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A proof-of-concept receptor-based assay for sulfonamides

Xiao Liang [1], Zhanhui Wang [1], Chunmei Wang, Kai Wen, Tiejun Mi, Jing Zhang, Suxia Zhang *

Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, China Agricultural University, Beijing 100193, People's Republic of China

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ABSTRACT

The gene encoding the dihydropteroate synthase (DHPS) of *Streptococcus pneumonia* has been cloned, sequenced, and expressed in *Escherichia coli.* The protein has been purified and used to develop a novel microplate assay for the detection of sulfonamides. The assay was based on the competition between sulfonamides and horseradish peroxidase (HRP)-labeled sulfonamide derivative, 4-(4-aminobenzenesulfonylamino) benzoic acid (CS) for the immobilized protein. Under optimized conditions, nine sulfonamides and *p*-aminobenzoic acid (PABA) could be detected below 100 ng/ml and 28 sulfonamides used in the study could be detected with IC_{50} values ranging from 426 to 50,000 ng/ml. It is concluded that this method offers a robust and rapid alternative to other methods for the screening of sulfonamides.

The sulfonamides are a family of synthetic antibiotics that have broad-spectrum antimicrobial activity and are widely used in veterinary medicine for the prophylaxis and therapy of infectious diseases [1]. In the European Union, sulfonamides represented approximately 15 to 20% of the total amount in 2004, whereas they represented only 5% of veterinary drugs used during the period from 2005 to 2007. In China, the consumption of sulfonamides was estimated to be 20,000 tons, and one-third of these sulfonamides were used in animal husbandry in 2003. The presence of sulfonamide residues in animal-derived food is of toxicological and regulatory concern because they could be carcinogenic and cause allergic hypersensitivity reactions and therapeutic ineffectiveness in human medicine [2]. To minimize the risk of sulfonamides, the maximum residue limit for edible tissues was set to be 100 ng/ml for total sulfonamide content in the European Union and China [3–6].

Traditional methods for sulfonamide residue analysis involving high-performance liquid chromatography [7], biosensor-based assays [8], and liquid chromatography–tandem mass spectrometry [9] are sensitive and reliable. In contrast, they require extensive sample cleanup before application to the assay and have limited sample throughput. Generic immunoassay [10] based on the specific interaction of antibody and antigen is proven to be simple, highly sensitive, and cost-effective without sophisticated instrumentation. However, development of antibodies with broad-specificity recognition for sulfonamide drugs was found to be surprisingly difficult [3]. The receptor binding assays do not use antibodies to bind antibiotics, although the principles of these tests are analogous to immunological methods. During recent years, several methods based on receptors were developed for detecting drug residues, including β-agonist [11] and β-lactam [12,13] antibiotics.

In the case of sulfonamides, little information is available on detection of sulfonamides using receptor. Dihydropteroate synthase (DHPS)[2] is one of the receptors of sulfonamides, and the mechanism of sulfonamides on DHPS is clear. The receptor is one of several crucial enzymes in the de novo biosynthesis of folate cofactors and is the target for important antimicrobial agents such as sulfonamides and dapsone, which are competitive inhibitors with respect to *p*-aminobenzoic acid (PABA). To date, a variety of DHPS apo- and holo-crystal structures have been deposited in the Protein Data Bank from many kinds of bacterial species (such as *Escherichia coli* [14], *Staphylococcus aureus* [15], *Mycobacterium tuberculosis* [16], and *Streptococcus pneumoniae* [17]) as well as one fungal species [18]. In *Streptococcus pneumonia,* the identical subunit of DHPS encoded by the gene *SulA* is

* Corresponding author. Fax: +86 1062731032.
E-mail address: suxia@cau.edu.cn (S. Zhang).
[1] The first two authors contributed equally to this work.

[2] *Abbreviations used:* DHPS, dihydropteroate synthase; PABA, *p*-aminobenzoic acid; DHPPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; PP_i, sodium pyrophosphate decahydrate; HRP, horseradish peroxidase; LB, Luria–Bertani; TMB, 3,3′,5,5′-tetramethylbenzidine; DMF, dimethylformamide; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; IPTG, isopropy-β-D-thiogalactoside; SCP, sulfachloropyridazine; SPA, sulfaphenazole; SMM, sulfamonomethoxine; SQX, sulfaquinoxaline; CM, chloramphenicol; KAN, kanamycin; AMP, ampicillin; CS, 4-(4-aminobenzenesulfonylamino) benzoic acid; TS, [2-(4-amino-benzenesulfonylamino)-1,3-thia-zol-4-yl] acetic acid; BS, 6-(4-aminobenzenesulfonylamino) butanoic acid; HS, 6-(6-aminobenzenesulfonylamino) hexanoic acid; PB, [4-(4-amino-benzenesulfonylamino)phenyl] acetic acid; ELISA, enzyme-linked immunosorbent assay; UV–vis, ultraviolet–visible; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; OD, optical density; CR, cross-reactivity.

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34 kDa [19]. The enzyme forms an α/β barrel structure, with a highly conserved binding pocket for recognition of the pterin substrate 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP). There is a fixed order of substrate binding: DHPPP binds first, followed by the second substrate, PABA. Binding of sodium pyrophosphate decahydrate (PP_i) also allows the enzyme to recognize PABA or sulfonamides, which act as PABA analogues [17].

The purpose of the current study was to develop a receptor-based microplate assay for the detection of sulfonamides. PP_i was used to replace the first substrate of the enzyme, DHPPP. Besides theoretical interest, this work has practical significance aimed at the development of a system for drug screening and assay.

Materials and methods

Reagents and chemicals

BL21(DE3) Chemically Competent Cell, anti-6×His monoclonal antibody, and *pEASYTM*-T1 Simple Cloning Vector were obtained from TransGen (Beijing, China). pET-28b expression plasmid was purchased from Novagen (Madison, WI, USA). *S. pneumoniae* strain R6 was purchased from American Type Culture Collection (ATCC, no. 49619). Goat anti-mouse immunoglobulin G (IgG)–horseradish peroxidase (HRP) conjugate was supplied by Bio-Rad (Hercules, CA, USA). Primer synthesis and DNA sequencing were performed by Invitrogen (Shanghai, China). Luria–Bertani (LB) and LB agar were obtained from Beijing Aoboxing (Beijing, China). Tributylamine, 3,3′,5,5′-tetramethylbenzidine (TMB), dimethylformamide (DMF), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), HRP, lysozyme, PP_i, isopropy-β-D-thiogalactoside (IPTG), sulfachloropyridazine (SCP), sulfaphenazole (SPA), sulfisoxazole (SIZ), sulfathiazole (STZ), sulfamonomethoxine (SMM), sulfaquinoxaline (SQX), sulfadimethoxine (SDM), sulfamethoxypyridazine (SMP), sulfamethylthiazole (ST), sulfamoxole (SMX), sulfasalazine (SPH), sulfamethizole (SMT), sulfapyridine (SPY), sulfamethoxazole (SMZ), sulfamethazine (SM_2), sulfamerazine (SMR), sulfameter (SMD), sulfaethoxypyridazine (SEP), sulfanilamide (SN), sulfadiazine (SDZ), sulfadoxine (SAA), sulfanitran (SAN), 5-sulfaminouracil (SAU), sulfabenzamide (SBA), sulfacetamide (SA), sulfaguanidine (SG), sulfanitran (SNT), phthalylsulfathiazole (PST), chloramphenicol (CM), kanamycin (KAN), ampicillin (AMP), and PABA were purchased from Sigma–Aldrich (St. Louis, MO, USA). Haptens—4-(4-aminobenzenesulfonylamino) benzoic acid (CS), [2-(4-amino-benzenesulfonylamino)-1,3-thia-zol-4-yl] acetic acid (TS), 6-(4-aminobenzenesulfonylamino) butanoic acid (BS), 6-(6-aminobenzenesulfonylamino) hexanoic acid (HS), and [4-(4-aminobenzenesulfonylamino)phenyl] acetic acid (PB)—were previously synthesized in our laboratory [20,21].

Instrument

Polystyrene microplates were purchased from Costar (Corning, NY, USA). The thermal cycler was purchased from Applied Biosystems (USA). The enzyme-linked immunosorbent assay (ELISA) plate reader was obtained from Tecan (Durham, NC, USA). The ultraviolet–visible (UV–vis) spectrometer was obtained from Shanghai Analytical Instrument (Shanghai, China). The ultramicro UV–vis spectrophotometer ND-1000 was purchased from NanoDrop (Chicago, IL, USA).

Standard solutions

Stock solutions were prepared by adding 10 mg of sulfonamide to 10 ml of methanol (1 mg/ml). The individual stock solutions were stored at −20 °C in glass bottles and were stable for at least 3 months. Working standards (32,000, 8000, 2000, 500, 125, and 31.25 ng/ml) of each sulfonamide were prepared by diluting the stock solution in 10 mM Tris–HCl (pH 8.0), 20 mM $MgCl_2$, and 2% skim milk.

Buffers and solutions

The following buffers were used in the receptor-based microplate assay: (i) coating buffer consisted of 12.5 mM Tris–HCl (pH 8.0) and 90 mM $MgCl_2$; (ii) blocking buffer consisted of phosphate-buffered saline (PBS, pH 7.4) with 5% (w/v) sucrose, 0.25% (w/v) casein, 5% (v/v) fetal calf serum, and 0.03% (v/v) Proclin 300; (iii) assay buffer consisted of 10 mM Tris–HCl (pH 8.0) with 20 mM $MgCl_2$, 2 mM PP_i, and 2% (v/v) skim milk; (iv) washing buffer consisted of PBS (pH 7.4) with 0.05% (v/v) Tween 20; (v) substrate was 0.1% TMB and H_2O_2 in 0.05 M citrate buffer (pH 4.5); (vi) 2 M H_2SO_4 was the stopping reagent.

Cloning of SulA gene

The *SulA* gene encoding DHPS was obtained by polymerase chain reaction (PCR) amplification using genomic DNA obtained from the R6 strain of *S. pneumoniae*. The design of the oligonucleotide primers was based on the sequence of the *SulA* gene deposited in the NCBI database (NC_003098.1). The primers used in this research were 5′-GGAATTCCATATGTCAAGTAAAGCCAAT-3′ and 5′-CCGCTCGAGTTATTTATATTGTTTTAAATC-3′. The oligonucleotides incorporated *Nde*I and *Xho*I sites (underlined).

Amplification reactions were performed with a thermal cycler using the following program: denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, annealing at 51 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The PCR products were analyzed on a 1% agarose gel to identify the amplified species of the appropriate size (925 bp). The PCR products were purified, subcloned into *pEASY*-T1 Simple Cloning Vector according to their instructions, and finally sequenced to ensure that the restriction sites were present and that no point mutations had been introduced during the PCRs.

Protein expression, purification, and characterization

The expression in *E. coli* of DHPS from *Bacillus anthracis* has been described previously [22]. A similar method was employed for expression of the enzyme in *E. coli* from *S. pneumoniae* with some modifications. The *SulA* gene was subcloned from the *pEASY*-T1 Simple Cloning Vector into the pET-28b expression plasmid that incorporated a 6×His Tag at the N terminus of the expressed protein. After sequence verification, the gene was expressed in *E. coli* BL21(DE3) Chemically Competent Cells. A 1-ml overnight culture was grown from a single colony of cells in LB containing 30 μg/ml KAN, and this culture was used to inoculate 100 ml of the same medium. Cultures were then subcultured in fresh medium and grown at 37 °C until OD_{600} reached approximately 0.6. IPTG (1 mM) was subsequently added, and expression was carried out at 30 °C for 3 h. The cells were harvested by centrifugation at 9000 rpm for 10 min. The supernatant was decanted, and the cell pellets were frozen overnight at −80 °C.

Purification of DHPS required two steps of Ni^{2+} chelation affinity chromatography and ion exchange. The cell pellets were resuspended in 5 ml of binding buffer (0.5 M NaCl, 0.05 M Tris–HCl [pH 7.9], and 0.001 M imidazole), and lysozyme (10 mg/ml solution) was also added to the cell suspension. After incubation at room temperature with mild agitation for 30 min, cells were lysed by sonication and the cell debris was sedimented by centrifugation at 9000 rpm for 10 min at 4 °C. The supernatant was recovered and filter sterilized with a 0.45-μm filter. The protein was eluted using