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COMMUNICATION

Selective alkylation of parallel G-quadruplex structure

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The selective alkylation of nucleic acids is important for a medicinal approach and biological study. We now report a novel selective alkylation of the parallel G-quadruplex structure using the conjugate of the macrocyclic hexaoxazole L2G2-6OTD-1M1PA and vinyl-quinazolinone-S(O)Me (6OTD-VQ-S(O)Me).

The G-quadruplex (G-4) is a higher-ordered structure of a nucleic acid that consists of the G-quartet, a four guanine bases plane formed in the presence of cation species (*e.g.*, potassium). The potential G4-forming sites have been shown to exist in the critical regions of DNA and RNA such as the telomere, promoter regions of the oncogenes, mRNA, and non-cording RNA.[1] Since the G-4 motifs are thought to be involved in the regulation of diverse biological processes, various types of G-4 targeting ligands have been developed for antitumor therapies and G-4 identification probes.[1-5]

The selective alkylation of nucleic acids is one of the most promising ways to strongly inhibit the target biological process. Chlorambucil derivatives have often been used as an alkylator for inhibition studies of the higher-ordered structures of nucleic acids.[6-8] While chlorambucil derivatives have a good reactivity, their selectivity and stability are not yet sufficient for a medicinal approach. For further improvement of the alkylation properties, many alkylating ligands have been developed.[4, 9-17]

In our previous study, we developed acridine-vinyl-quinazolinone(VQ)-S(O)Me as the G-4 reactive OFF-ON type alkylating agent (Fig. 1A).[18] The sulfoxide group was generated from the oxidation of VQ-SMe by MMPP and this spontaneous elimination provided the highly reactive VQ species (Fig. 1B). VQ reacted at the *N*3 position of the T or U base of DNA or RNA, respectively. The alkylation of acridine-VQ-S(O)Me showed a high selectivity to the higher-ordered structure such as G-4. However, since acridine can generally bind to the double strand DNA (dsDNA) in addition to G-4, a more selective binder is required for the selective alkylation in the presence of dsDNA. In this study, we utilized the macrocyclic hexaoxazole, L2G2-6OTD-1M1PA (6OTD), as the selective G-4 binder (Fig. 1A).[19, 20]

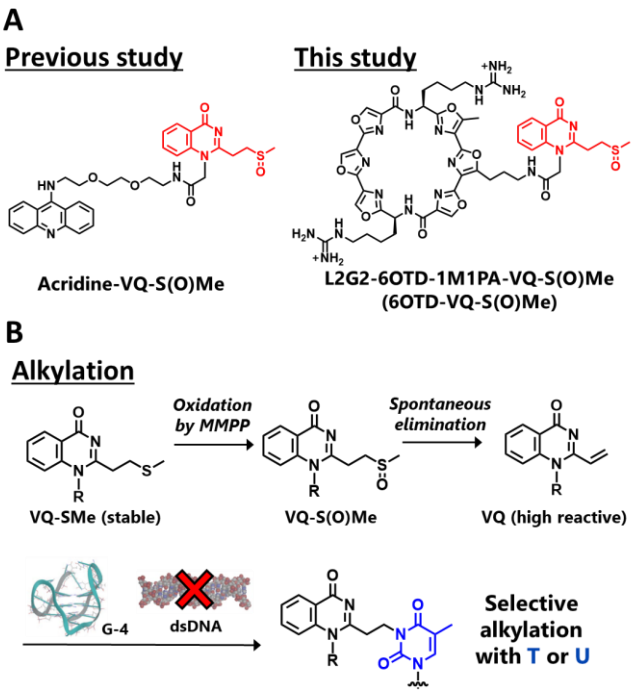

Figure 1. (A) The structure of the VQ-S(O)Me ligands. (B) Alkylation process of VQ.

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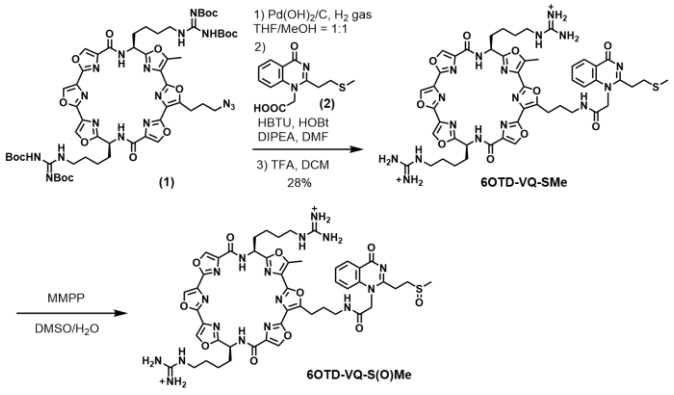
Scheme 1. Synthesis of 6OTD-VQ-S(O)Me.



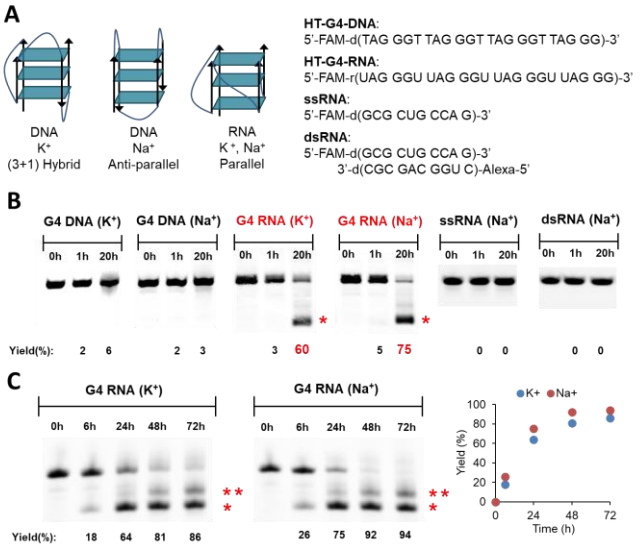


Figure 2. Alkylation using 6OTD-VQ-S(O)Me. (A) G-4 topologies and sequences used for alkylation screening. (B) PAGE analysis. The alkylation was carried out using 6OTD-VQ-S(O)Me (0.50 µM), target DNA or RNA (0.25 µM) in phosphate buffer (pH 7.0) at 37 °C. K^+ and Na^+ denote K^+ and Na^+ phosphate buffers, respectively. The asterisk indicates the alkylated product. (C) Time course of G4-RNA alkylation.

6OTD selectively stabilizes G-4 via a π-π interaction with the G-quartet even in the presence of an excess of dsDNA due to the high planarity of the core structure of the macrocyclic moiety in 6OTD. We designed the 6OTD and VQ-S(O)Me conjugate molecule, 6OTD-VQ-S(O)Me, and expected a highly G-4 selective alkylation performance in the presence of dsDNA.

6OTD-VQ-SMe was synthesized from compounds (1)[19] and (2)[18] which we previously reported (Scheme. 1). 6OTD-VQ-SMe was oxidized by MMPP and the produced 6OTD-VQ-S(O)Me was used for the alkylation without further purification. The target screening of the alkylation was performed using 6OTD-VQ-S(O)Me and the human telomere (HT) G-4 DNA or RNA (Fig. 2). HT-G4-DNA forms different topologies in the K^+ and Na^+ buffers, *i.e.*, the (3+1) hybrid and anti-parallel structure, respectively (Fig. 2A). HT-G4-RNA forms a parallel structure in both the K^+ and Na^+

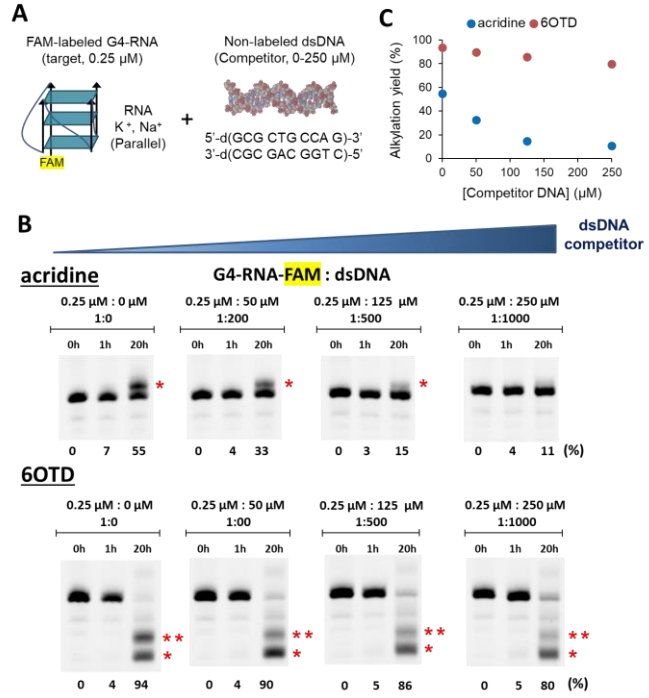


Figure 3. Competitive alkylation assay using acridine- or 6OTD-VQ-S(O)Me. (A) FAM-labelled HT-G4-RNA and non-labelled dsDNA were used for the assay. (B) PAGE analysis. The alkylation was carried out using acridine- or 6OTD-VQ-S(O)Me (1.25 µM), HT-G4-RNA (0.25 µM) and dsDNA (0-250 µM) in phosphate buffer (pH 7.0). (C) The alkylation yield changes in the presence of the increasing concentration of the non-labelled dsDNA (0-250 µM).

buffers. The reaction was analysed by denaturing polyacrylamide gel electrophoresis (PAGE). Unexpectedly, the reaction efficiently proceeded to only G-4 RNA (Fig. 2B). The clear band with a faster mobility was observed when G-4 RNA was the target, on the other hand, clear product bands were not observed when G-4 DNA was used as the target. No reaction occurred with the single stranded (ss) and double stranded (ds) RNAs, ss and dsDNA, dsDNA with dangling T and ssDNA with 23 mer T (Figs. 2B and S1). The time course of the alkylation to G-4 RNA was confirmed by 72 h (Fig. 2C). After 24 h, first product band (*) was clearly observed in a good yield. After 48 and 72 h, the second product band (**) located between the starting target G-4 RNA and first adduct bands gradually appeared (Fig. 2C). The two products extracted from the gel bands were analysed by MALDI-TOF MS measurements (Fig. S2). The first and second products corresponded to the one and two adducts, respectively. We rationalize that the stabilization of the G-4 structure by alkylation produced a faster mobility of the adducts compared to the non-alkylated G-4 DNA and RNA because the highly-stabilized G-4 was not denatured on a gel.

To confirm the generality of the parallel structure alkylation, we performed alkylation on other parallel G-4 sequences (Fig. S3). The NRAS G-4 RNA showed some alkylation occurrences although the reaction was slower than that of HT-RNA. Presumably, the U base number and position are

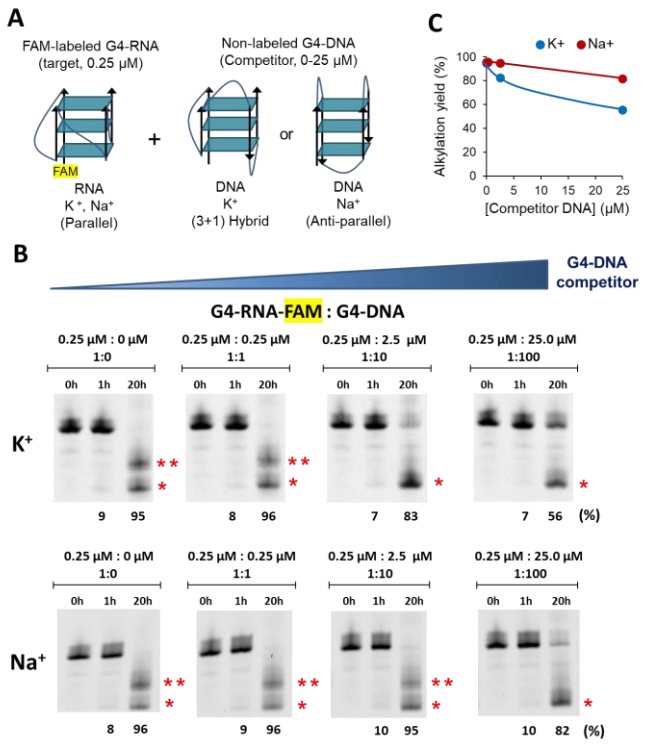


Figure 4. Competitive alkylation assay using 6OTD-VQ-S(O)Me. (A) G-4 topologies used for the assay. FAM-labelled HT-G4-RNA and non-labelled HT-G4-DNA were used. (B) PAGE analysis. The alkylation was carried out using 6OTD-VQ-S(O)Me (1.25 μM), HT-G4-RNA (0.25 μM) and DNA (0-25 μM) in phosphate buffer (pH 7.0). (C) The alkylation yield changes in the presence of the increasing concentrations of the non-labelled G4-DNA (0-25 μM).

important for an efficient alkylation. $(UGGU)_6$ with only two G-tetrad planes also underwent alkylation (Fig. S1). Besides the parallel G-4 RNA, we observed a moderate alkylation yield on a parallel G-4 DNA (*c-myc*) (Fig. S3). These data suggest that 6OTD-VQ-S(O)Me enables selective alkylation on the T or U-containing parallel G4 structure.

To confirm the alkylation of both acridine-VQ-S(O)Me and 6OTD-VQ-S(O)Me in the presence of dsDNA, we performed competitive alkylation assays by incubating 6OTD-VQ-S(O)Me (1.25 μM) with a constant amount of FAM-labelled HT-G4-RNA (0.25 μM) in the presence of increasing concentrations of the non-labelled dsDNA (0-250 μM) (Fig. 3A). When acridine-VQ-S(O)Me was used, the alkylation band appeared as a slower mobility band as we previously reported because the G-4 structure was denatured on a gel.[18] While the alkylation of acridine-VQ-S(O)Me dramatically decreased with the increasing dsDNA concentration, the change in 6OTD-VQ-S(O)Me was significantly small. The 80% alkylation was observed even when 1000 times the competitor dsDNA was used, indicating that the selective and strong 6OTD binder enables an efficient G-4 target alkylation even in the excess presence of other non-target structures.

With the aim to further confirm the recognition ability of 6OTD-VQ-S(O)Me toward G-4 RNA, we carried out a competitive alkylation assay using the FAM-labelled G-4 RNA and non-labelled G-4 DNA in the Na^+ or K^+ phosphate buffer (Fig. 4). After 1 and 20 h, the adducts with the labelled G-4 RNA were analysed and quantified by PAGE (Fig. 4B). The results depicted in the quantification graph showed that over 100 equivalents of the G-4 DNA competitor were required to decrease the yield to 50% under the K^+ buffer conditions (Fig. 4C). In the Na^+ buffer, 100 equivalents of the G-4 DNA competitor slightly competed with the adduct formation of G-4 RNA. When we used *c-myc* G-4 DNA as a competitor, the alkylation yields dramatically decreased (Fig. S4). These results indicated that 6OTD-VQ-S(O)Me selectively binds to the parallel structure, and the order of the binding preference is G-4s (parallel) > G-4 DNA (hybrid) > G-4 DNA (anti-parallel) >> dsDNA. Based on this selectivity order, we could argue that the loop position of the G4-topology would be the key factor for the binding selectivity, that is, the upper site loops of the G-4 structure would prevent the binding of 6OTD-VQ-S(O)Me.

To gain additional insight into the parallel selectivity, the CD spectra were measured using the different ligand concentrations (Fig. S5). As a ligand, the less reactive 6OTD-VQ-SMe was used. The alkylation yield of 6OTD-VQ-SMe was only 5% after 24 h (Fig. S6). Regarding HT-G4-RNA (K^+), significant changes in the CD spectra were not observed by the addition of the ligand, indicating that the ligand did not change the G-4 topology. In the Na^+ buffer, the positive Cotton effect at 267 nm increased and a slight blue shift was caused by the addition of the ligand, suggesting that the ligand would stabilize the parallel structure. Regarding HT-G4-DNA, significant changes in the CD spectra were observed by the addition of the ligand. The transition state was between 1.5 μM and 5.0 μM, suggesting that the apparent dissociation constant would be in this range. In the K^+ buffer, the positive Cotton effect decreased with the increasing ligand concentration. In the Na^+ buffer, the positive Cotton effect increased and a blue shift occurred. These CD results of G4-DNA suggest that big changes in the G4-topology were induced by the ligand binding.

In the alkylation assay, low concentrations of G-4 (0.25 μM) and the ligand (0.50 μM) were used. Since the CD results suggest that the ligand could not easily bind to G-4 DNA in this ligand concentration, the alkylation was carried out using higher concentrations of HT-G4-DNA (2.5 μM) and the ligand (12.5 μM) (Fig. S7). The alkylated band was observed after 24 h, suggesting that the parallel selectivity would mainly come from the low binding affinity of the ligand for the other G-4 topologies.

We also checked the G-4 stabilizing effect of the ligand by measuring the T_m values (Table. S1 and Fig. S8). For HT-G4-RNA, the 4 and 3 °C T_m value increases were observed by 2 equivalents of ligand addition in the K^+ and Na^+ buffers, respectively. For the HT-G4 DNA, the increase in the T_m value was just 1°C. This result also indicated that this ligand prefers the HT-G4-RNA over the HT-G4 DNA. Additionally, the T_m values of the alkylated HT-G4-RNA were measured (Table S2 and Fig. S8). Unlike our previous studies of alkylation using acridine derivatives[10, 12], significant increases in the T_m values were not

observed after the alkylation. Additionally, no significant changes in the CD spectra occurred before and after the alkylation (Fig. S9). In contrast, the stabilization of the parallel G-4 structure by alkylation was observed on a gel, suggesting that the alkylation by 6OTD-VQ-S(O)Me would highly stabilize the parallel G-4 structure at room temperature, but not under high temperature conditions.

In conclusion, we have developed a highly selective parallel G-4 alkylator, 6OTD-VQ-S(O)Me. The alkylation easily proceeded even in the presence of an excess amount of dsDNA and other topologies of G-4 DNA. This is the first example of topology-selective alkylation using the VQ reactive moiety. Recently, several topology-selective G-4 binders have been reported.[3, 21-26] In addition to 6OTD, the different G-4 binder conjugates with VQ would provide a new selectivity for the G-4 alkylation. The selective alkylation would lead to the strong inhibition of G-4 RNA-related biological processes without interfering with the telomeric G-4 DNA. Research is currently underway along these lines.

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Conflicts of interest

There are no conflicts to declare.

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