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A new ligand binding to G–G mismatch having improved thermal and alkaline stability

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Abstract—Naphthyridine dimer (**ND**) specially binds to guanine–guanine (G–G) mismatch in duplex DNA. In order to improve the thermal and alkaline stability and binding ability of the ligand, we have examined structural modification of the linker. A new ligand (**NNC**) possessing 2-amino-1,8-naphthyridines and a carbamate linker is much more thermally stable than **ND**. The half-life of **NNC** is 2.5 times longer than that of **ND** at 80 °C. **NNC** is also much more stable than **ND** under alkaline conditions. In addition, **NNC** binds to G–G mismatch more strongly than **ND**. The improved stability and the binding of **NNC** to the G–G mismatch would be suitable for the practical use of **NNC**-immobilized sensor.

Since a draft sequence of the human genome was determined,[1,2] some 1.6 million human single nucleotide polymorphisms (SNPs) have been found in the human genome and deposited to public databases.[3] SNPs became extremely important as a genetic marker for the identification of disease genes and detection of genetic mutations.[4,5] Thus, simple and rapid detection of a single nucleotide difference in the DNA sequences is an indispensable technique for both SNP mapping and typing. Although a number of methods have been developed for SNPs typing,[4,6,7] there is still a great need for designing new typing methods that are simple in operation, rapid and accurate in analysis, and low in cost.

We have recently reported a novel approach for the detection of SNPs by sensing guanine–guanine (G–G) mismatches in duplex DNA.[8] We have developed a sensor chip that can detect G–G mismatches in duplex DNA by means of surface plasmon resonance (SPR).[9,10] The sensor was prepared by immobilizing mismatch binding ligand naphthyridine dimer (**ND**) onto the carboxylated dextran matrix on the gold surface. We have reported that **ND** binds selectively to G–G mismatches in duplex DNA.[8,11] During the regeneration process of the **ND**-immobilized surface under alkaline conditions after each mismatch analysis, it was observed that the immobilized **ND** was slowly degraded under the conditions. We also found that high temperature necessary for denaturing the bound duplex on **ND**-immobilized sensor induced the **ND** degradation. Improving the thermal and alkaline stability of the mismatch-binding ligand eventually leads to a prolonged sensor lifetime. We report here a novel G–G mismatch binding ligand (**NNC**) that has not only greatly improved thermal and alkaline stability but also the higher affinity and selectivity to the G–G mismatch compared to **ND** (Fig. 1).

To gain insights into the degradation pathway, we first examined the thermal reaction of **ND** at 80 °C in 100 mM sodium cacodylate (pH 7.0) by HPLC (Fig. 2).

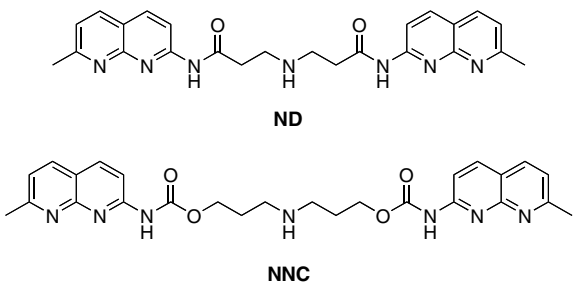

Figure 1. **ND** and **NNC**.

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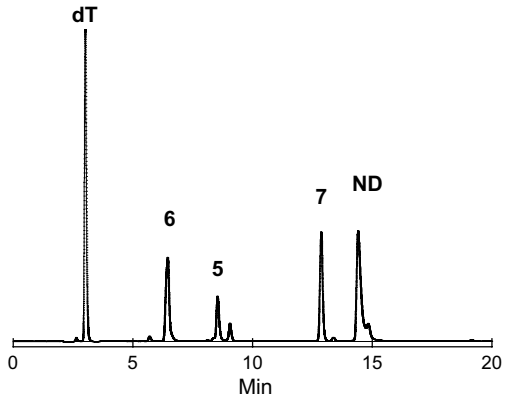


Figure 2. HPLC profile for the thermolysis of **ND** (0.71 mM) in 100 mM sodium cacodylate buffer (pH 7.0) for 45 min at 80 °C. dT was added as an internal standard.

The dT was selected as an internal standard for the thermolysis so that the reproducible and quantitative data could be obtained from the chromatographs. We detected three major products, which were identified as 2-amino-7-methyl-1,8-naphthyridine (**5**), 3-amino-*N*-(7-methyl-1,8-naphthyridin-2-yl)-propionamide (**6**), and *N*-(7-methyl-1,8-naphthyridin-2-yl)-acrylamide (**7**). The formation of **5** suggested the hydrolysis of the amide linkage, where as β-elimination was another degradation pathway producing **6** and **7** (Fig. 3).

To suppress both degradation processes and retain the binding ability to the G–G mismatch, a new molecule **NNC**, where amide linkage was substituted by a carbamate linkage, was synthesized. In addition, the alkyl chain length was further extended by one carbon for each side to slow down the nucleophilic addition of the secondary amino group in the linker to the carbonyl group leading to a release of **5**.

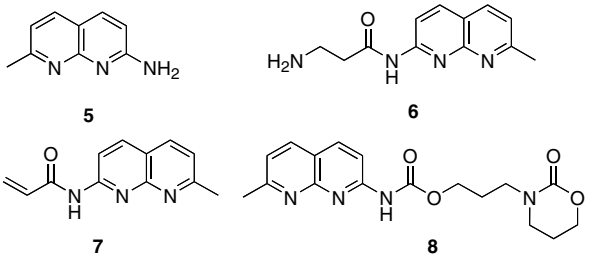


Figure 3. The products derived from thermolysis of **ND** and **NNC**.

NNC was synthesized as shown in Scheme 1. *N*-Boc-dipropanolamine was reacted with *N*,*N*′-disuccinimidyl carbonate (DSC) in dry acetonitrile to produce carbonate,[12] which was then reacted with 2-amino-7-methyl-1,8-naphthyridine to afford Boc-protected **NNC**. Deprotection by hydrogen chloride in ethyl acetate gave hydrochloride salt of **NNC**.[13]

The thermal reaction of **NNC** was examined under the same condition as that of **ND** (Fig. 4).

The major products of the **NNC** degradation after incubating at 80 °C for 120 min were identified as **5** and (7-methyl-1,8-naphthyridin-2-yl)-carbamic acid 3-(2-oxo-1,3-oxazinan-3-yl)-propyl ester (**8**) (Fig. 3). After a periodic incubation, the amount of **ND** and **NNC** were analyzed by HPLC. The rate of thermolysis could be determined from the decrease of the ligands. The half-life curves for the thermolysis of **NNC** and **ND** were shown in Figure 5. It is clearly shown that the half-life of **ND** is about 40 min at 80 °C, whereas the half-life of

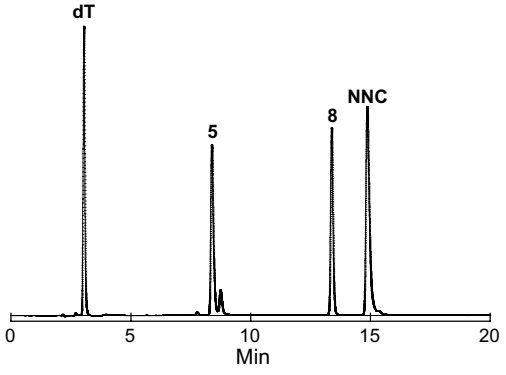
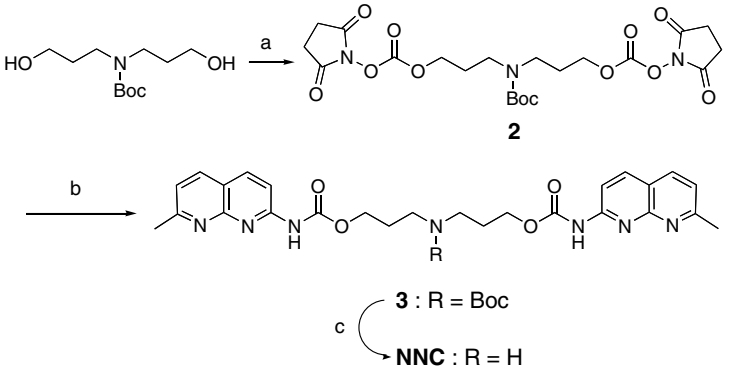


Figure 4. HPLC profile for the thermolysis of **NNC** (0.71 mM) in 100 mM sodium cacodylate buffer (pH 7.0) for 120 min at 80 °C.



Scheme 1. Reagents and conditions: (a) *N*,*N*′-disuccinimidyl carbonate, CH_3CN, Et_3N; (b) 2-amino-7-methyl-1,8-naphthyridine, CH_2Cl_2, Et_3N, 49% for two steps; (c) HCl, AcOEt, $CHCl_3$, quantitative.

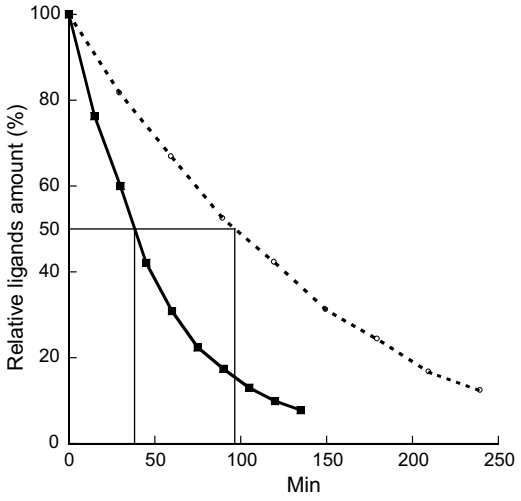


Figure 5. The half-life curves of **ND** (—) and **NNC** (···) in 100 mM sodium cacodylate buffer (pH 7.0) at 80 °C. *Y*-Axis represents the relative amount of ligands remained after incubation.

NNC is about 100 min. These results showed that the ligand **NNC** is much more thermally stable than **ND**.

The stabilities of the ligands in alkaline conditions were also examined incubating at room temperature in 50 mM sodium hydroxide (Fig. 6). Under the alkaline conditions, only 29% of **ND** remained after 2 h incubation, whereas 86% of **NNC** still remained under the same conditions. After incubation for 4 h, the amount of **ND** and **NNC** remaining was 7% and 79%, respectively. It is very clear that **NNC** is much more stable than **ND** in an alkaline solution.

Having confirmed the improved stability of **NNC** under the thermal and alkaline conditions, we then looked at

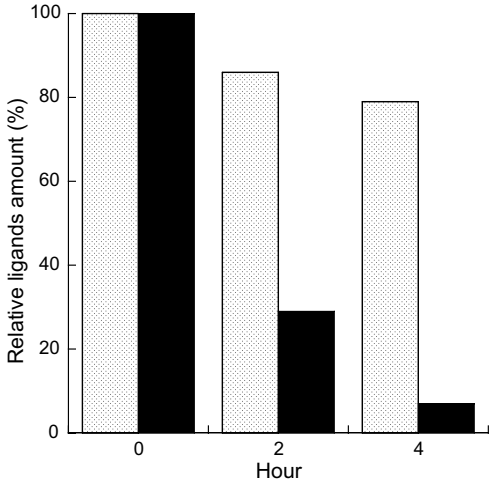


Figure 6. The amount of **ND** (solid bar) and **NNC** (shaded bar) (100 μM) remaining after incubation in 50 mM sodium hydroxide and 100 mM sodium chloride at room temperature. The amount was obtained by HPLC relative to the dT added as an internal standard. *Y*-Axis represents the relative amount of ligands remaining after incubation.

Table 1. ΔT_m of mismatch-containing duplexes in the presence ligands[a]

X*–*Y	T_m	ΔT_m[b]	
		NNC	**ND**
A–A	17.8 (1.4)	1.5 (0.2)	−0.8 (1.1)
A–C	16.1 (0.8)	4.1 (0.2)	2.1 (0.2)
C–C	18.2 (0.2)	6.1 (0.3)	6.7 (0.6)
G–A	25.7 (0.2)	7.0 (0.2)	8.6 (1.3)
G–G	25.6 (1.3)	29.1 (0.2)	23.7 (1.2)
G–T	28.3 (0.2)	1.2 (0.8)	10.2 (1.3)
T–C	18.6 (0.2)	3.7 (0.3)	5.5 (0.7)
T–T	25.1 (0.2)	0.7 (0.7)	0.6 (1.2)
A–T	34.3 (0.3)	0.0 (0.3)	−1.5 (0.3)
G–C	40.3 (0.0)	−2.0 (0.4)	2.0 (0.9)

[a] The UV-melting curve was measured for a duplex of d(CTA AC***X*** GAA TG)/d(CAT TC***Y*** GTT AG) at a total base concentration of 100 μM in a 10 mM sodium cacodylate buffer (pH 7.0) containing 0.1 M NaCl. A mismatch (***X*–*Y***) is produced in the middle of the duplex. Temperature was increased at a rate of 1 °C/min. All measurements were taken three times, and standard deviations are shown in the parentheses.

[b] ΔT_m is calculated as a difference of T_m in the presence and absence of drugs (100 μM), respectively.

the selective binding of **NNC** to the G–G mismatch. The assay was carried out by measuring the melting temperature (T_m) of 11-mer duplexes d(CTA AC***X*** GAA TG)/d(CAT TC***Y*** GTT AG) (where ***X***, ***Y*** = A, G, T, or C) containing mismatches in the absence and presence of **NNC** (Table 1). T_m increase (ΔT_m) of the duplex containing a G–G mismatch was 23.7 °C in the presence of **ND** (100 μM). Under identical conditions, ΔT_m of 29.1 °C was recorded in the presence of **NNC**. The difference of ΔT_m ($\Delta\Delta T_m$) between **NNC** and **ND** for the G–G mismatch was 5.4 °C, suggesting that the modification of the linker structure of **ND** to that of **NNC** has a positive effect for the thermodynamic stabilization of the G–G mismatch by **NNC**. This is most likely due to an expanded π-surface in a carbamate linkage and a release of the linker strain involved in the bound DNA–**ND** complex. The affinity of **NNC** to the G–G mismatch was calculated by the curve fitting of the UV-melting curve obtained in the absence and presence of **NNC** to the theoretical equation.[14] The K_a obtained for the assumed 1:1 binding between **NNC** to the G–G mismatch was $>10^7\,M^{-1}$, that is larger than the K_a we reported for the **ND** binding to the G–G mismatch.

All of the data presented here showed that the ligand **NNC** is a better ligand than **ND** in terms of the affinity and selectivity to the G–G mismatch, and the thermal and alkaline stability. The use of **NNC** for the SPR sensor would enhance the sensitivity to the G–G mismatch by eliminating the unnecessary binding to DNA containing other mismatches. Furthermore, with a higher thermal and alkaline stability, **NNC** can be applied more expansively than **ND**.

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13. The data of the hydrochloride salt of **NNC**: ^{1}H NMR (CD_3OD, 400 MHz): $\delta = 8.15$ (m, 6H), 7.35 (d, 2H, $J = 8.4$ Hz), 4.34 (t, 4H, $J = 6.0$ Hz), 3.15 (t, 4H, $J = 7.2$ Hz), 2.71 (s, 6H), 2.11 (br s, 4H). ^{13}C NMR (CD_3OD, 400 MHz): $\delta = 163.0$, 154.5, 154.3, 154.0, 139.1, 137.6, 121.4, 118.2, 113.1, 62.7, 45.4, 26.5, 23.8. HR-FABMS calcd for $C_{26}H_{30}N_7O_6$ [$(M+H)^+$], 504.2359. Found: 504.2361.
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