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Unconventional surface plasmon resonance signals reveal quantitative inhibition of transcriptional repressor EthR by synthetic ligands

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ABSTRACT

EthR is a mycobacterial repressor that limits the bioactivation of ethionamide, a commonly used anti-tuberculosis second-line drug. Several efforts have been deployed to identify EthR inhibitors abolishing the DNA-binding activity of the repressor. This led to the demonstration that stimulating the bioactivation of Eth through EthR inhibition could be an alternative way to fight *Mycobacterium tuberculosis*. We propose a new surface plasmon resonance (SPR) methodology to study the affinity between inhibitors and EthR. Interestingly, the binding between inhibitors and immobilized EthR produced a dose-dependent negative SPR signal. We demonstrate that this signal reveals the affinity of small molecules for the repressor. The affinity constants (K_D) correlate with their capacity to inhibit the binding of EthR to DNA. We hypothesize that conformational changes in EthR during ligand interaction could be responsible for this SPR signal. Practically, this unconventional result opens perspectives onto the development of an SPR assay that would at the same time reveal structural changes in the target upon binding with an inhibitor and the binding constant of this interaction.

Tuberculosis is one of the most frightening illnesses on the planet. There were an estimated 8.6 million new TB[1] cases in 2012, including 1.1 million cases among people infected with HIV, and 1.3 million people died from TB that year [1]. Next to HIV infection, demographic factors, poor living conditions, and neglected TB control in many countries caused this pandemic. There is an urgent need for new drugs and new strategies to eliminate more efficiently sensitive or drug-resistant strains. We have recently validated the mycobacterial transcriptional repressor EthR as a new target to fight tuberculosis. EthR belongs to the TetR/CamR family of transcriptional regulators and negatively regulates the expression of *ethA* in *Mycobacterium tuberculosis* [2]. The *ethA* gene encodes a flavin-containing monooxygenase that catalyzes the bioactivation of ethionamide, one of the most commonly used anti-TB second-line drugs [3,4].

We have shown that in vitro and in vivo allosteric inhibition of the interaction between EthR and the *ethA* promoter with a drug-like compound was able to improve ethionamide efficacy, which in mice allowed for a therapeutic dose reduction [5–7]. In the past few years, several efforts have been made to identify and optimize EthR inhibitors [5–10]. To that end, interactions between EthR and synthetic inhibitors have been studied using a large panel of biological and biophysical assays such as X-ray crystallography [7], thermal shift assay detecting the thermostabilization effect of the ligand on the protein [8,11], and finally surface plasmon resonance (SPR) [2,5–7].

Using the SPR biosensor assay, the interaction between biomolecules immobilized on a sensor surface and biomolecules or small

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[1] *Abbreviations used:* SPR, surface plasmon resonance; TB, tuberculosis; NTA, nitrilotriacetic acid; CM5 chip, carboxymethylated chip; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; NHS, *N*-hydroxysuccinimide; TSA, thermal shift assay; HTH, helix-turn-helix; SA, streptavidin; EDC, *N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide hydrochloride; RU, resonance unit

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chemical entities flowing over the chip in an aqueous buffer is quantified by measuring variations in refractive index. Interactions on the gold surface of the biosensor chip between two partners increase the electron density around the sensor surface and in most cases induce positive changes in the refractive index, translated as a real-time SPR signal called a sensorgram (resonance unit as function of time) [12]. SPR is an ideal tool to generate high-quality data on the interactions between biomolecules, as well as between small ligands and biomolecules. Direct binding assay based on SPR presents many advantages: it is label-free, has a low target consumption, and provides real time analysis and detection of low-affinity target binders [13].

As an efficient tool to study inhibitors of EthR, we previously designed a bioanalytical assay based on SPR. SPR was used to measure the capacity of inhibitors to disturb the interaction between EthR and its DNA operator (DNA/EthR SPR methodology) [2,5–7]. Although very useful to quantify the capacity of EthR ligand to inhibit the DNA binding function of EthR, this approach was unsuitable for revealing the binding constants that drive the direct interactions between EthR and the inhibitors, which allowed only fragmentary comparisons between compounds. Then, to further characterize our EthR inhibitors by accessing binding constants (K_D), we immobilized the transcriptional repressor EthR on a CM5 carboxymethylated sensor chip and monitored the interaction with EthR inhibitors in solution (ligand/EthR SPR methodology). We report here an SPR assay focusing on the direct ligand/transcriptional repressor interactions to compare chemical series of new EthR inhibitors. This assay produced unconventional negative SPR signals that we demonstrate to be specific for the ligand/protein interaction. We discuss the hypothesis that this signal could be predictive of the allosteric structural changes undergone during ligand/protein interaction and examine the implications of this observation in the context of target-based drug screening.

Materials and methods

SPR experiments were carried on a BIAcore 3000 instrument (GE HealthCare). Thermal shift assay (TSA) experiments were performed on a LightCycler 480 instrument (Roche). Research-grade CM5, SA (streptavidin), and NTA (nitrilotriacetic acid) sensors were purchased from GE HealthCare. HBS-EP (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) was purchased from GE HealthCare and used with various sodium chloride concentrations. All chemicals used (Tris–HCl, NaCl, EDTA, DTT (dithiothreitol), NHS (*N*-hydroxysuccinimide), EDC (*N*-ethyl-*N*′-(3-diethylaminopropyl)carbodiimide hydrochloride), ethanolamine, and Sypro orange) were of the highest grade available commercially.

Plasmid construction and His$_6$-EthR and His$_6$-EthR-mutant expression and purification

Construction of plasmid pET-15b-ethR

The N-terminally hexahistidine-tagged EthR was produced in *Escherichia coli* C41 (pET-15b-*ethR*) and purified as described. The DNA coding for EthR was amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as template and oligonucleotides O-183, 5′-CATATGACCACCTCCGCGGCCAGT-3′, and O-184, 5′-GGATCCGAGCACCCCCGACCGAGT-3′, as primers. The PCR product was inserted into pCR2.1Topo (Invitrogen) to generate pCR2.1-*ethR*. The EthR-encoding fragment was sequenced on both strands and then isolated from pCR2.1-*ethR* by digestion with *Nde*I and *Bam*HI and inserted into pET-15b (Novagen), yielding pET-15b-*ethR*. This plasmid encodes EthR containing an extra amino-terminal tag with the following sequence: MGSSH$_6$SSGLVPRGSHM.

Construction of plasmid pET-15b-ethR$_{G106W}$

A first PCR product was obtained by amplification using *M. tuberculosis* H37Rv chromosomal DNA as template and oligonucleotides O-275 (5′-AGGACCGTCCGCTGGCCGATA-3′) and O-276 (5′-AACACGTTGAT**CCA**GGTGCGCCACA-3′) as primers, thus introducing a point mutation at codon 106 of *ethR* (underlined in oligonucleotide O-276), resulting in the replacement of glycine 106 by a tryptophan residue. This 216-bp fragment was used as a 5′ primer in association with oligonucleotide O-278 (5′-GCTTCCTTTCGGGCTTTGTTAGCAG-3′) to amplify a 594-bp fragment, which was subsequently inserted into PCRII-Topo (Invitrogen) to generate pCRIIethRG106W. The 275-bp *Sal*I–*Sal*I fragment of pET-15b-*ethR* [2] was then exchanged for the equivalent fragment of pCRIIethRG106W to produce pET-15b-*ethR*$_{G106W}$. The orientation of the fragment was checked by restriction digestion and the DNA sequence was confirmed by sequencing of the entire open reading frame.

Production and purification of His$_6$-EthR and mutant His$_6$-EthR$_{G106W}$

N-terminally hexahistidine-tagged EthR WT or G106W was produced in *E. coli* C41 using the protein expression plasmids pET-15b-*ethR* and pET-15b-*ethR*$_{G106W}$, respectively, as previously described [2]. Bacteria were grown in 100 ml LB broth to an $OD_{600\ nm}$ of 0.6–0.7. Isopropylthiogalactoside was then added to a final concentration of 1 mM and the culture was grown for 3 h. The cells were harvested by centrifugation at 12,000*g* at 4 °C, resuspended in 10 ml of lysis buffer (50 mM Tris–HCl, 300 mM NaCl, pH 7.5, 10 mM imidazole), and lysed by two passages at 6.2 MPa through a French press cell. After centrifugation (20,000*g*, 25 min, 4 °C), the supernatant was recovered and His$_6$-EthR or His$_6$-EthR$_{G106W}$ was separated from the whole-cell lysate by Ni–NTA agarose chromatography (Qiagen). After three washing steps with lysis buffer, His$_6$-tagged proteins were eluted from the resin with 250 mM imidazole in lysis buffer, dialyzed overnight against EthR buffer (10 mM Tris–HCl, 300 mM NaCl, pH 7.5, 1 mM DTT, 0.1 mM EDTA), and concentrated to 14 mg/ml for EthR and 7 mg/ml for EthR$_{G106W}$. Protein purity was controlled by Coomassie blue staining after SDS–PAGE on a 12% polyacrylamide gel. Protein concentration was evaluated using the Bio-Rad protein assay kit. Purified proteins His$_6$-EthR and His$_6$-EthR$_{G106W}$ were stored in EthR buffer at 4 °C.

Production of biotinylated double-stranded DNA fragments

The 106-bp fragment overlapping the *ethA-R* intergenic region ($DNA_{etha\text{-}r}$) was obtained from *M. tuberculosis* H37Rv chromosomal DNA using O-270, 5′-CGGTCATGGATCCACGCTATCAAC-3′, and O-271, 5′-biotin-CTGACTGGCCGCGGAGGTGGT-3′. The biotinylated double-stranded 113-bp-long irrelevant DNA fragment (+14 to +127) of the *E. coli bla* gene was PCR amplified using oligonucleotides O-343, 5′-TTTCCGTGTCGCCCTTATTCC-3′, and O-344, 5′-CCACTCGTGCACCCAACTGAT-3′, and pUC18 as substrate ($DNA_{nonspecific}$). The amplified biotinylated fragments were purified on a QiaQuick column (Qiagen), cloned, and sequenced.

SPR assay for determining EthR/ligand affinity

Immobilization of EthR and EthR$_{G106W}$ on CM5 sensor chip

All SPR experiments were performed at 25 °C. Capture of EthR and EthR$_{G106W}$ was performed on CM5 sensor chips with a carboxymethylated dextran matrix using an HBS-EP running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20). The chip was first activated by injecting a fresh mixture (35 µl, 50/50, v/v) of 0.1 mM NHS and 0.4 mM EDC at a flow rate of 5 µl/min. Several 5-µl injections of EthR or EthR$_{G106W}$, respectively at 15 and 30 µg/ml, in 10 mM acetate buffer, pH 4.5, were performed so as to reach the desired level of covalently attached