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Cooperative binding of a quinoline derivative to an RNA stem loop containing a dangling end

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ABSTRACT

The binding of a quinoline derivative (QD2) to a small RNA stem loop containing a 3′-dangling end (RNA1) has been studied. The compound was identified by first performing a similarity search of the NCI database of 250,000 compounds and then using computational docking with AUTODOCK to evaluate the binding of the resulting compounds to RNA1. Binding experiments using fluorescence and ITC methods revealed that QD2 binds cooperatively to four binding sites on RNA1 with equilibrium binding dissociation constants ranging from 8.2 (±0.3) to 12.5 (±4.2) μM. CD and UV titration experiments suggested that binding of QD2 changes the conformation of both RNA1 and the QD2 chromophore and stabilizes RNA1.

RNA plays important and versatile roles in gene expression by both carrying and regulating the information used to direct protein synthesis.[1,2] Therefore, small molecules able to bind to RNA and alter these biological processes would be of great utility.[3–5] The diverse secondary and tertiary structures of RNA provide many different binding environments for targeting by small molecule ligands. We have been investigating small molecule recognition of junctions between double stranded and single stranded regions of RNA, including stem loops, bulges and duplexes with dangling single stranded sequences.[6–10] These junctions are common binding sites for proteins and form distinct structures for recognition by small molecules. Here we report a quinoline derivative (QD2) that binds with unusual cooperativity to four sites on a stem loop RNA containing a 3′-dangling end.

Quinoline derivatives have antimalarial, antiviral, analgesic, hypotensive, antitumour, and anti-inflammatory activities and are important in the field of agriculture because of their insecticidal, fungicidal, and pesticidal properties.[11–16] Quinolines are also known intercalators of DNA.[17–20] Investigations of quinolines as ligands for RNA are more limited.[21,22] However, a series of helix threading peptides containing quinolines have been developed to bind double stranded RNA.[23–25] We have recently reported the identification of a quinoline derivative that binds to the 3′-dangling end of RNA1 shown in Figure 1.[6] To investigate quinoline recognition of RNA1 more generally, we report here an investigation of the ability of related quinoline derivatives to bind RNA1.

To identify molecules related to QD1 that bind RNA1, we performed a computational substructure and 3D search of the NCI database of 250,000 compounds. Out of this search, 20 molecules containing a quinoline substructure with diverse chemical scaffolds with molecular weights between 200 and 500 and calculated log *P* values less than or equal to 5 were selected for computational docking using the program AUTODOCK 3.[26,27] AUTODOCK uses a scoring function that includes van der Waals, electrostatic, desolvation, hydrogen bonding, and ligand torsional energies. AUTODOCK has been validated by several investigators as an effective tool to identify small molecule ligands for RNA.[28–32] We have previously used AUTODOCK in combination with DOCK to identify ligands for a tetraloop and stem loop 3 of the Ψ RNA of HIV.[7,9]

The NMR structure of RNA1 was used for the computational docking with AUTODOCK 3.[33] The docking site was centered on the loop region between G5 and A6 bases and included the entire RNA. The 10 quinoline derivatives with a predicted binding energy of better than −10 kcal/mol were selected for experimental studies (Fig. 2).

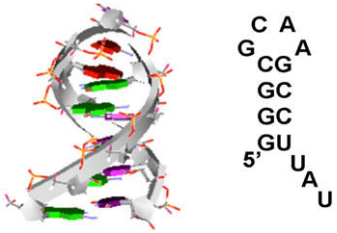

Figure 1. Structure of RNA1 determined by NMR. (PDB code: 1ZIH).[33]

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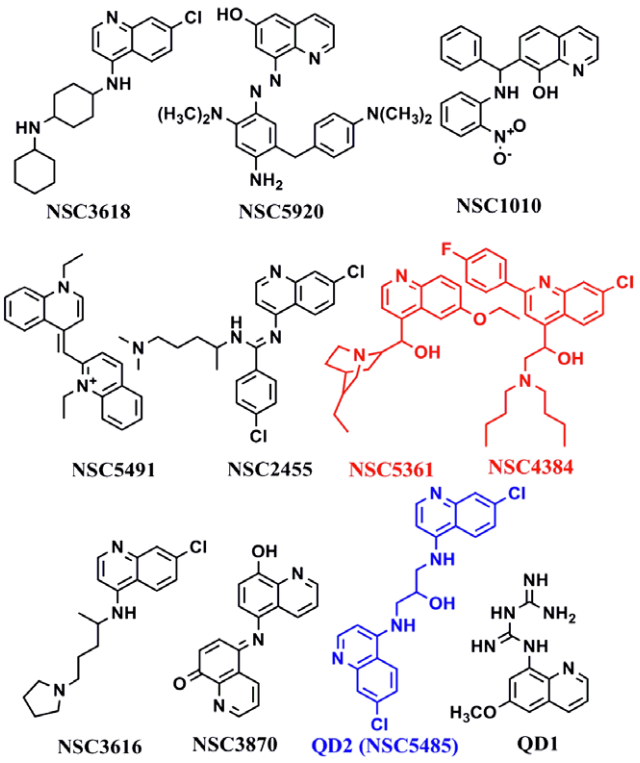

Figure 2. Compounds from the NCI studied experimentally. The identities of the compounds in red (NSC5361 and NSC4384) could not be confirmed by mass spectrometry or NMR spectroscopy. QD2, shown in blue, bound with highest affinity to RNA1.

The selected 10 quinoline derivatives were obtained from the NCI, and their purity evaluated using NMR and mass spectrometry. The NMR and mass spectra of 2 of the 10 molecules (NSC5361 and NSC4384) were not consistent with reported structures. The affinities of the remaining 8 molecules for RNA1 were determined using fluorescence spectroscopy by monitoring the quenching of the fluorescence of a 5′-fluorescein-labeled RNA1 upon titration with each selected ligand. This is a common approach for measuring small molecule–RNA affinities.[5,34–36] Only one of the eight molecules (NSC5485 or QD2) was shown to bind with low micromolar affinity to RNA1. The other seven molecules bound with greater than 50–150 μM affinities to RNA1. QD2 contains two quinolines linked by a flexible diaminohydroxypropane group that can form hydrogen bonds with the RNA target. The flexible and highly functionalized linker may enable QD2 to use both quinoline groups and the hydrogen bond donors and acceptors of the linker to form a greater number of interactions with the RNA than the other selected molecules.

A typical titration curve for the binding of QD2 to RNA1 is shown in Figure 3. This type of S-shaped binding curve usually results from positive cooperative interactions between bound ligand molecules, although a mixed mode of binding may also occur.[37,38] It is possible that once the first molecule binds to RNA, the second molecule can bind with higher affinity because it can stack and form hydrogen bonds with the already bound ligand. A similar binding geometry has been proposed for the related quinolones, which bind cooperatively to DNA.[39–41]

The data were analyzed using the following equation, which assumes cooperative binding to identical interacting sites.

$$(F - F_0)/(F_f - F_0) = (KL^N)/(1 + KL^N) \quad (1)$$

F is the fluorescence intensity of the sample, F_0 is the initial fluorescence intensity, F_f is the final fluorescence intensity, K is the association constant, N is the number of identical sites, and L is the ligand concentration. Using this equation, a dissociation constant of 8.2 (±0.3) μM with four identical sites was obtained from the average of five titrations.

The experiments performed with QD2 included 3.5% DMSO in order to improve the solubility of QD2. The addition of DMSO alone to the 5′-fluorescein-labeled RNA1 resulted in an increase in fluorescence intensity. The change of the signal of the 5′-fluoresein-labeled RNA due to the addition of DMSO was subtracted from that observed upon addition of QD2 in order to determine the K_d of the RNA–QD2 complex. The addition of QD2 to fluorescein did not result in a significant change in fluorescence signal, suggesting that QD2 does not interact directly with fluorescein in the binding experiments with RNA.

The binding of small molecule aggregates to the RNA target can give false positive results because the aggregates may be responsible for the observed binding rather than the isolated molecules.[42,43] To confirm that the measured binding affinity of QD2 for RNA1 was not that of QD2 aggregates, we performed experiments in 0.01% Triton X-100, which is typically sufficient to prevent aggregation.[42] The dissociation constant obtained in the presence of Triton X-100 was 12 (±0.5) μM, which is similar to the binding constant in the absence of Triton X-100, 8.2 (±0.3) μM. These results suggest that pre-formed aggregates of QD2 are not responsible for the observed binding of QD2 to RNA1.

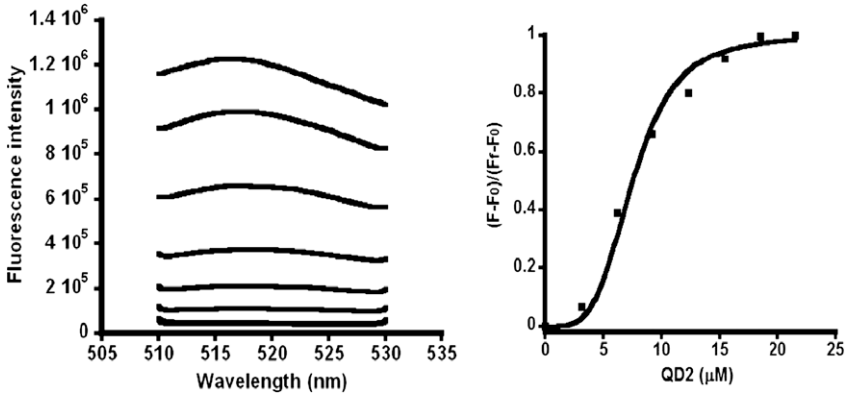

Figure 3. (a) Fluorescence spectra for 5′-fluorescein-labeled RNA1 titrated with increasing concentrations of QD2 (3–21 μM). The intensity of the fluorescence signal decreased upon addition of QD2. (b) Plot for the fraction of RNA fluorescence signal quenched versus QD2 assuming a 1:4 binding stoichiometry. The fraction of RNA bound was calculated using the fluorescence emission at 520 nm by excitation at 490 nm, and the data were fit to Eq. 1. The experiments were performed with 100 nM RNA1 and 0–21 μM QD2 in a buffer comprised of 10 mM sodium phosphate buffer (pH 6) and 50 mM NaCl.

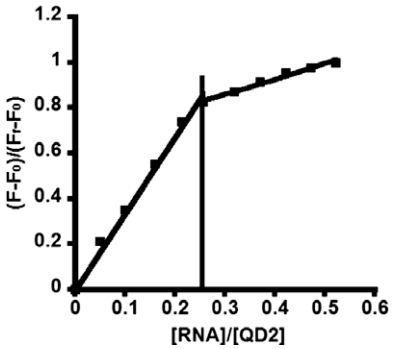

Figure 4. A plot of the fraction of RNA bound vs molar ratio ([RNA]/[ligand]) that was used to determine the stoichiometry of the QD2–RNA1 complex. QD2 (25 μM in 3.5% v/v DMSO) was titrated with RNA1 in a buffer comprised of 10 mM sodium phosphate (pH 6) and 50 mM NaCl. The excitation and emission wavelengths were 310 nm and 369 nm, respectively.

To provide additional support for the observed 1:4 stoichiometry, increasing concentrations of RNA were added to a constant concentration of QD2 above the measured K_d (Fig. 4). This experiment was performed as a reverse titration in which QD2 was held constant because QD2 precipitates at higher concentrations. In these experiments the fluorescence of QD2 was monitored from 355 to 390 nm. The stoichiometry of binding was found to be 1:4, which agrees with the number of binding sites determined from the fluorescence binding assay.

The binding affinity and stoichiometry of QD2 for RNA was investigated using isothermal titration calorimetry (ITC). A reverse titration was performed in which a constant concentration of QD2 was titrated with RNA1. An example of an ITC experiment is shown in Figure 5. The binding curve obtained was fit with a sequential

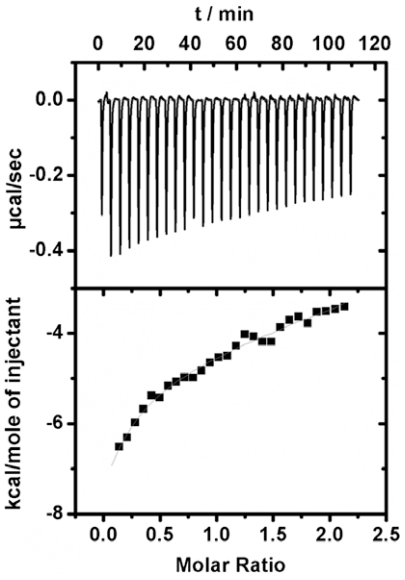

Figure 5. Plots of data from a reverse titration ITC experiment. QD2 (20 μM in 3.5% v/v DMSO) in 10 mM sodium phosphate buffer (pH 6, 50 mM NaCl) was titrated with increasing concentrations of 200 μM RNA at 25 °C. A standard experiment consisted of titrating 20 μM of QD2 (1.42 mL in sample cell) with 10 μL of 200 μM of RNA solution. A control experiment was performed which involved addition of aliquots (10 μL) of buffer into QD2 (20 μM). The duration of each injection was 24 s, and the spacing between two injections was 240 s. The initial delay prior to first injection was 60 s. The heat of ligand binding for each injection was determined by subtracting the heat of ligand solvation from that of the ligand–RNA injection to yield the heat due solely to ligand binding for each injection. Binding constants were determined from plots of heat of ligand binding as a function of RNA–ligand molar ratio. The data was fit with a sequential four-site binding model.

four binding site model. The absence of biphasicity in the binding curve indicates cooperativity between the four ligand binding sites.[44,45] The binding constants obtained from fluorescence and ITC experiments are compared in Table 1.

The effect of the binding of four QD2 molecules on the conformation of RNA1 was investigated using CD spectroscopy (Fig. 6). The CD spectrum of RNA1 resembles that of A-form RNA, with a strong negative band at 208, a weak negative band at 240 and a strong positive band at 266 nm, and a slight shoulder at 280 indicating a loop structure.[46–48] The ligand titration was monitored from 240 to 320 nm because the DMSO present in the buffer interferes with the signal below 240 nm. The decrease of RNA band intensity at 266 nm with increasing ligand concentration suggests that there is a significant change in the conformation of RNA1 upon binding to ligand.

The effect of the binding of RNA1 on QD2 was investigated in a UV reverse titration experiment in which the UV signal of QD2 was monitored from 310 to 350 nm upon addition of RNA. A displacement of maximum absorption from 329 to 335 nm (a bathochromic shift of 6 nm) and a large hypochromism of 48.1% (0.316 to 0.152) were observed. The large hypochromism suggests a perturbation of the complexed chromophore system upon binding to RNA1. Taken together these experiments suggest that cooperative binding of QD2 to RNA1 changes the molecular conformation of both QD2 and the RNA1.

In conclusion, the results reported here indicate an unusual 1:4 stoichiometry of binding between RNA1 and QD2. Both the fluorescence and ITC binding experiments provide evidence for cooperative binding to four sites. Cooperative 1:4 binding is rare, especially to a small RNA target, although cooperative binding of more than one quinolone and 1:3 stoichiometry of bis-netropsin to DNA have been studied previously and 1:2 cooperative binding for the glycine-dependent riboswitch has been observed.[40,49–51] The CD and UV experiments support a change in conformation of the RNA upon binding the four ligands. Cooperative interactions are likely to contribute to the better binding affinity of this molecule than that of QD1 or the other molecules selected in the screening of the NCI database.

Table 1
Dissociation constants determined from fluorescence and ITC experiments

RNA1	Fluorescence	Fluorescence (0.01% Triton X-100)	ITC
K_d1 (μM)			10.8 ± 1.2
K_d2 (μM)			9.2 ± 2.4
K_d3 (μM)	8.2 ± 0.3	12.0 ± 0.5	8.1 ± 1.8
K_d4 (μM)			12.5 ± 4.2

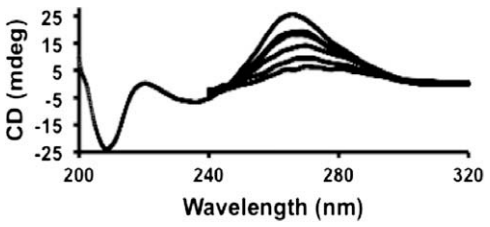

Figure 6. Circular dichroism spectra of RNA1 (15 μM in 400 μL of buffer) titrated with increasing concentrations of QD2 (7.5–50 μM in 3.5% v/v DMSO) to RNA in 10 mM sodium phosphate buffer (pH 6, 50 mM NaCl). The CD spectrum of the free RNA is shown from 200 to 320 nm, while those that include QD2 are shown from 240 to 320 nm because of the presence of DMSO in these samples. The intensity of the CD signal decreased upon addition of QD2.

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