SUPPORTING INFORMATION

Bioanalytical Screening of Riboflavin Antagonists for Targeted Drug Delivery – A Thermodynamic and Kinetic Study

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EXPERIMENTAL SECTION

Materials. Riboflavin, riboflavin binding protein,[1] and the riboflavin antagonists 2-chlorophenothiazine, chlorpromazine, lumiflavin, perphenazine, and quinacrine were all purchased from Sigma-Aldrich. The riboflavin antagonist chloroquine was purchased from Fisher. See the Supporting Information for the dissolution of each antagonist.

General synthetic methods. Each of the solvents and reagents used for the reaction or purification process were purchased from a commercial supplier and used as received. These include ethyl bromoacetate (Sigma-Aldrich), and *N*-Boc-1,3-diaminopropane (TCI America). Each reaction was run under nitrogen atmosphere unless noted otherwise. Progress of the reactions was monitored by thin layer chromatography on Merck® TLC plates (250 μm thick), and compound spots were detected by UV illumination at 254 or 365 nm, or by staining with either phosphomolybdic acid reagent (20% w/v in ethanol) or ninhydrin solution (5% w/v in 3% acetic acid/ethanol). Flash column chromatography was performed by using silica gel (200-400 mesh). Structural characterization for each reaction product was carried out by NMR spectroscopy (^{1}H, ^{13}C) and mass spectrometry. For the NMR measurement, each of the samples was dissolved in a deuterated solvent (D_2O, DMSO-d_6), and NMR spectra were acquired with a Varian nuclear magnetic resonance spectrometer at 500 MHz or 400 MHz for ^{1}H NMR spectra, and at 125 MHz for ^{13}C NMR spectra under standard observation conditions. Mass spectrometric identification of compounds was performed by electrospray ionization mass spectrometry (ESI-MS) with a Micromass AutoSpec Ultima spectrometer.

3: 3-Carboxymethylriboflavin was synthesized as described elsewhere [2, 3] in three consecutive steps that comprise of the exhaustive acetylation of (−)-riboflavin, the *N*-alkylation of 2',3',4',5'-tetra-*O*-acetylriboflavin to the ethoxycarbonyl methyl derivative, and complete removal of ester protecting groups. It was obtained as yellow-brown solid. R_f (20% MeOH/CH_2Cl_2) = 0.19. ^{1}H NMR (399.97 MHz, $CDCl_3$): δ = 8.01 (s, 1H), 7.99 (s, 1H), 5.05-5.02 (m, 1H), 4.72-4.68 (d, 1H), 4.61 (s, 2H), 4.32-4.29 (d, 1H), 3.67-3.50 (m), 2.55 (s, 3H), 2.44 (s, 3H) ppm; MS (ESI): *m/z* (relative intensity, %) = 435.2 (100) $[M+H]^+$, 392.1 (6) $[M-CO_2]^+$; HRMS (ESI) calcd for $C_{19}H_{22}N_4O_8$ 435.1516 $[M+H]^+$, found 435.1511.

4: To a solution of 3-carboxymethylriboflavin (500 mg, 1.15 mmol) dissolved in DMF (30 mL) was added *N*-hydroxysuccinimide (NHS; 154 mg, 1.34 mmol), 4-dimethylaminopiridine (DMAP; 154 mg, 1.26 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide

hydrochloride (EDCI; 154 mg, 1.26 mmol) in a sequence. The reaction mixture was stirred at ambient temperature for 24 h prior to the addition of *N*-Boc-1,3-diaminopropane (200 mg, 1.15 mmol). The final mixture was stirred for an additional 24 h period, and concentrated *in vacuo*, yielding a yellow oily residue. It was dissolved in a small volume of methanol (~5 mL), and loaded onto a silica column (~100 mL of silica gel by dry volume). After the flash column chromatography performed by eluting with 5% to 20% MeOH/CH_2Cl_2, 3-(*N*-Boc-3'-aminopropylamidocarbonyl)methylriboflavin, the product was obtained as yellow-brown solid (308 mg, 45%). R_f (20% MeOH/CH_2Cl_2) = 0.87. ^{1}H NMR (399.97 MHz, DMSO-d_6): δ = 8.01 (s, 1H), 7.98 (s, 1H), 5.01 (br, 1H), 4.77-4.72 (m, 1H), 4.50 (s, 2H), 4.32-4.30 (br m, 1H), 3.52 (m, 1H), 3.12-3.06 (t, 2H), 2.96-2.92 (t, 2H), 2.63 (s, 3H), 2.44 (s, 3H), 1.54-1.51 (m, 2H), 1.40-1.36 (s, 9H) ppm; MS (ESI): *m/z* (relative intensity, %) = 613.2 (100) $[M+Na]^+$, 1203.5 (8) $[2M+Na]^+$; HRMS (ESI) calcd for $C_{27}H_{38}N_6O_9$ 613.2598 $[M+Na]^+$, found 613.2612.

1: To a stirred suspension of 3-(*N*-Boc-3'-aminopropylamidocarbonyl)methylriboflavin (52 mg, 88.0 μmol) in dichloromethane (1 mL) was added trifluoroacetic acid (1 mL). The mixture was stirred for 30 min, and then titrated into ethyl ether (30 mL), causing brown precipitates. The precipitates were collected by centrifugation and dried, yielding 3-(3-aminopropylamidocarbonyl)methylriboflavin as brown powder. Analysis by ^{1}H NMR spectroscopy indicated a complete deprotection of the *N*-Boc group. ^{1}H NMR (499.91 MHz, D_2O): δ = 7.84 (s, 2H), 7.77 (s, 2H), 5.02-4.97 (m, 1H), 4.38-4.34 (m, 1H), 3.92-3.91 (m, 2H), 3.86-3.85 (d, 1H), 3.74-3.72 (m, 1H), 3.39-3.37 (t, 2H), 3.13-3.10 (t, 1H), 3.07-3.04 (t, 2H), 2.53 (s, 3H), 2.40 (s, 3H), 1.94-1.91 (quin, 2H) ppm; ^{13}C NMR (125.7 MHz, D_2O): δ = 21.62, 23.82, 29.68, 39.18, 39.56, 39.99, 47.17, 65.58, 72.27, 75.34, 76.16, 119.76, 133.53, 136.48, 138.22, 153.87, 159.73, 164.14, 172.90 ppm; MS (ESI): *m/z* (relative intensity, %) = 491.3 (100) $[M+H]^+$, 981.5 (6) $[2M+H]^+$; HRMS (ESI) calcd for $C_{22}H_{30}N_6O_7$ 491.2254 $[M+H]^+$, found 491.2250.

Calorimetric Measurements. All experiments were carried out using a Nano ITC Standard Volume from TA Instruments (Lindon, UT). Binding conditions were optimized when a 40 μM ligand (RF) was injected into 4μM protein (RfBP), except for chloroquine, where 202 μM chloroquine was injected into 20 μM RfBP. The pH dependency studies were carried out with four buffers (0.1M phosphate, PBS, 0.1M sodium acetate and 0.1M tris). The PBS buffer (Sigma-Aldrich) used in all of the studies consisted of 0.1 M sodium phosphate, 0.1 M NaCl, pH

7.4. Control experiments were performed with 40 µM RF injected into 0.1M of the appropriate buffer. The six antagonists used were 2-chlorophenothiazine, chlorpromazine, chloroquine, lumiflavin, perphenazine, and quinacrine. Experiments with the antagonists were all performed in PBS buffer. Control experiments with the antagonists were performed using 40 µM of the antagonist in PBS injected into PBS buffer (except for chloroquine, see above). Each buffer was degassed prior to an experiment for 25 mins at room temperature with a stir rate of 610 rpm. In a multiple injection mode, the experiment was set up using the following parameters: 25°C, 250 rpm, 500 seconds between injections, 20 injections of 10 µL each. A control experiment was run for each buffer and each antagonist to account for the heat of dilution. Upon filling the cell and syringe, the cell was stirred and allowed to equilibrate for 30-45 minutes. The experiment was initiated when the baseline power difference was < 0.4 µW within 5 minutes. All data were recorded with the TA instrument software while further analysis was performed using TA NanoAnalyze Version 2.1.6. The area under each peak was integrated and the resulting data was modeled using an independent model. The independent site model used for obtaining values for *n*, *K*, and Δ*H* were determined by the following equations:

$$\Delta q = \left\{\frac{(nC_LK+C_MK+1)-\sqrt{(nC_MK+C_LK+1)^2-4nKC_MC_LK}}{2K}\right\} - [ML]_{n-1}\Delta H\Delta V_{cell} \quad (1)$$

Where Δq represents the change in heat for the reaction, n is the number of binding sites, *K* is the association binding constant, C_M is the bulk concentration of the protein, C_L is the bulk concentration of the ligand, Δ*H* is the molar enthalpy change, ΔV_{cell} represent the change in cell volume, and [ML], the total concentration of the complex is defined by equation 2.

$$[ML] = \frac{(nC_LK+C_MK+1)-\sqrt{(nC_MK+C_LK+1)^2-4nKC_MC_LK}}{2nK} \quad (2)$$

Equation 1 is fit to the ITC data by optimizing *n*, *K*, and Δ*H* to obtain the best fit between measured and calculated Δq values.[4, 5] Statistics were then performed on the thermodynamic parameters with a desired confidence interval of 95%. Each experiment was repeated 2-5 times.

Surface Plasmon Resonance (SPR) Spectroscopy. SPR experiments were performed using a Biacore® X (Pharmacia Biosensor AB, Uppsala, Sweden). A CM5 sensor chip was layered with the riboflavin ligand **1** in a reaction where 3-(3'-aminopropylamidocarbonyl) methylriboflavin **1** (2 mM) was covalently conjugated to a carboxymethylated dextran-coated layer on gold

following a standard amide coupling protocol (0.4 M EDC/0.1 M NHS in water).[6] The process for the immobilization of the RF ligand was performed on flow cell 1, and it led to 1100 response unit (RU) equivalent to ~0.11 ng/mm^2 (ΔRU = 110 after subtraction of the response unit from flow cell 2). Flow cell 2 treated in a similar manner to the control surface but the activated surface reacted with ethanolamine (1 M) alone without **1**. SPR binding studies of riboflavin binding protein (RfBP) were performed with or without competitive RF ligands in the solution as indicated in the figures. Typically the SPR sensorgrams were acquired by injecting each of the protein solutions prepared in HBS-EP buffer (pH 7.4) at a flow rate of 20 μL/min. After each measurement, the surface of the chip was regenerated by injection of 10 μL of 10 mM glycine-HCl (pH 2.5).

Response unit at steady state (RU_{eq}) was determined to calculate the amount of RfBP bound at the surface. It was calculated from each of the SPR sensorgrams by correcting bulk effect. In this analysis, we performed theoretical curve fittings for each experimental sensorgram at the dissociation phase according to an exponential dissociation model as established in the literature[7, 8]: $RU_t = RU_{t=0}\, e^{-(t-t_0)k_{off}}$ where t = 0 (t_0) refers to the time when dissociation begins. A series of theoretical curves were generated for each sensorgram by varying $RU_{t=0}$, and then, R^2 (the square of the correlation coefficient) values obtained from such curve fittings were plotted as a function of $RU_{t=0}$ in order to determine the value for an optimal $RU_{t=0}$ (RU_{eq}) that provides the best fit (Figure S1). The values of RU_{eq} that were acquired by this fitting method were used in Scatchard analysis for the determination of the dissociation constant of RfBP, as well as of the fractional inhibition in the ligand competition assay.

Kinetic binding parameters for RfBP that include the rate of association (k_{on}) and the rate of dissociation (k_{off}) were determined by fitting each of the binding curves using the Langmuir kinetic model as described[6] in Biacore evaluation software. These parameters were used to calculate the steady-state dissociation constant K_D (= k_{off}/k_{on}), and the value for K_D is provided on a mean basis measured from at least three sets of the measurement per injection concentration ($\chi^2 = 2.5$).

The inhibition constant K_i was derived for the added ligand according to the solution competition equation:[9, 10]

$$F = \frac{[I]}{([I] + K_i(1 + \frac{[L]_{surface}}{K_d}))}$$

The parameters are defined as: F is fractional inhibition of RfBP adsorption, [I] refers to the concentration of the inhibitor (ligand) added in the solution, $[L]_{surface}$ refers to the concentration of immobilized RF ligand on the surface, and K_d is the dissociation constant of RfBP for the surface.

Table S1. Dissolution of RF and RF Antagonists

Solute	Dissolution Procedure	Final Concentration
2-chlorophenothiazine	Cannot be dissolved in inorganic solvents.	NA
Chloroquine	To ~1 mg chlorpromazine, 10 mL of PBS buffer was added.	201.6 µM
Chlorpromazine	To ~1 mg chlorpromazine, 1 mL of .1 M HCl was added. PBS buffer was added 1 mL at a time, adding 1 mL more of .1 M HCl when chlorpromazine started to come out of solution. A total of 8 mL of PBS and 2 mL of .1 M HCl were added.	292.7 µM
Lumiflavin	To ~1 mg lumiflavin, 6 mL of PBS buffer was added. 1.5 mL of .1 M NaOH and 300 µL of 1 M NaOH were then added. 2.2 mL of PBS were added for a total volume of 10 mL.	344.3 µM
Perphenazine	To ~1 mg perphenazine, 1 mL of .1 M HCl and 9 mL of PBS buffer were added.	247.5 µM
Quinacrine	To ~1 mg quinacrine, 10 mL of PBS buffer was added.	215.2 µM
Riboflavin	To ~1 mg riboflavin, 10 mL of water were added. Solution was warmed to between 40-60 deg C for the dissolution of RF and then cooled to room temperature.	276 µM
RfBP	To 2 mg of RfBP, 2 mL of degassed PBS buffer was added.	34.2 µM

Table S2. Inhibition constants (K_i) for riboflavin, **1**, and quinacrine measured by the surface plasmon resonance (SPR) spectroscopic method.[a]

	Riboflavin (RF)	**1**	Quinacrine
K_i (M)	3.54×10^{-7}	2.56×10^{-6}	6.67×10^{-6}
Relative inhibition activity (SPR)[b]	1	0.14 (7.2-fold[c])	0.053 (18.8-fold[c])
Relative binding activity (ITC)[b]	1	-	0.019 (52.8-fold[c])

[a]Inhibition constant K_i calculated by the solution competition equation; [b] K_i(RF) ÷ K_i(RF, **1** or quinacrine); [c]Decrease in inhibition activity relative to RF.

Scheme S1. [a]Synthesis of 3-carboxymethylriboflavin, and its primary amine-containing amide derivative [a]*Reagents and conditions*: i) Ac_2O, AcOH, cat. H_2SO_4, 80°C; ii) K_2CO_3, Ethyl bromoacetate, DMF, 85°C; iii) 6M HCl, reflux; iv) *N*-Boc-1,3-diaminopropane, EDC, NHS, DMF, rt; v) TFA, CH_2Cl_2, 30 min.

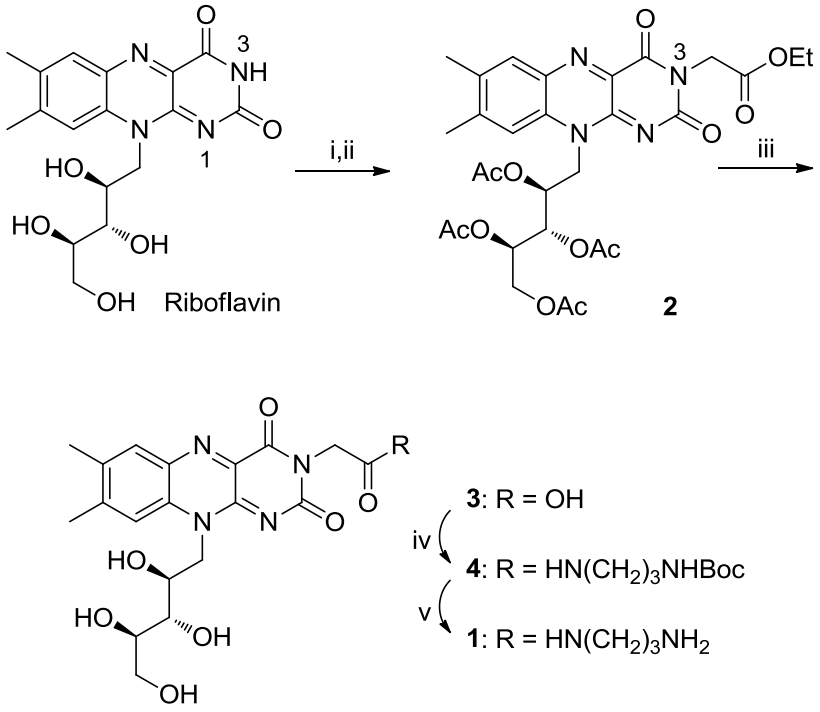
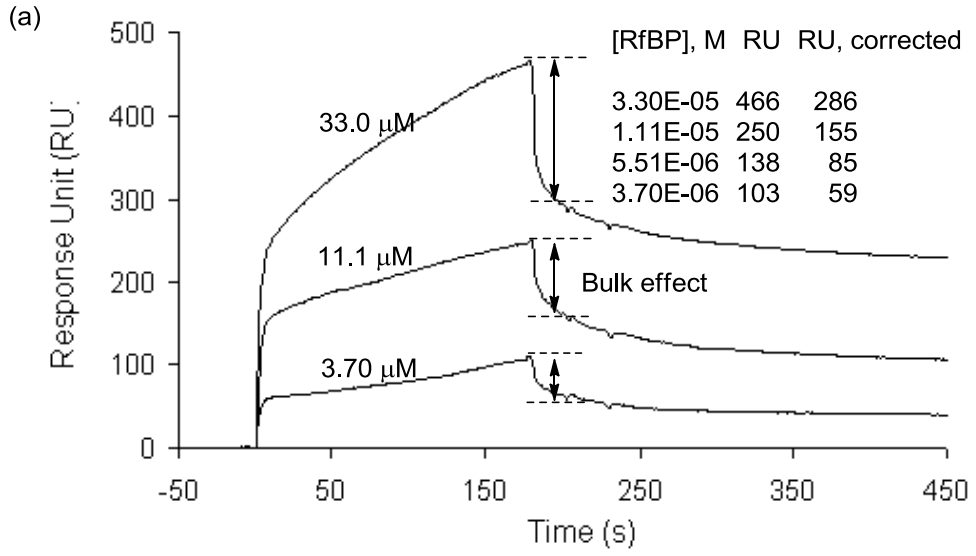
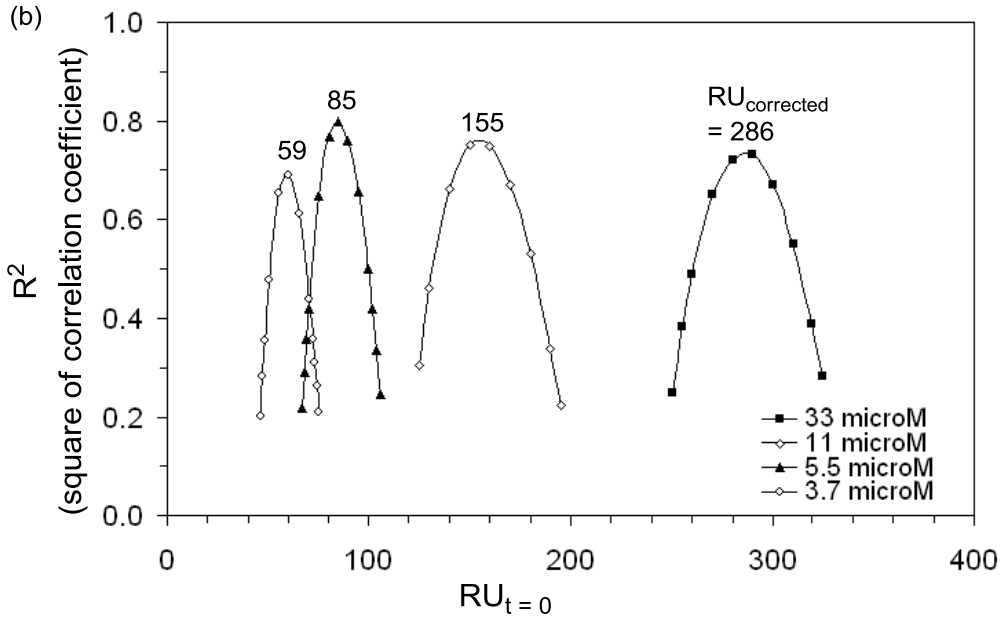


Figure S1. (a) A set of representative SPR sensorgrams for the concentration-dependent binding of riboflavin binding protein (RfBP) to the riboflavin ligand **1** immobilized to a CM5 sensor chip surface, illustrating the level of the bulk effect corrected for the estimation of RU_{eq}. (b) A plot of R^2 values as a function of $RU_{t = 0,\ dissociation}$ where $RU_{corrected}$ (RU_{eq}) refers to the value of $RU_{t = 0}$ that provides the best curve fitting (maximal correlation) at the dissociation phase (see Experimental Section). (c) Scatchard plots for RfBP binding based on RU either uncorrected or corrected.


(a)
Response Unit (RU)
Time (s)
[RfBP], M RU RU, corrected
3.30E-05 466 286
1.11E-05 250 155
5.51E-06 138 85
3.70E-06 103 59
33.0 μM
11.1 μM
3.70 μM
Bulk effect


(b)
R2 (square of correlation coefficient)
RUt = 0
59
85
155
RUcorrected = 286
33 microM
11 microM
5.5 microM
3.7 microM

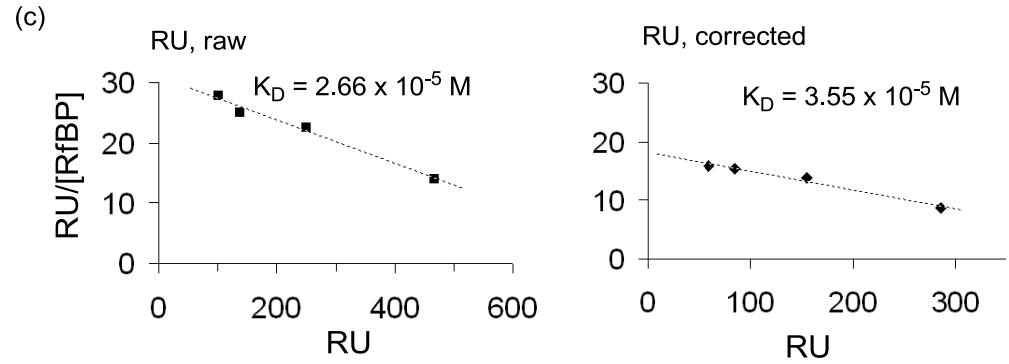

(c)
RU, raw
KD = 2.66 x 10-5 M
RU/[RfBP]
RU
RU, corrected
KD = 3.55 x 10-5 M
RU

Figure S2. A set of representative SPR sensorgrams obtained from the competitive inhibition experiments for RfBP (5 μM) binding to the RF (**1**)-immobilized CM5 chip surface by the addition of soluble ligands to the RfBP solution: (a) riboflavin; (b) quinacrine. The concentrations of the added RF ligand are indicated in the plot of the sensorgrams. Each of the RfBP sensorgrams (RfBP + ligand) was corrected against the contribution by the soluble ligand measured at the designated concentration (ligand alone).

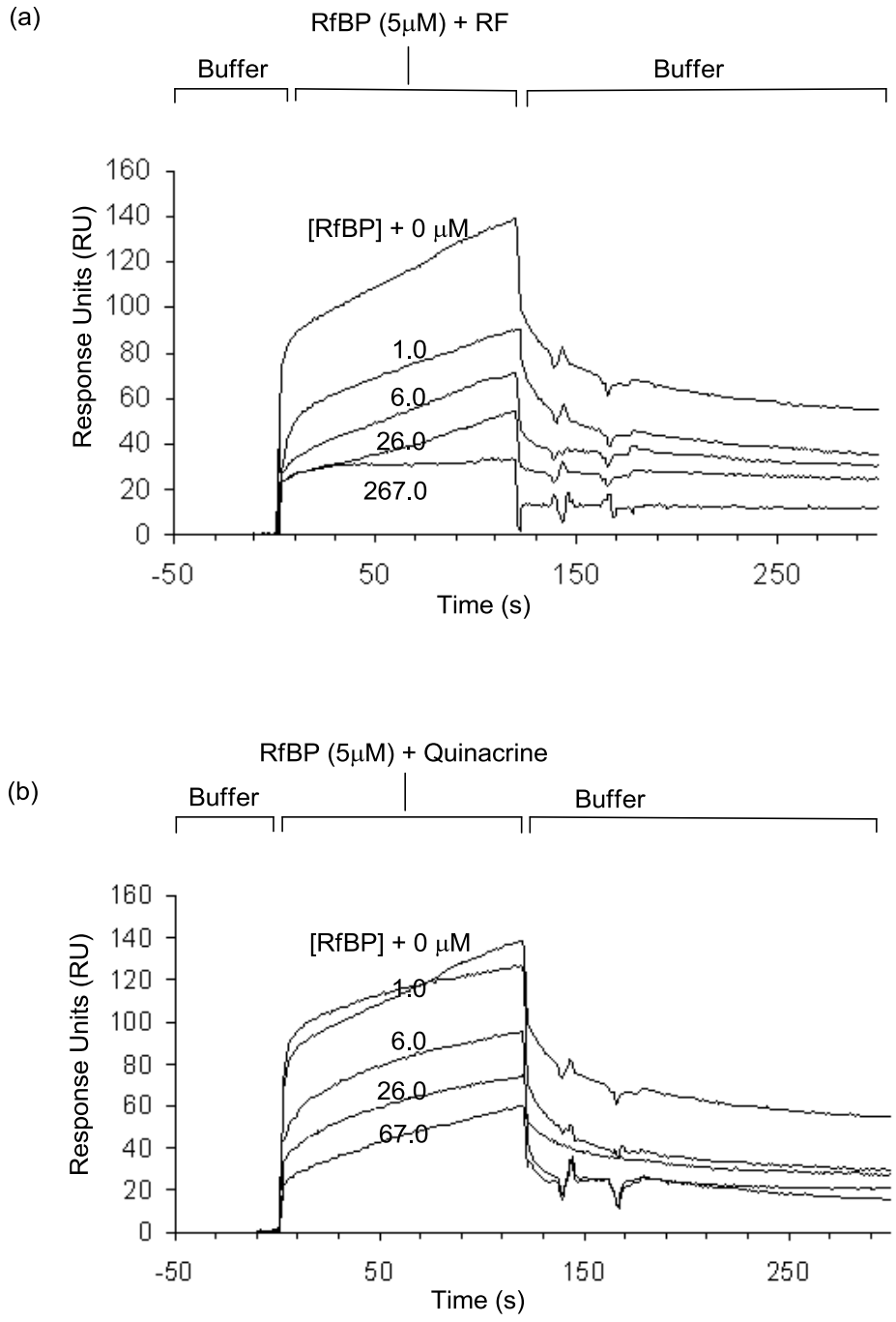
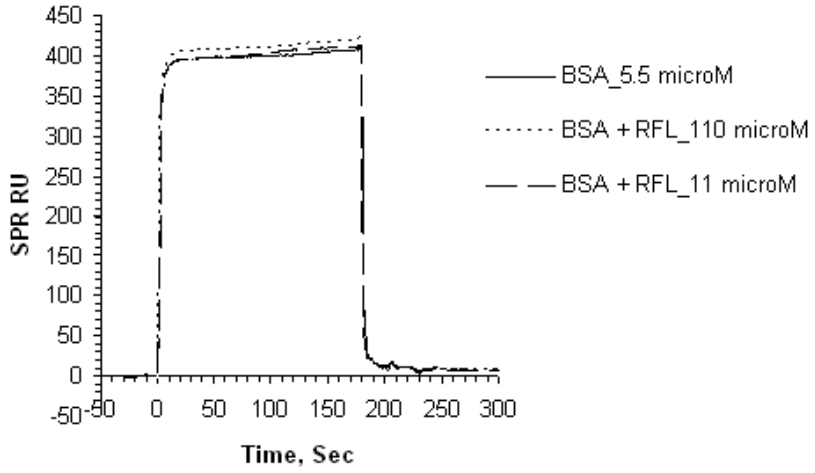


Figure S3. SPR sensorgrams from the control experiments performed by the injection of bovine serum albumin (BSA, 5.5 μM) to the CM5 sensor chip surface immobilized with riboflavin ligand **1**. The protein injection led to non-specific interaction and bulk effects (full and rapid protein desorption observed right after the buffer flow). This non-specific weak adsorption was further confirmed by the competitive inhibition experiments where the co-injection of soluble RFL (**5**) to the BSA solution did not make any effects on the resulting sensorgrams.



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