Pharmacophore-Based Strategy for the Development of General and Specific scFv Biosensors for Abused Antibiotics

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We developed fluorescent biosensor systems that are either general or selective to fluoroquinolone antibiotics by using a single-chain variable-fragment (scFv) as a recognition element. The selectivity of these biosensors to fluoroquinolone antibiotics was rationally tuned through the structural modification on the pharmacophore of fluoroquinolone antibiotics and the subsequent selection of scFv receptor modules against these antibiotics-based antigens using phage display. The resulting A2 and F9 scFv's bound to their representative antigen with a moderate affinty (K_D in micromolar range as determined by surface plasmon resonance). A2 is a specific binder for enrofloxacin and did not cross-react with other fluoroquinolone antibiotics including structurally similar ciprofloxacin, while F9 is a general fluoroquinolone binder that likely bound to the antigen at the common pyridone−carboxylic acid pharmacophore. These scFv-based receptors were successfully applied to the development of one-step fluorescent biosensor which can detect fluoroquinolone antibiotics at concentrations below the level suggested in animal drug application guidelines. The strategy described in this report can be applied to developing convenient field biosensors that can qualitatively detect overused/misused antibiotics in the livestock drinking water.

INTRODUCTION

Antibiotic overuse is a threat to humans because it may cause growth of bacterial strains that are resistant to the antibiotics; this is a global public health problem (*1, 2*). Nowadays, the prescription of antibiotics in hospitals has significantly decreased, but the problem of food contamination by antibiotics still persists (*3*). Antibiotics are routinely and nontherapeutically added to the feed and drinking water in order to prevent diseases or to promote the growth of the livestock, rather than to treat infections (*4*). Therefore, it is important to develop a practical method for detecting antibiotics that are either overused or misused in the livestock drinking water. However, a number of antibiotic detection methods described in the literature still require electronic instruments or involve time-consuming analytical processes, which make real-time monitoring of misused antibiotics difficult (*5, 6*).

Various classes of antibiotics are currently in use, and fluoroquinolones especially are frequently overused to prevent many diseases, e.g., typhoid fever and infectious diarrhea, in animals such as chickens, pigs, fish, and shellfish, due to their broad-spectrum activity with good Gram-positive and Gram-negative coverage (*7*). As shown in Figure 1, enrofloxacin **1**, norfloxacin **2**, ciprofloxacin **3**, ofloxacin **4**, and flumequine **5** are categorized as fluoroquinolone antibiotics having a pyridone−carboxylic acid moiety as a common pharmacophore (red in Figure 1a) (*8, 9*). Among them, enrofloxacin **1** is representative of the fluoroquinolones that are overused in livestock farms (*10, 11*). To develop a novel biosensor for overused fluoroquinolones antibiotics, we envisioned utilization of the phage-display selection of a single-chain variable-fragment (scFv) library for the discovery of a general or specific recognition module for antibiotics. Recombinant scFv is a small (~25 kDa) protein composed of antibody variable heavy (V_H) and light (V_L) chains that are connected by a peptide linker (*12*). The key advantages of using an scFv as the recognition element are its excellent selectivity to antigen and low operation cost; further, target-specific scFv clones can be easily selected from an scFv library by phage display (*13, 14*). In the antibody phage-display technology, an antibody fragment (scFv or Fab) is displayed on the surface of the filamentous phage M13 as a fusion to the minor coat protein pIII (*15*). The phage particle also harbors the gene that encodes this fusion protein, thus establishing a link between the genotype and the phenotype; this link is essential for the rapid enrichment and selection of target-specific scFv clones (*16*). Because the phage-display selection process is carried out in vitro, various experimental parameters such as antigen concentration, mode of antigen presentation, temperature, binding time, wash stringency, and negative selection can be controlled as desired (*17*). This is particularly advantageous when a binding molecule with a very specific binding profile, such as one that can distinguish minor differences in the antigen structure, is desired (*18*).

To develop a general or selective scFv-based biosensor for antibiotics, it was required to prepare proper antigens that can differentiate the binding profile toward fluoroquinolones antibiotics in a general or selective fashion. Therefore, we designed and synthesized two sets of biotinylated antibiotics (Figure 1b, **6** and **8**) to use them as antigens for the phage-display selection of an scFv library. The key factor in designing antibiotic analogues as an antigen was the portion of antibiotics displayed as a recognition element, which we named the pharmacophore-based strategy. As shown in Figure 1b, biotin analogue **6** and Cy3 analogue **7** have a unique structural element that is typical

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10.1021/bc1004153
Published on Web 12/10/2010

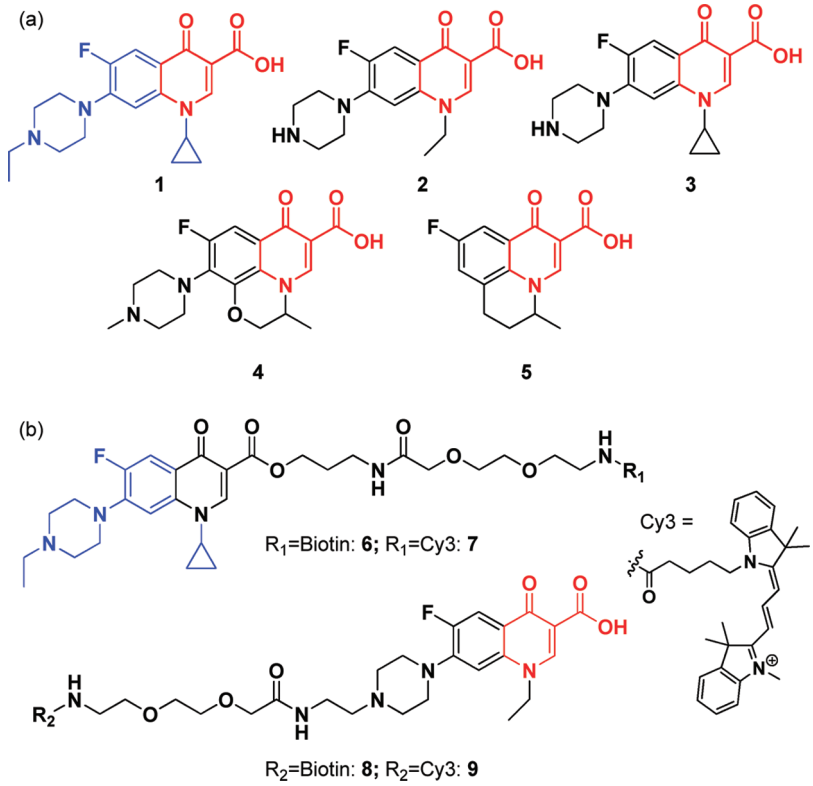


Figure 1. Structures of (a) representative fluoroquinolones, enrofloxacin **1**; norfloxacin **2**; ciprofloxacin **3**; ofloxacin **4**; and flumequine **5**; and (b) biotin-analogues (**6** and **8**) and Cy3-analogues (**7** and **9**) of fluoroquinolones.

of enrofloxacin (blue in Figure 1), and this feature enables the use of **6** and **7** for developing a selective scFv-based recognition module for top-selling and most overused antibiotic **1** (*19*). In the case of biotin analogue **8** and Cy3 analogue **9**, the common pharmacophore of fluoroquinolone antibiotics, pyridone−carboxylic acid (red in Figure 1), can be displayed as a recognition element by the modification of piperazine on **2**; this modification is based on the structure−activity relationship (*9*) and this pharmacophore-based antigen **8** can be utilized on the scFv selection of general recognition module for a series of fluoroquinolones antibiotics. Herein, we described a pharmacophore-based strategy for developing modular single-chain variable-fragment (scFv)-based biosensor systems using a fluorescent (optical) detection method and demonstrated this strategy through the development of one-step general or selective biosensors for overused/misused fluoroquinolone antibiotics.

EXPERIMENTAL PROCEDURES

Synthesis of a Biotin-Analogue of Enrofloxacin (6). To a solution of 3-(2-(2-(2-aminoethoxy)ethoxy)acetamido)propyl 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (**6S**, 77 mg, 137 μmol) and (+)-biotin *N*-hydroxysuccinimide ester (biotin-NHS, 47 mg, 137 μmol) in DMF (2 mL), added a *N,N*-diisopropylethylamine (DIPEA, 48 μL, 274 μmol). The reaction mixture was stirred at room temperature for 1 h. When the reaction was completed, ddH_2O was added and the organic material was extracted with chloroform. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo after filtration. Purification of the residue by silica-gel flash column chromatography (ethyl acetate/chloroform/methanol = 7:7:3) provided the desired product **6** (67 mg, 62%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.14 (bt, J = 5.5 Hz, 1H), 8.80 (s, 1H), 7.99 (d, J = 13.0 Hz, 1H), 7.34 (d, J = 7.0 Hz, 1H), 7.00 (bt, J = 5.5 Hz, 1H), 6.58 (s, 1H), 5.87 (s, 1H), 4.49 (m, 1H), 4.29 (m, 3H), 4.19 (s, 2H), 3.72 (m, 2H), 3.67 (m, 2H), 3.60−3.52 (m, 4H), 3.50 (m, 1H), 3.43 (m, 2H), 3.36 (m, 4H), 3.12 (m, 1H), 2.88 (m, 1H), 2.71 (m, 5H), 2.56 (m, 2H), 2.22 (t, J = 7.5 Hz, 2H), 2.01 (m, 4H), 1.75−1.59 (m, 4H), 1.42 (m, 2H), 1.33 (m, 2H), 1.16 (t, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.68, 173.74, 170.84, 165.47, 164.41, 152.68, 147.00, 145.11, 138.72, 121.93, 112.73, 111.24, 105.06, 71.07, 70.26, 70.21, 68.63, 63.15, 62.02, 60.45, 55.78, 52.52, 52.38, 49.90, 40.75, 39.37, 36.30, 36.07, 35.02, 28.99, 28.42, 28.26, 25.80, 11.86, 9.05; HRMS (FAB): calcd for $C_{38}H_{55}FN_7O_8S$ $[M+H]^+$ 788.3817; found 788.3813.

Synthesis of Cy3-Analogue of Enrofloxacin (7). To a solution of 2-((1*E*,3*E*)-3-(1-(4-carboxybutyl)-3,3-dimethylindolin-2-ylidene)prop-1-enyl)-1,3,3-trimethyl-3*H*-indolium (Cy3-CO_2H, 22 mg, 50 μmol), 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium (HATU, 23 mg, 59 μmol) and DIPEA (24 μL, 135 μmol) in DMF (1 mL), added 3-(2-(2-(2-aminoethoxy)ethoxy) acetamido)propyl 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquino-line-3-carboxylate (**6S**, 25 mg, 45 μmol). The reaction mixture was stirred at room temperature for 3 h. When the reaction was completed, the reaction mixture was concentrated in vacuo. Purification of the residue by prep-HPLC provided the desired product **7** (7 mg, 16%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.82 (s, 1H), 8.55 (t, J = 13.5 Hz, 1H), 8.00 (d, J = 13 Hz, 1H), 7.62 (d, J = 4.2 Hz, 1H), 7.53 (m, 2H), 7.44 (m, 2H), 7.34 (m, 2H), 7.30 (m, 2H), 6.43 (d, J = 13.5 Hz, 2H), 4.16 (t, J = 6.5 Hz, 2H), 4.10 (s, 2H), 3.94 (m, 2H), 3.80−3.47 (m, 19H), 3.38−3.30 (m, 5H), 2.31 (bt, J = 7 Hz, 2H), 1.83 (m, 6H), 1.75 (s, 12H), 1.48−1.33 (m, 5H), 1.19 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ 175.53, 174.79, 174.27, 171.18, 170.58, 165.82, 156.40, 154.58, 152.61, 150.94, 147.39, 143.38, 142.88, 142.17, 141.00, 140.91, 138.86, 128.82, 128.75, 125.61, 125.54, 122.34, 122.19, 111.88, 111.24, 111.10, 110.36, 106.72, 102.63, 102.45, 71.10, 69.95, 69.27, 68.00, 62.68, 56.28, 52.09, 51.22, 49.45, 43.74, 39.14, 36.00, 35.25, 35.06, 30.23,

29.57, 28.60, 27.14, 26.96, 26.61, 22.88, 8.36, 7.37; HRMS (FAB): calcd for $C_{57}H_{73}FN_7O_7^+$ $[M]^+$ 986.5556; found 986.5567.

Synthesis of Biotin-Analogue of Norfloxacin (8). To a solution of 7-(4-(2-(2-(2-(2-aminoethoxy)ethoxy)acetamido)ethyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**8S**, 2.6 mg, 5 μmol) and (+)-biotin-NHS (1.7 mg, 5 μmol) in DMF (1 mL), added a DIPEA (1.8 μL, 10 μmol). The reaction mixture was stirred at 70 °C overnight. When the reaction was completed, the reaction mixture was concentrated in vacuo. Purification of the residue by prep-HPLC provided the desired product **8** (1.5 mg, 41%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.92 (s, 1H), 8.09 (d, J = 13 Hz, 1H), 7.29 (d, J = 6.5 Hz, 1H), 4.58 (m, 2H), 4.49 (m, 2H), 4.32 (m, 2H), 4.09 (s, 2H), 3.76 (m, 3H), 3.69 (m, 4H), 3.58 (t, J = 5.5 Hz, 2H), 3.46 (m, 4H), 3.40 (t, J = 5.5 Hz, 2H), 3.24 (m, 2H), 2.93 (m, 2H), 2.69 (d, J = 12.5 Hz, 2H), 2.24 (t, J = 7.5 Hz, 2H), 1.68 (m, 2H), 1.56 (t, J = 7 Hz, 2H), 1.45 (m, 2H), 1.32 (t, J = 7.5 Hz, 3H); HRMS (FAB): calcd for $C_{34}H_{49}FN_7O_8S$ $[M+H]^+$ 734.3347; found 734.3321.

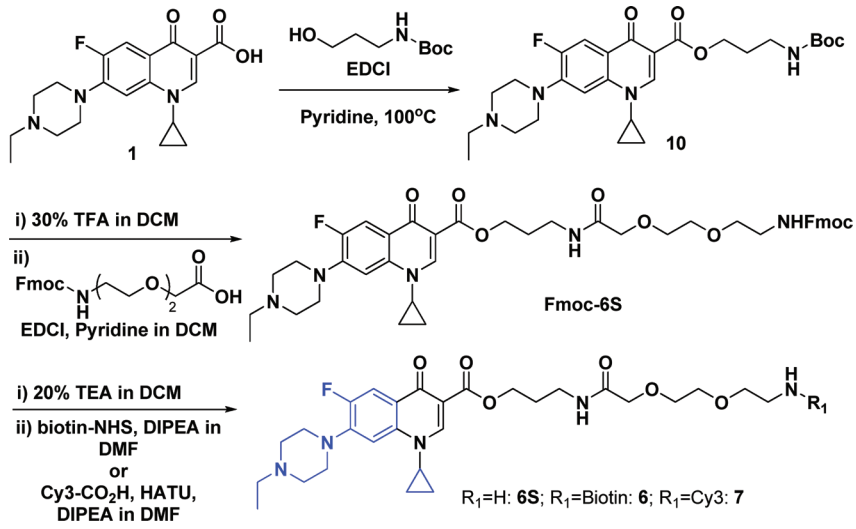
Synthesis of Cy3-Analogue of Norfloxacin (9). To a solution of Cy3-CO_2H (81 mg, 182 μmol), HATU (82 mg, 215 μmol), and DIPEA (90 μL, 497 μmol) in DMF (3 mL), added 7-(4-(2-(2-(2-(2-aminoethoxy)ethoxy)acetamido)ethyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**9S**, 84 mg, 166 μmol). The reaction mixture was stirred at room temperature overnight. When the reaction was completed, the reaction mixture was concentrated in vacuo. Purification of the residue by prep-HPLC provided the desired product **9** (19 mg, 12%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.84 (s, 1H), 8.54 (t, J = 13 Hz, 1H), 7.96 (d, J = 14 Hz, 1H), 7.54 (d, J = 7 Hz, 2H), 7.44 (m, 2H), 7.35 (d, J = 8 Hz, 2H), 7.31 (m, 2H), 7.23 (m, 1H), 6.43 (d, J = 13.5 Hz, 2H), 4.54 (bs, 2H), 4.16 (t, J = 7 Hz, 2H), 4.10−3.33 (m, 25H), 2.32 (t, J = 7.5 Hz, 2H), 1.84 (m, 2H), 1.78 (m 2H), 1.763 (s, 6H), 1.759 (s, 6H), 1.53 (m, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 175.58, 174.77, 174.35, 173.12, 168.17, 152.66, 150.98, 148.53, 142.86, 142.16, 141.00, 140.91, 137.56, 128.81, 125.65, 125.55, 122.37, 122.20, 112.02, 111.85, 111.20, 111.12, 107.43, 106.41, 102.65, 102.42, 70.75, 70.12, 69.45, 56.67, 51.99, 49.78, 49.45, 46.94, 43.74, 39.10, 35.10, 33.64, 30.59, 27.14, 26.96, 26.72, 22.85, 13.61; HRMS (FAB): calcd for $C_{53}H_{67}FN_7O_7^+$ $[M]^+$ 932.5086; found 932.5085.

Selection of Antibiotic-Specific scFv Clones by Phage Display. Panning of the phage display scFv library was performed as described in previous reports with some modifications (*15*). The biotinylated antibiotics (**6** or **8**, 1.5 μg) was added to a 50 μL suspension of M-280 streptavidin-conjugated paramagnetic beads (Invitrogen, USA) preblocked with mPBS (phosphate-buffered saline with 3% [w/v] skim milk) and incubated for 15 min. After washing the antigen-immobilized beads with PBST (phosphate buffered saline with 0.1% Tween-20) three times, one library equivalent (10^{13} cfu) of a human synthetic scFv phage-display library (*15*) in 1 mL mPBS was added to the beads and the suspension was incubated with slow rotation for 1 h at room temperature. The beads were subsequently washed with PBST using a magnetic separator (twice in the first round of the selection and three times in the subsequent rounds), and the bound phage particles were eluted with 100 mM aqueous triethylamine solution (10 min, room temperature). Eluted phages were neutralized with 1 M Tris-HCl (pH 7.4). Midlog phase ER2537 was added to the neutralized phage and incubated for 1 h at 37 °C with gentle shaking. Infected bacteria were plated on a LB-agar plate supplemented with ampicillin and 2% glucose and incubated overnight at 37 °C. The next day, cells were scraped from the plate with 5 mL SB (super broth, 3% [w/v] bactotryptone, 2% [w/v] yeast extract, and 1% MOPS, pH adjusted to 7.0) to make a panning output stock. Twenty milliliters of SB-ampicillin was inoculated with 50 μL of panning output stock and grown until OD_{600} reached 0.5−1.0. VCSM13 helper phage (10^{12}−10^{13} pfu) was added and the culture was gently shaken (120 rpm) for 1 h at 37 °C. Kanamycin (70 μg/mL) was subsequently added and the culture was incubated overnight at 30 °C. The next day, 5 mL of 5× PEG precipitation buffer (20% PEG8000, 15% NaCl in water) was added to the overnight culture supernatant and incubated in ice for 30 min. The precipitated phage was collected by centrifugation and resuspended in 0.5 mL PBS, which was used in the next round of selection as previously described (*15*). A total of three rounds of panning were performed, and the resulting output clones were subjected to ELISA screening.

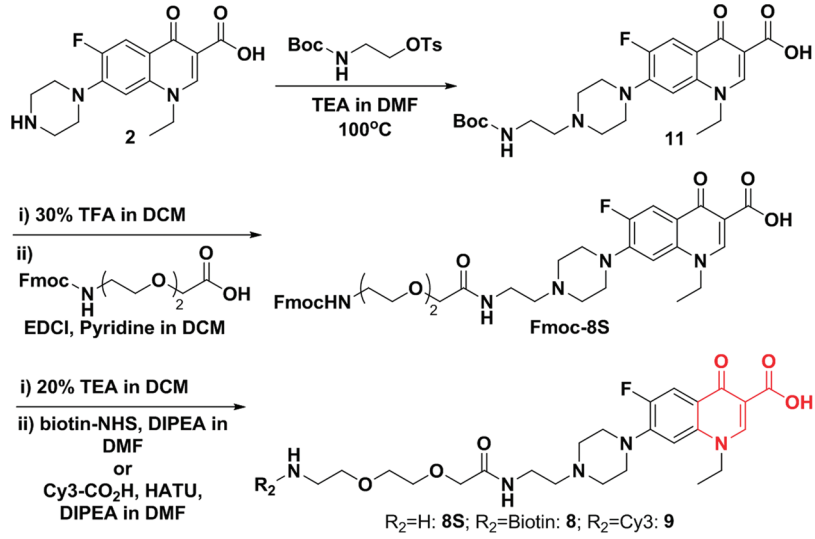
Screening of the Selected Clones by ELISA. Single colonies from the output agar plate were inoculated into 200 μL of SB-ampicillin in the wells of a 96-well microtiter culture plate and grown at 37 °C with shaking (400 rpm) until turbid. IPTG (1 mM final concentration) was added to each well, and the induced cultures were incubated at 30 °C with shaking for 12−16 h. The next day, the plate was centrifuged (3000 rpm, 15 min) and the supernatant was removed. The bacterial pellet in each well was resuspended in 40 μL of ice-cold 1× TES buffer (20% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0), 60 μL of 0.2× TES was subsequently added to each well, and the plate was incubated on ice for 30 min. After centrifugation at 3000 rpm for 15 min, the supernatants (periplasmic fractions) containing soluble scFv were carefully recovered. Avidin (10 μg/mL in PBS) was immobilized on the wells of an ELISA plate (Corning Costar 3690, 25 μL/well) and the wells were then blocked with mPBS. The biotinylated antigen (**6** or **8**, 1 μg/mL in PBS) was added to the plate (25 μL/well) and incubated at room temperature for 15 min. After washing off the unbound antigen, 25 μL of the periplasmic extract was added to each well of the ELISA plate and incubated for 1 h at room temperature. The plate was washed three times with PBST, and an anti-HA-HRP antibody (Santa Cruz Biotechnology, 1:3000 in mPBS) was added to each well. The plate was incubated for 1 h at room temperature, washed three times with PBST, and the bound scFv clones were colorimetrically detected by measuring the absorbance change at 450 nm caused by the horseradish peroxidase (HRP)-mediated oxidation of 3,3′,5,5′-tetramethylbenzimidine (TMB). The ELISA-positive scFv clones were sequenced (*20*), and the unique sequences were expressed and purified as described (*15*).

ELISA-Based Binding Study and Competition Assay. The binding of A2 or F9 to the biotin-analogues **6** or **8** was confirmed by ELISA using Synergy HT Microplate reader (BioTek Instruments, Inc., Winooski, VT) for the measurement of absorbance at 450 nm. The biotin-analogue **6** or **8** was added to an avidin-coated ELISA plate and incubated at room temperature for 15 min. After washing off the unbound antigen, serially diluted A2 or F9 was added to each well and incubated for 1 h at room temperature. The plate was washed three times with PBST, and an anti-HA-HRP antibody was added to each well. The plate was incubated for 1 h at room temperature, washed three times with PBST, and the bound scFv was colorimetrically detected using the TMB substrate. For the competition assay, 50 μL of A2 or F9 (20 μg/mL in PBS) was added to the **6**- or **8**-coated plates, respectively, and incubated for 1 h at room temperature. After washing off unbound scFV three times with PBST, serially diluted analyte **1** or **2** was added to each well and incubated for 1 h at room temperature. The plate was washed three times with PBST, and the bound scFv was colorimetrically detected using the anti-HA-HRP antibody and the TMB substrate. To confirm the selectivity or generality of biosensors, 100 μM aliquots of various analytes were treated in the same manner. Interestingly, the scFv F9 did not bind **6** (an enrofloxacin derivative) in ELISA screening, but the free,

Scheme 1. Preparation of Biotin-Analogue 6 and Cy3-Analogue 7 for the Development of Selective ScFv Biosensor of Enrofloxaxin 1



Scheme 2. Preparation of Biotin-Analogue 8 and Cy3-Analogue 9 for the Development of General ScFv Biosensor of Fluoroquinolones Antibiotics



underivatized enrofloxacin (**1**) or other fluoroquinolone antibiotics could compete with **8** for the binding to F9 (see below).

Surface Plasmon Resonance. To determine the dissociation constant of scFv and antibiotics, surface plasmon resonance (SPR) analysis using Biacore T100 instrument (Biacore, Sweden) was performed. A streptavidin (SA) sensor chip was purchased from Biacore. One molar NaCl/50 mM NaOH solution was injected to the flow cell three times for 60 s to activate the SA sensor chip surface, followed by the injection of the biotin-analogue of **6** or **8** (500 pM in 5% DMSO containing PBS). After capturing biotin-analogues onto the SA sensor chip, various concentrations of scFv solution (serially diluted from 1.5 μM to 100 nM in PBS) were injected for 60 s with a flow rate of 30 μL/min. Then, the dissociation phase was followed for 600 s with PBS buffer (30 μL/min). The results were analyzed using Biacore T100 Evaluation software.

RESULTS

Design and Synthesis. To develop general or selective antibiotics sensors using the pharmacophore-based strategy, we prepared two sets of biotinylated fluoroquinolone antibiotics (**6** and **8**, Schemes 1 and 2) with the proper understanding of structure−activity relationship (*9*) and used them as antigens for the phage-display selection of an scFv library. For the specific recognition of scFv toward enrofloxacin (**1**), the top-selling and most overused antibiotic, a biotin analogue of antibiotic (**6**) was designed to present the pharmacophore, the key structural motif of enrofloxacin (blue in Scheme 1), as a recognition element. As shown in Scheme 1, the synthesis of biotin-analogue **6** and Cy3-analogue **7** was initiated with the ester formation of enrofloxacin **1** with *N*-Boc-protected aminopropylalcohol to yield **10**, followed by Boc deprotection and the introduction of PEG linker via amide bond formation. The resulting **Fmoc-6S** was subjected to the biotinylation and Cy3 labeling after Fmoc deprotection to provide **6** and **7**, respectively. In contrast, we designed a biotinylated fluoroquinolone antibiotic from norfloxacin **2** to display the common pharmacophore of fluoroquinolone antibiotics, pyridone−carboxylic acid (red in Scheme 2) for the general recognition of scFv toward a series of fluoroquinolone antibiotics. The synthesis of biotin-analogue **8** and Cy3-analogue **9** shares the synthetic route for **6** and **7**; the *N*-alkylation of piperazine on **2** does not affect the efficacy as antibiotics based on the structure−activity relationship study (*9*); therefore, *N*-alkyl-substituted norfloxacin (**11**) was used to present the common pharmacophore as a recognition element for the phage-display selection of scFv library (Scheme 2).

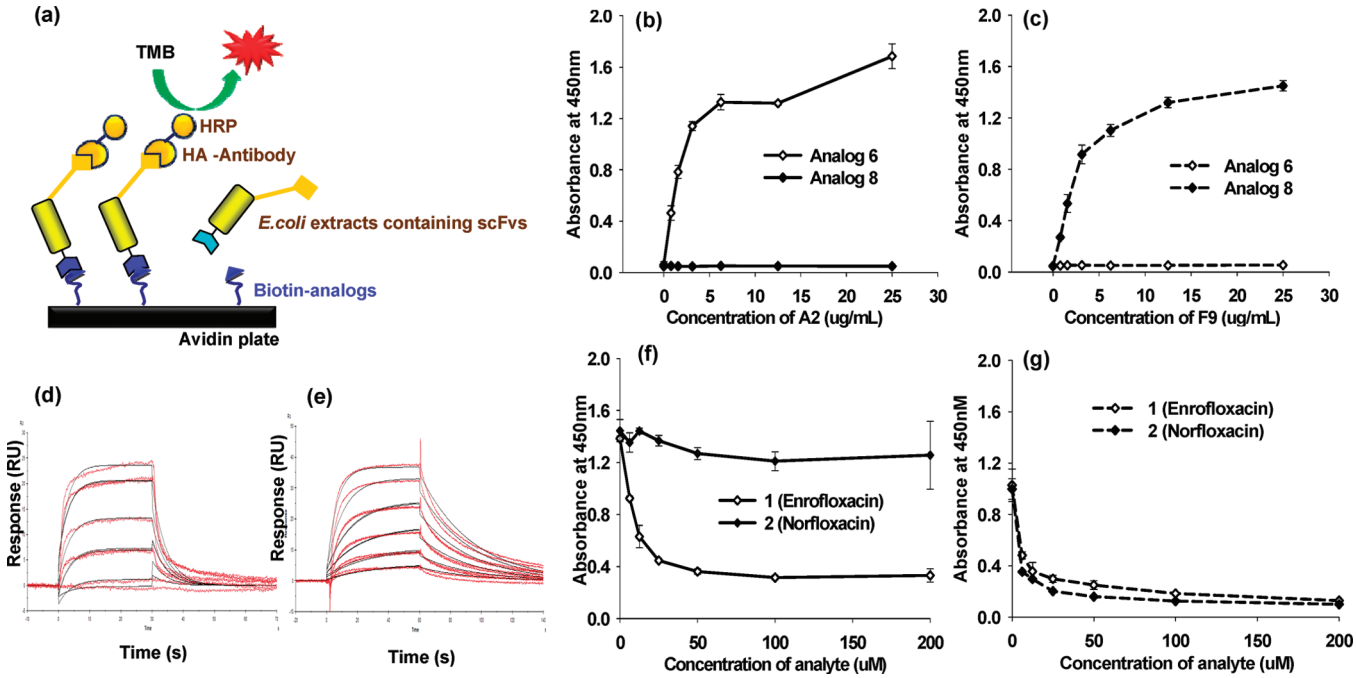


Figure 2. (a) Schematic illustration of scFv screening using biotin-analogues. (b) ELISA binding assay of A2 with **6** or **8**. (c) ELISA binding assay of F9 with **6** or **8**. (d) SPR analysis of A2 for direct interaction with analogue **6**. (e) SPR analysis of F9 for direct interaction with analogue **8**. (f) Competitive binding of **6**, with analyte **1** (enrofloxacin) or **2** (norfloxacin), to A2. (g) Competitive binding of **8**, with analyte **1** (enrofloxacin) or **2** (norfloxacin), to F9.

Selection of scFv's and SPR. Biotin-analogues (**6** or **8**) were separately immobilized on streptavidin-coated magnetic beads, and antigen-specific phage antibody clones were enriched by three rounds of panning. The selected scFv clones were individually screened by ELISA (see Figure 2a). A total of 19 clones that bound to **6** (10 clones) or **8** (9 clones) were found to bind selectively to the corresponding antibiotics and did not cross-react with the other antigen (Figure S1, Supporting Information). Among these clones, we chose two scFv clones for further investigation on the basis of the binding assay data with **1** and **2** as competitors (Figure S2, Supporting Information). The selected clones A2 and F9 (Figure S3, Supporting Information) bind to biotin-analogues **6** and **8**, respectively, as confirmed by ELISA (Figure 2b,c). We then performed surface plasmon resonance spectroscopy (SPR, Figure 2d,e) and confirmed the direct binding of A2 with **6** (K_D = 2.5 μM) and F9 with **8** (K_D = 1.58 μM).

As we discussed earlier, we designed these biotin-analogues **6** and **8** for the presentation of a particular portion of molecules as a recognition element using the pharmacophore-based strategy and aimed to develop a selective and general biosensor system for fluoroquinolone antibiotics as a model system to validate our pharmacophore-based strategy. Therefore, we subjected A2 and F9 to the ELISA-based competition assay by using **1** and **2** as analytes. A2 immobilized on a **6**-coated 96-well plate was selectively washed off by treatment with incremental concentration of **1**, but not **2** (Figure 2f). In contrast, both fluoroquinolone antibiotics were equally effective in washing off F9 immobilized on an **8**-coated 96-well plate (Figure 2g). Thus, we successfully demonstrated the validity of our pharmacophore-based approach through the discovery of A2, an enrofloxacin-selective scFv-based recognition element, and F9, a general biosensor for fluoroquinolone antibiotics.

Modification of scFv's on Solid Supports. After the discovery of selective and general scFv-based recognition elements (A2 and F9, respectively), we focused on the development of biosensor systems for fluoroquinolone antibiotics. A simple colorimetric/fluorescent system was designed to obtain an enrofloxacin-selective biosensor (A2 biosensor) and a general biosensor for a series of fluoroquinolone antibiotics (F9 biosensor). This sensor system is based on the competitive displacement of Cy3-analogues (**7** and **9**) by antibiotics in analytes using the scFv-based recognition elements that are immobilized on solid supports (see Figure 3a). Agarose resins (note: cyanogenbromide-activated agarose matrix (Sigma C9210) was used as a solid support for the modification of A2 and F9 clones and the modification was followed by the manual protocol) was chosen in preference to other solid supports including polystyrene, TentaGel, and HiCore resins, because the agarose resin did not bind nonspecifically to Cy3-labeled analogues (Figure S4, Supporting Information).

Competition by Analytes. As shown in Figure 3b−g, the fluorescence decolorization was observed on the surface of enrofloxacin-selective A2-immobilized agarose resins; this decolorization was caused by the dissociation of Cy3-analogue **7** upon treatment with incremental concentrations of **1**, but not **2** (Figure 3h). In contrast, the general F9-biosensor can detect the presence of both fluoroquinolone antibiotics in the analyte (Figure 3i). The detection limits of both biosensors are low (in the micromolar range), considerably lower than the application concentration of antibiotics as specified in the instruction guide for poultry farms (enrofloxacin, 139 μM; norfloxacin, 313 μM) (available at: (a) http://www.accessdata.fda.gov/cms_ia/importalert_111.html; (b) http://www.synap.co.kr/preview/txtview2.php?seq=1416993; (c) http://poultry.chonbuk.ac.kr/Poultry/Lecture/Rear/Broiler/Biosecurity/safe.html.); therefore, this scFv-based fluorescence biosensor can be utilized for one-step detection of overused fluoroquinolone antibiotics in livestock and poultry in water on the field.

Selectivity and Generality of A2 and F9 Sensor. Next, we confirmed the selectivity of the A2 biosensor and the generality of the F9 biosensor with various fluoroquinolone-based antibiotics such as **3**, **4**, and **5** and nonfluoroquinolone-based β-lactam antibiotics such as cefepime and faropenem. The selectivity of these biosensors was tested by ELISA assay. As shown in Figure 4, the A2 biosensor showed excellent selectivity to enrofloxacin

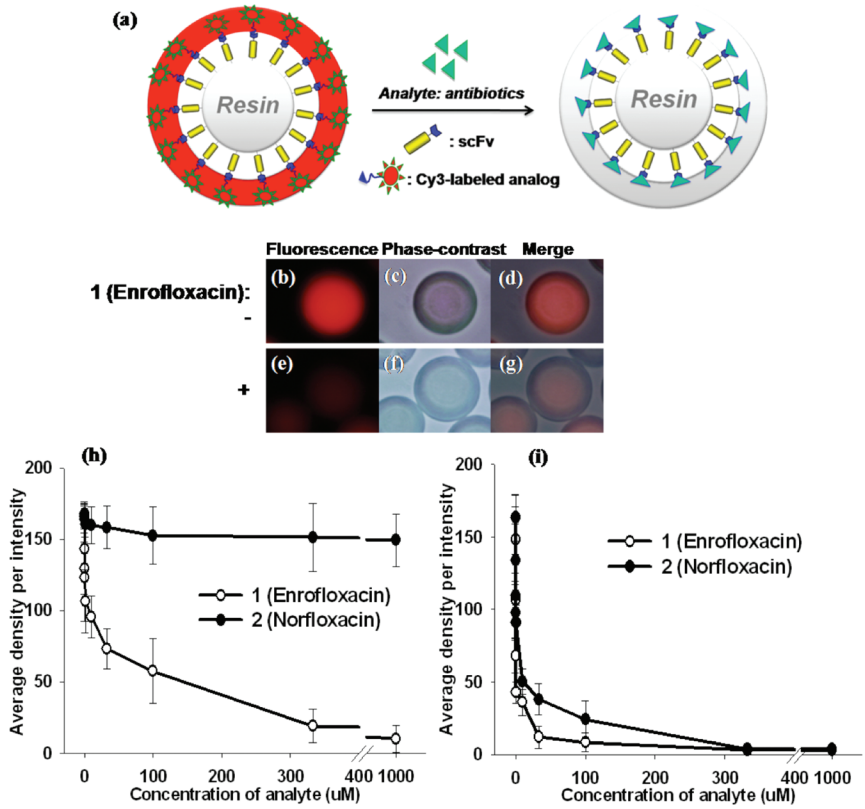


Figure 3. (a) Schematic illustration of scFv-based fluorescent biosensor system for fluoroquinolone antibiotics. Images of the resin for A2 biosensor with **7** before (b−d) and after (e−g) treatment with analyte **1** (100 μM): (b,e) fluorescence images (exposure time: 1/5000 s); (c,f) phase-contrast images (exposure time: 1/300 s); (d,g) merged images. (h) The ratio of average density and fluorescence intensity of the resins upon treatment with analytes **1** and **2** on A2-biosensor with **7** (n = 13). (i) The ratio of average density and fluorescence intensity of the resins upon treatment with analytes **1** and **2** on F9-biosensor with **9** (n = 13).

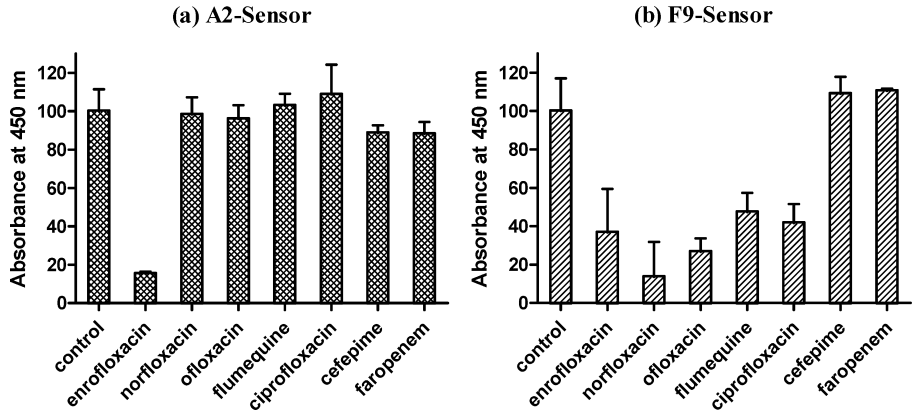


Figure 4. ELISA competitive assay of scFv-based biosensors with fluoroquinolone antibiotics **1**, **2**, **3**, **4**, and **5**, and β-lactams (cefepime and faropenem) as analytes for (a) enrofloxacin-selective A2 biosensor and (b) general fluoroquinolone F9 biosensor.

(**1**) over various analytes, although the structures of enrofloxacin (**1**) and ciprofloxacin (**3**) are very similar (see Figure 1). In contrast, the F9 biosensor can detect the presence of enrofloxacin (**1**) and norfloxacin (**2**) and many fluoroquinolone antibiotics such as ciprofloxacin (**3**), ofloxacin (**4**), and flumequine (**5**); however, this is not a nonspecific binding to any type of organic molecule. In fact, the F9 biosensor is sensitive enough to differentiate fluoroquinolone antibiotics from other classes of antibiotics such as β-lactam antibiotics whose chemical structures differ from those of fluoroquinolone antibiotics.

DISCUSSION

In our endeavor to develop modular biosensor systems that can selectively detect **1** or broadly detect a series of fluoroquinolone antibiotics, we designed pharmacophore-based antigens and fabricated the appropriate recognition elements for these antigens using scFv-based antibody phage-display technique. After the synthesis of biotin-analogues of fluoroquinolone antibiotics by adopting a pharmacophore-based strategy, we successfully obtained a novel scFv-based biosensor system with either generality or selectivity for fluoroquinolone antibiotics. The selectivity of the biosensors for the fluoroquinolone antibiotics can be modulated by modifying the structures of antibiotics on the basis of their structure−activity relationship. The resulting A2 recognition element can selectively detect enrofloxacin (**1**), which is the most widely overused and misused antibiotics in the livestock and poultry industry. Notably, A2 scFv can discriminate **1** from ciprofloxacin (**3**), which is almost

identical to enrofloxacin in chemical structure (Figure 1a) except for an ethyl group. While the presence of an ethyl group contributes about −6.5 kcal/mol of free energy to the protein−ligand binding (*21*), which may be large enough to confer the observed selectivity, the scFv has to provide a binding site with optimal noncovalent interaction and correct orientation. We also demonstrated the development of a general biosensor, F9, for detecting a series of fluoroquinolone antibiotics. In ELISA assay, F9 selectively binds to the norfloxacin derivative **8** and not to the enrofloxacin derivative **6**. However, its binding to **8** competes with free enrofloxacin (**1**) or other fluoroquinolone (Figure 4b), suggesting that F9 recognizes the fluoroquinolones at the pyridone-carboxy-side (Figure 1) which is blocked in **6**. On the other hand, A2 seems to bind to the antigen at the piperazine side, resulting in a highly selective scFv toward enrofloxacin. These results show that biosensors with an exquisite specificity toward the analyte can be developed using a combination of the pharmacophore-based antigen design and the phage-display antibody selection.

We envision that this strategy can be applied to the development of novel biosensors to detect various analytes, and the rational design of antigens combined with the combinatorial approach of phage display can provide a powerful selection tool via the presentation of pharmacophore—the key structural motif of antibiotics—as a recognition element. The development of biosensors for field use is currently being investigated and will be reported in due course.

ACKNOWLEDGMENT

This study was supported by (1) the National Research Foundation of Korea (NRF); (2) the WCU program funded by NRF and the Korean Ministry of Education, Science and Technology (MEST); and (3) the Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fisheries. H.Y.L and Y.J.K are grateful for the fellowships awarded by the BK21 Program.

Supporting Information Available: Chemistry general information, ELISA-based selection of scFv's (19 clones and A2 and F9 selection from 19 clones), characterization of A2 and F9 (Gel electrophoresis, MALDI data and DNA sequences) and images of agarose resins immobilized without and with A2 biosensor. This material is available free of charge via the Internet at http://pubs.acs.org.

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BC1004153