

Article

Chloro-Substituted Naphthyridine Derivative and Its Conjugate with Thiazole Orange for Highly Selective Fluorescence Sensing of an Orphan Cytosine in the AP Site-Containing Duplexes

Chun-xia Wang [†], Yusuke Sato , Takashi Sugimoto, Norio Teramae and Seiichi Nishizawa *

Department of Chemistry, Graduate School of Science, Tohoku University, Sendai 980-8578, Japan;
cxwang@iccas.ac.cn (C.-x.W.); yusuke.sato.a7@tohoku.ac.jp (Y.S.); sgmt6600@gmail.com (T.S.);
norio.teramae.d3@tohoku.ac.jp (N.T.)

* Correspondence: seiichi.nishizawa.c8@tohoku.ac.jp

[†] College of New Energy and Materials, China University of Petroleum Beijing, Beijing 102249, China.

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Abstract: Fluorescent probes with the binding selectivity to specific structures in DNAs or RNAs have gained much attention as useful tools for the study of nucleic acid functions. Here, chloro-substituted 2-amino-5,7-dimethyl-1,8-naphthyridine (CINaph) was developed as a strong and highly selective binder for target orphan cytosine opposite an abasic (AP) site in the DNA duplexes. CINaph was then conjugated with thiazole orange (TO) via an alkyl spacer (CINaph–TO) to design a light-up probe for the detection of cytosine-related mutations in target DNA. In addition, we found the useful binding and fluorescence signaling of the CINaph–TO conjugate to target C in AP site-containing DNA/RNA hybrid duplexes with a view toward sequence analysis of microRNAs.

Keywords: fluorescent probe; conjugate; abasic site; DNA; microRNA

1. Introduction

Much attention has been paid to the design of fluorescent probes capable of selective binding to specific structures in DNAs and RNAs [1,2]. This class of fluorescent probes has been designed to bind abasic (apyrimidinic or apurinic; AP) sites [3–8], bulges [9,10], mismatched sites [11,12] and overhanging structures [13–15], by which the biological functions of these noncanonical base-pairs have been examined. Moreover, these fluorescent probes have great potential to be applied for the analysis of target DNAs and RNAs based on their binding-induced fluorescence signaling. These probes also serve as the affinity-labeling agents in the label-free assays for detecting various analytes [16–18].

We developed AP site-binding ligands (APLs) that are conjugated with thiazole orange (TO) for the fluorescence sensing of orphan nucleobases in DNA/DNA duplexes (Figure 1A) [19,20]. APLs can form the pseudo-base pairing with target orphan nucleobases, which allows the selective binding to the target nucleobase opposite the AP site [3–7]. On the other hand, TO unit connected with the APL unit through an appropriate linker can function as a fluorescent intercalator [21,22]. The TO unit alone shows negligible fluorescence, but its fluorescence is greatly enhanced upon intercalation into the base pairs near the AP site. APL–TO conjugates thus enable the light-up sensing of target orphan nucleobase in the AP site-containing DNA (AP–DNA) duplexes. Such light-up probes are useful for a more sensitive analysis compared to fluorescence quenching probes. We succeeded in the detection of single base mutation in target DNA sequences based on the binding and light-up functions of APL–TO conjugates in combination with an AP–DNA probe (cf. Figure 1A). In addition, these conjugates were applicable to the sequence-selective analysis of microRNAs, a class of small

non-coding RNAs associated with various diseases and cancers [23], by the selective binding of conjugates to target orphan nucleobases in the AP site-containing DNA/RNA (AP–DNA/RNA) hybrids. The properties of nucleobase-selectivity and fluorescence emission wavelength are tunable by adopting suitable APLs and cyanine dyes, respectively, which enables the development of multiplex analysis of target sequences by the simultaneous use of these conjugates in a single solution [19]. In this context, 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND, Figure 1B) was previously used as an APL unit in the conjugate with TO unit for the light-up sensing of target orphan C in the AP–DNA and AP–DNA/RNA hybrid duplexes [19]. The binding selectivity of ATMND toward target C can be rationalized by the complementary base pairing of the N1-protonated form of ATMND with target C through hydrogen bonding (Figure 1B) [3]. However, ATMND inherently has only moderate selectivity for C over T because of the possible protonation at N8 that allows the recognition of T (Figure 1B). This should limit the application of the conjugate for the sequence analysis of target DNAs and microRNAs such as the discrimination of C against T or U.

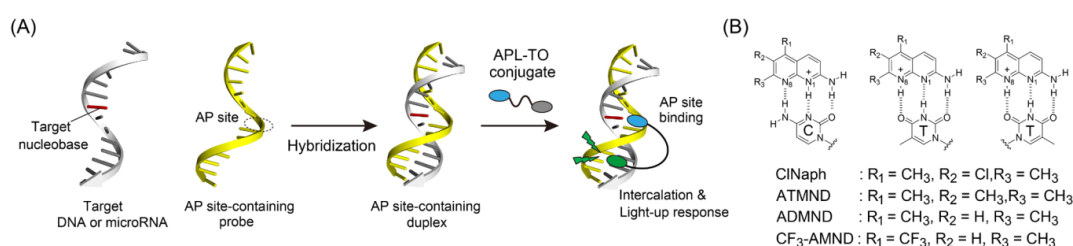


Figure 1. (A) Schematic illustration of the analysis of target DNA or microRNA sequences based on the binding of abasic (AP) site-binding ligands conjugated with thiazole orange conjugates (APL–TO) in combination with an AP site-containing probe for hybridization; (B) Chemical structures of chloro-substituted 2-amino-5,7-dimethyl-1,8-naphthyridine (ClNaph) and its related derivatives. Proposed binding of these ligands with cytosine and thymine (two possible patterns) are also shown.

This work describes the enhanced binding selectivity of a 2-amino-1,8-naphthyridine derivative for orphan C by the incorporation of a chloro group. We previously found that the introduction of the electron-withdrawing trifluoromethyl group into 2-amino-7-methyl-1,8-naphthyridine (AMND) led to remarkably enhanced C/T selectivity due to more favorable protonation at N1 compared to N8 [24]. However, the obtained CF₃-AMND (Figure 1B) showed much weaker affinity compared to ATMND. Herein, 2-amino-5,7-dimethyl-1,8-naphthyridine (ADMND), an AMND derivative with an additional methyl group, was explored as the scaffold for the introduction of an electron-withdrawing group considering the fact that ADMND can bind more strongly to target C than AMND [3]. The resulting compound carrying the chloro group at position 6 in the naphthyridine ring (ClNaph) was found to exhibit the strong binding affinity as well as the high selectivity for target C. The use of ClNaph as an APL unit in the conjugate with TO was shown to be useful for the design of a light-up probe toward target C over other nucleobases in the AP–DNA duplexes and AP–DNA/RNA hybrids with a view toward the detection of C-related mutations in DNA and microRNA sequences.

2. Materials and Methods

2.1. Materials

All of the DNAs and RNAs were purchased from Nihon Gene Research Laboratories, Inc. (Sendai, Japan) and Sigma-Genosys (Hokkaido, Japan), respectively. The other reagents were commercially available and used without further purification. The concentration of DNAs and RNAs were determined from the molar extinction coefficient at 260 nm (ϵ_{260}) according to the literature [25]. Water was deionized (≥ 18.0 M Ω cm specific resistance) by an Elix 5 UV water purification system and a Milli-Q synthesis A10 system (Millipore Corp., Bedford, MA, USA). The other reagents were purchased from standard suppliers and used without further purification. ¹H NMR spectra were measured with a JEOL

ECA-600 spectrometer at 500 MHz. High-resolution ESI-MS spectra were measured with a Bruker APEX III mass spectrometer.

Unless otherwise mentioned, all measurements were performed in 10-mM sodium cacodylate buffer solutions (pH 7.0) containing 100-mM NaCl, 1.0-mM EDTA and ethanol (<2%). Before the measurements, target duplex-containing samples were annealed as follows: heated at 75 °C for 10 min and gradually cooled to 5 °C (3 °C/min), after which the solution temperature was raised again to 20 °C. The probe was then added to the samples.

2.2. Probe Synthesis

ClNaph: 2,6-diaminopyridine (3.17 g, 29.1 mmol) in phosphoric acid (50 mL) was added to 3-chloropentane-2,4-dione (3.99 g, 29.6 mmol) and the reaction mixture was stirred at 90 °C for 24 h. After neutralization with NaOH, the mixture was filtered. The brown residue was extracted with 300 mL of CHCl₃ three times and the organic phase was dried over Na₂SO₄. The solvent was evaporated *in vacuo*, affording ClNaph as a dark brown solid (3.57 g, 17.2 mmol, 59.2%). ¹H-NMR (CDCl₃): 8.03 (d, 1H, *J* = 8.4 Hz), 6.76 (d, 1H, *J* = 9.2 Hz), 2.75 (s, 3H), 2.64 (s, 3H). High resolution ESI-MS calcd ([M + H]⁺) 207.0563; found, 207.0569.

ClNaph-TO and ClNaph-TO2 conjugates: The conjugates were synthesized according to our previous report [19]. Briefly, aminoethyl group was incorporated into ClNaph and the resulting derivative was attached with the carboxylate-terminated decanyl (C10) spacer-containing TO derivative [26,27]. ClNaph-TO conjugate, ¹H-NMR (DMSO-*d*₆) 8.80 (d, 1H, *J* = 8.8 Hz), 8.60 (d, 1H, *J* = 7.2 Hz), 8.13 (d, 1H, *J* = 8.8 Hz), 8.05 (d, 1H, *J* = 7.6 Hz), 8.00 (t, 1H, *J* = 7.6 Hz), 7.80 (d, 1H, *J* = 8.4 Hz), 7.76 (t, 1H, *J* = 8.0 Hz), 7.63 (t, 1H, *J* = 8.4 Hz), 7.41 (t, 1H, *J* = 8.0 Hz), 7.36 (d, 1H, *J* = 7.6 Hz), 6.93 (s, 1H), 6.78 (d, 1H, *J* = 9.2 Hz), 4.58 (t, 2H, *J* = 7.2 Hz), 3.44–3.47 (q, 4H), 2.58 (s, 3H), 2.54 (s, 3H), 2.05 (t, 2H, *J* = 7.2 Hz), 1.84 (t, 2H), 1.42 (t, 2H), 1.34–1.16 (q, 14H). High resolution ESI-MS calcd ([M + H]⁺) 707.3299; found, 707.3292. ClNaph-TO2 conjugate, ¹H-NMR (DMSO-*d*₆) 8.72 (d, 1H, *J* = 8.8 Hz), 8.61 (d, 1H, *J* = 6.8 Hz), 8.07–8.01 (m, 4H), 7.78–7.74 (m, 2H), 7.62–7.58 (t, 1H, *J* = 7.6 Hz), 7.43–7.37 (m, 2H), 6.92 (s, 1H), 6.77 (d, 2H, *J* = 4.0 Hz), 4.61 (t, 2H), 4.17 (s, 3H), 3.50–3.42 (m, 4H), 2.58 (s, 3H), 2.55 (s, 3H), 2.00 (t, 2H, *J* = 7.2 Hz), 1.59–1.12 (m, 18H). High resolution ESI-MS calcd ([M + H]⁺) 707.3299; found, 707.3286.

2.3. Fluorescent Measurements

Fluorescence spectra were measured with a JASCO model FP-6500 spectrofluorophotometer equipped with a thermoelectrically temperature-controlled cell holder (Japan Spectroscopic Co., Ltd., Tokyo, Japan) using a 3 × 3 mm quartz cell.

The dissociation constant (*K*_d) of the probe was determined at 20 °C by fluorescent titration experiments. The changes in fluorescence intensity were analyzed by nonlinear least-squares regression based on a 1:1 binding isotherm [19]. Errors in the *K*_d values are the standard deviations obtained from three independent experiments (*N* = 3).

3. Results and Discussion

First, we examined the binding ability of ClNaph (500 nM) to target orphan nucleobases in model 21-meric AP-DNA duplexes (500 nM; 5'-d(GCA GCT CCC AXA GTC TCC TCG)-3'/3'-d(CGT CGA GGG TNT CAG AGG AGC)-5', X = AP site (Spacer C3, a propyl residue), N = target nucleobase; G, C, A or T) by the fluorescence measurements. As shown in Figure 2, ClNaph showed the emission with the maximum at 393 nm in the absence of DNAs. The addition of AP-DNA duplexes caused a decrease in the fluorescence intensity of ClNaph. The largest fluorescence quenching response was observed for target C, which indicates that ClNaph shows the preferential binding to target C compared to target G, A and T. The binding affinity of ClNaph was assessed by the fluorescence titration experiments (Inset of Figure 2). ClNaph showed the quenching response for target DNA duplexes in a concentration-dependent manner and the resulting titration curves were analyzed by a

1:1 binding model for the determination of the dissociation constants (K_d). The K_d value for C was obtained as 70 ± 5.3 nM. Significantly, this affinity is much stronger compared to the parent ADMND and the previously developed CF₃-AMND and is almost comparable to ATMND (Table 1). In addition, ClNaph showed useful binding selectivity toward target C, in which the K_d value for target C was two orders of magnitude smaller than those for other target nucleobases (K_d /nM: T, 2020 ± 240 , G and A > 5000). The observed selectivity for C over T is much superior to ADMND and ATMND [3] whereas it is slightly moderate in comparison with CF₃-AMND [24]. We reason that this is due to the more favorable protonation of ClNaph at the N1 position for the complementary base-pairing with orphan C compared to the N8 protonation (Figure 1B), where the electron-withdrawing effect of the chloro group would be essential as was observed for CF₃-AMND [24]. We note that the type of the AP site affects the binding of ClNaph. The use of Spacer C3 (propyl residue) allowed the stronger binding to target C compared to the tetrahydrofuranyl residue (dSpacer), presumably due to less steric hindrance (Figure S3). The observed strong and highly selective binding properties for target C should render ClNaph a useful APL unit in the conjugate with TO unit.

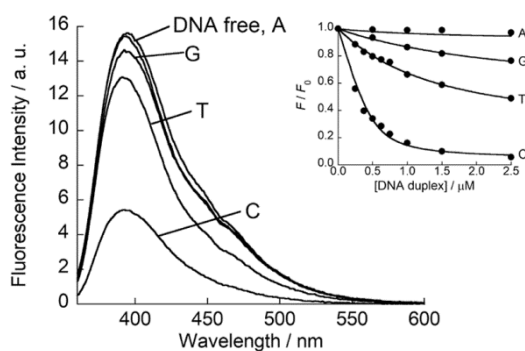


Figure 2. Fluorescence response of ClNaph (500 nM) to target AP-DNA duplexes (500 nM). Inset: Titration curves for the binding of ClNaph (500 nM) to target nucleobases. Measurements were done in solutions buffered to pH 7.0 (10-mM sodium cacodylate) containing 100-mM NaCl and 1.0-mM EDTA. F and F_0 denote the fluorescence intensities of ClNaph in the presence and absence of DNA duplexes. Excitation, 350 nm. Analysis, 393 nm. Temperature, 20 °C.

Table 1. Dissociation constants (K_d) of ClNaph and its related derivatives for target C in 21-meric AP-DNA duplexes ^a.

	K_d /nM
ClNaph	70 ± 5.3
ADMND ^b	164
ATMND ^b	53
CF ₃ -AMND ^c	1400

^a K_d values measured in solutions buffered to pH 7.0 (10-mM sodium cacodylate) containing 100-mM NaCl and 1.0-mM EDTA. Temperature, 20 °C. ^b Values taken from [3]. ^c Value taken from [24].

ClNaph was coupled with the quinolone ring of the TO unit through a long alkyl (C10)-linker according to our previous report [19], which affords the ClNaph-TO conjugate (Figure 3A). We measured the fluorescence response of the TO unit of the conjugate (500 nM) for the same AP-DNA duplexes (500 nM) as those used for the examination of ClNaph (cf. Figure 2). As shown in Figure 3A, TO unit shows negligible emission in the absence of DNAs due to the free rotation of the benzothiazole and quinolone rings [21]. In contrast, we observed a remarkable light-up response of the TO unit for target C-containing AP-DNA duplex, in which the fluorescence intensity at 526 nm increased by 207-fold. This can be explained by the restriction of the rotation of the TO unit by intercalation into the duplex region (cf. Figure 1A) [19]. In addition, the fluorescence titration experiments revealed that the K_d value of ClNaph-TO conjugate for target C reached 6.6 ± 0.3 -nM (Figure S4). This affinity is

one order of magnitude stronger than ClNaph itself (cf. Table 1). Apparently, the conjugation with the TO unit led to the enhanced binding affinity for target C in the AP–DNA duplexes. It should be noted that ClNaph–TO conjugate has high binding selectivity for target C over other three nucleobases (Figure S4: K_d values for T, G and A > 1500 nM). Considering that TO lacks the selectivity for the orphan nucleobases [19], the ClNaph unit is responsible for the observed C-selectivity of the conjugate. This was confirmed by the fluorescence quenching response of the ClNaph unit with high selectivity to target C (Figure S5). These results showed the useful binding and light-up functions of the ClNaph–TO conjugate for the detection of C-related mutations in target DNA sequences. It is also noteworthy that the strong emission of ClNaph–TO conjugate for target C can be seen even with the naked eyes under UV light irradiation (Figure 3B). Fluorescence response for target C is clearly distinguishable from those for other target nucleobases, which thus facilitates a simple and rapid analysis of target DNA sequences.

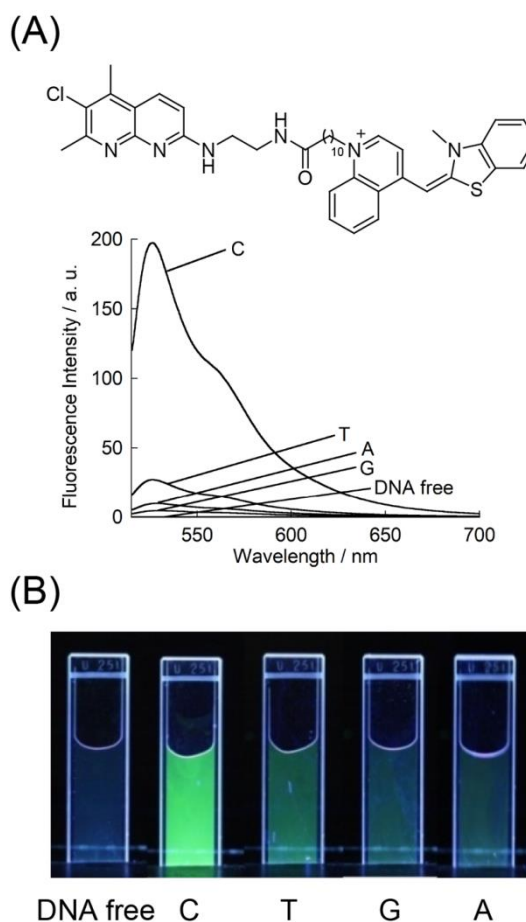


Figure 3. (A) Fluorescence response of ClNaph–TO conjugate (500 nM) to target AP–DNA duplexes (500 nM). Excitation, 504 nm. Temperature, 20 °C. (B) Images of fluorescence emission of ClNaph–TO conjugate (500 nM) in the absence and presence of target AP–DNA duplexes (500 nM) under excitation light with the wavelength of 365 nm, obtained by a digital camera at room temperature. Other solution conditions are the same as those given in Figure 2.

We found that the kind of heterocycles of the TO unit, benzothiazole or quinolone rings, that connect to ClNaph unit in the conjugate affected the binding and fluorescence signaling abilities for the binding to AP–DNA duplexes. When the C10-linker was appended to the benzothiazole ring, the resulting conjugate (ClNaph–TO2) showed the selective light-up response for target C in the AP–DNA duplexes (Figure S6); however, the degree of the response was smaller compared to ClNaph–TO (cf. Figure 3A). This is attributable to the reduced binding affinity of ClNaph–TO2 conjugate to target C

($K_d = 71$ nM). This indicates that the conjugation of ClNaph unit into the benzothiazole ring hinders the effective intercalation of the TO unit for DNA duplexes as reported in the literature [27]. In addition, we observed moderate selectivity of ClNaph–TO2 conjugate for target C over other three nucleobases in AP–DNA duplexes (Figure S6) while the reason for this is unclear yet. Hence, the connection of ClNaph unit to the quinolone ring of the TO unit is effective for the strong binding and large light-up response of the conjugate for target C in the AP–DNA duplexes.

As described above, the ClNaph–TO conjugate can serve as a useful light-up probe for target C in the AP–DNA duplexes. Meanwhile, we noticed weak binding of the conjugate for AP–RNA duplexes (Figure 4A and Figure S7). The binding affinity was estimated as > 1300 -nM for target C in the AP–RNA duplex (5′-r(GCA GCU CCC AXA GUC UCC UCG)-3′/3′-r(CGU CGA GGG UCU CAG AGG AGC)-5′, X = AP site (Spacer C3), C = target nucleobase), which was three orders of magnitude larger than that for the AP–DNA duplex (cf. Figure S4). We consider that this arises from the preferential binding of the ClNaph–TO conjugate to B-formed AP–DNA duplexes relative to A-formed AP–RNA duplexes [6]. However, when targeting C in single stranded RNA, the use of AP–DNA probe is highly effective. The resulting duplex is the hybrid between RNA and AP–DNA ((5′-d(GCA GCT CCC AXA GTC TCC TCG)-3′/3′-r(CGU CGA GGG UCU CAG AGG AGC)-5′, X = AP site (Spacer C3), C = target nucleobase) that can adopt A-form/B-form intermediate structure [28], under the same measurement conditions used to examine AP–RNA duplexes. As shown in Figure 4B, we observed the significant light-up response of the conjugate for the AP–DNA/RNA hybrid, in which the response was 22-fold larger compared to that for the AP–RNA duplex. The K_d for target C in the hybrid was obtained as 22 nM (Figure S7), where the affinity is much superior to that for AP–RNA duplexes. Importantly, the ClNaph–TO conjugate retains its selectivity for target C over U opposite the AP site in the DNA/RNA hybrid, as was observed in AP–DNA duplexes (cf. Figure 3B). These results suggest the potential use of ClNaph–TO conjugate for the detection of microRNAs in combination with AP–DNA hybridization probe (cf. Figure 1A).

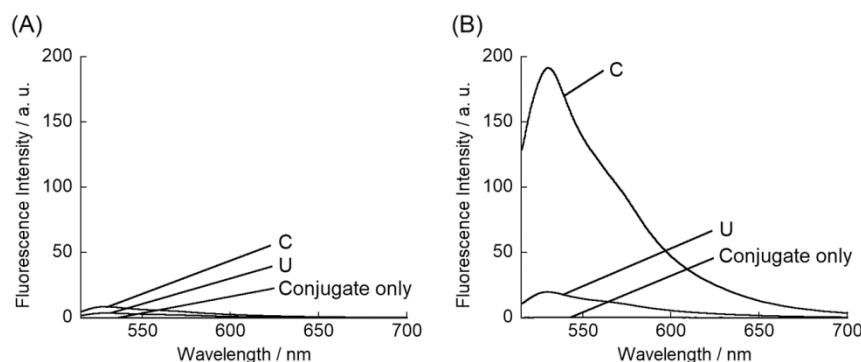


Figure 4. Fluorescence response of ClNaph–TO conjugate (500 nM) for target C or U in AP site-containing duplexes (500 nM): (A), AP–RNA duplex and (B) AP–DNA/RNA hybrid. Other solution conditions are the same as those given in Figure 2. Excitation, 504 nm. Temperature, 20 °C.

We performed the preliminary experiments for detection of microRNAs based on the binding-induced light-up response of ClNaph–TO conjugate for AP–DNA/RNA hybrids. A 21-meric AP–DNA probe was designed for the selective detection of the let-7d sequence among let-7 family [29], as shown in Figure 5A. Hybridization between this probe and let-7d allows the construction of the AP–DNA/RNA hybrid containing an orphan C opposite an AP site. Meanwhile, the hybridization with other let-7 sequences leads to the formation of orphan U-containing hybrids with several mismatch base pairs (Table S1). We observed the significant light-up response of ClNaph–TO for the let-7d-containing hybrid (Figure 5A). This response is much larger than those for other let-7 sequences, which clearly shows that ClNaph–TO conjugate enables the selective detection probe for let-7d over other let-7 members. The binding affinity of the conjugate for the let-7d-containing hybrid ($K_d = 23$ nM) was

found to be comparable to that for the model AP–DNA/RNA hybrid (cf. Figure S8). Our assay can be applied to the analysis of various microRNAs by using the AP site-containing DNA probe whose sequence is designed so as to be complementary to the target sequence. Figure 5B shows the fluorescence response of the conjugate in combination with the 22-meric AP–DNA probe for let-7i. Selective light-up detection for let-7i was achieved due to the selective binding of the conjugate to target C in the AP–DNA/RNA hybrid formed between the AP–DNA probe and let-7i. These results show the applicability of the CINaph–TO conjugate for the selective detection of target microRNA sequences based on the hybridization for the construction of the AP–DNA/RNA hybrid as well as the binding-induced light-up response of the conjugate for the orphan C in the resulting hybrid.

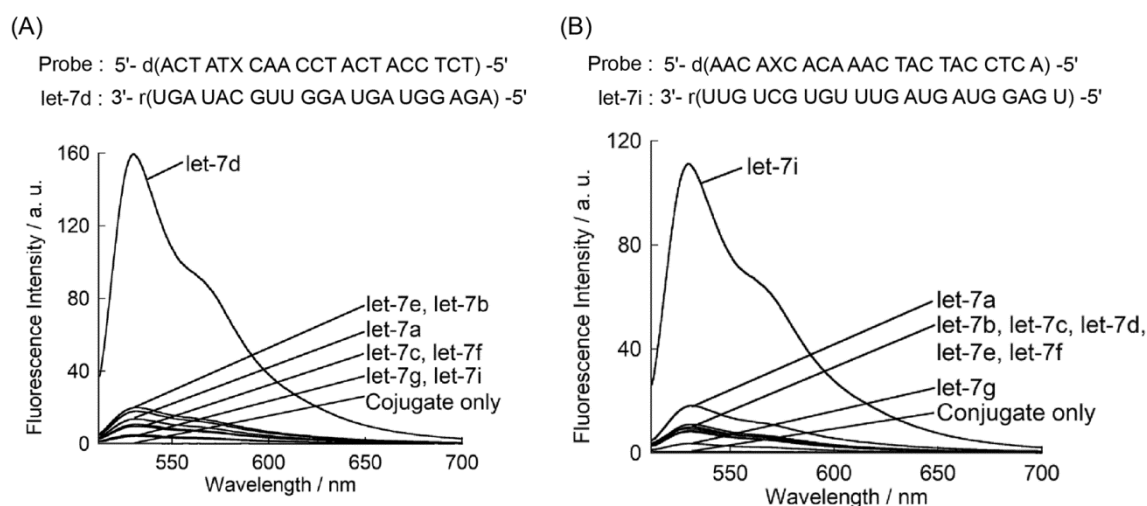


Figure 5. Selective detection of (A) let-7d and (B) let-7i sequences by CINaph–TO conjugate in combination with AP–DNA probe (X = AP site (Spacer C3)). Sequences of other let-7 members are shown in Table S1. [CINaph–TO], [target microRNA], [AP–DNA probe] = 500 nM. Other solution conditions for the fluorescence response of the conjugate are the same as those given in Figure 2. Excitation, 504 nm. Temperature: 20 °C.

4. Conclusions

In summary, we report that CINaph served as a strong and highly selective binder for the orphan C opposite an AP site in DNA duplexes. In addition, we demonstrated the usefulness of CINaph as the APL unit in the conjugate with a TO unit for the design of light-up probes for the detection of C-related mutations in DNA and microRNA sequences. These results obtained could provide insights needed to design this class of light-up conjugates suitable for the analysis of DNA and microRNA sequences. As shown in our previous works [19,20], the spacer length and structure have a large impact on the binding and fluorescence sensing abilities of APL–TO conjugates. We will examine these concerns for further improvement of the APL–TO conjugates in order to develop the practical assays.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3417/10/12/4133/s1>, Figure S1: ¹H NMR spectra of the conjugates; Figure S2: ESI-MS spectra of the conjugates; Figure S3: Fluorescence response of CINaph to target dSpacer-containing AP–DNA duplexes. Inset: Titration curves for the binding of CINaph to target nucleobases; Figure S4: Titration curves for the binding of CINaph–TO conjugate to target nucleobases; Figure S5: Fluorescence response of CINaph unit in the conjugate to target AP–DNA duplexes; Figure S6: Chemical structure of CINaph–TO2 conjugate and its fluorescence response to target AP–DNA duplexes; Figure S7: Titration curves of the binding of CINaph–TO conjugate to target C in the AP–DNA/RNA hybrid and AP–RNA duplex; Table S1: Sequences of let-7 members used in this study. Figure S8. Titration curves for the binding of CINaph–TO conjugate to the let-7d/DNA probe hybrid.

Author Contributions: C.-x.W. and Y.S. conceived and designed the experiments. C.-x.W. and T.S. performed the experiments. C.-x.W. and Y.S. wrote the study. N.T. and S.N. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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