2+} content of the enzyme solutions in different buffers was compared using PAR. The results and detailed experimental protocol are provided in the Supplementary information (Fig. S3).

2.4. Preparation of benzylpenicillin

Benzylpenicillin was weighed and dissolved in 100 mM K-phosphate buffer (pH 7.0). It was used within 1 h of preparation to avoid spontaneous hydrolysis over time.

2.5. Fluorescence spectroscopy

Fluorescence emission spectra (excitation wavelength—470 nm) of MTR alone and in the presence of $E_{[MBL]}$ were recorded in a Perkin Elmer LS55 luminescence spectrometer at 30°C using cuvettes of 1 cm path length. The reported spectra are an average of 4 scans. Excitation and emission slit widths were 10 nm each. Fluorescence emission intensity of MTR at 540 nm was used to get the binding isotherm reported below. The experimental points for the binding isotherm were fitted using the equation

$$C_0(\Delta F/\Delta F_{max})^2 - \left(C_0 + C_p + K_d\right)(\Delta F/\Delta F_{max}) + C_p = 0 \qquad (1)$$

where, C_0 is the initial concentration of MTR, ΔF is the change in fluorescence at 540 nm for each addition of $E_{[MBL]}$; ΔF_{max} is the same parameter when MTR is totally bound to $E_{[MBL]}$ and C_p is the concentration of $E_{[MBL]}$ added. The X-intercept value at $\Delta F / \Delta F_{max} = 0.5$ (corresponding to 50% binding) gives the dissociation constant, K_d. ΔF_{max} was determined from the double reciprocal plot of 1 / ΔF against $1 / (C_P - C_0)$ using the equation

$$1/\Delta F = 1/\Delta F_{max} + 1/\left(C_p - C_0\right) \qquad (2)$$

2.6. Isothermal titration calorimetry (ITC)

Calorimetric titration for the association of MTR with $E_{[MBL]}$ was performed with VP-ITC microcalorimeter (MicroCal Inc., U.S.A.). The binding isotherms were generated with the help of built-in VPViewer 2000 software with Origin 7.0. MTR (650 μM) was taken in syringe and aliquots of MTR were dropped into the calorimetric cell containing 10 μM $E_{[MBL]}$ at 30 °C in 50 mM Tris–HCl, pH 7.0 (stirring speed: 307 rpm, injected volume: $(1 \times 1\ \mu l) + (44 \times 6\ \mu l) = 265\ \mu l$). Corrected enthalpies were calculated after subtracting the enthalpy change due to dilution of MTR in 50 mM Tris–HCl, pH 7.0. The resultant thermograms were analyzed using two sets of binding sites model of Levenberg–Marquardt non-linear least squares curve fitting algorithm, inbuilt in the MicroCal LLC software. Same protocol was followed to examine the binding of MTR to apo $E_{[MBL]}$.

2.7. Circular dichroism spectroscopy (CD)

All CD spectra were recorded in a Jasco J-715 spectropolarimeter. Far UV CD spectra were recorded to monitor changes in the secondary structure of $E_{[MBL]}$ (3 μM) upon interaction with increasing concentration of MTR. Spectra for MTR bound $E_{[MBL]}$ in the far UV region were generated after linear subtraction of the recorded CD signal of MTR in the same wavelength region. Near UV and visible range (250 nm–550 nm) CD spectra of MTR alone and in the presence of increasing concentrations of $E_{[MBL]}$ were recorded. CD spectra of $E_{[MBL]}$