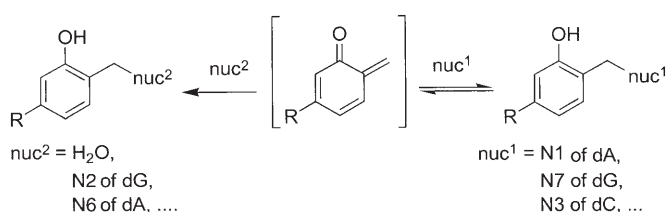


Immortalizing a Transient Electrophile for DNA Cross-Linking**

Huan Wang, Manvinder S. Wahi, and Steven E. Rokita*

Quinone methide intermediates (QMs) are integral to the activity of numerous natural products and synthetic compounds that, for example, may function as anticancer drugs, mechanism-based inactivators, or self-immolative dendrimers.^[1] The potential for these intermediates to act reversibly has a significant influence on their effective longevity, the resulting product distribution, and their general utility. For example, a parent *ortho*-QM forms adducts with strong DNA nucleophiles under kinetic control, but ultimately only adducts of weak nucleophiles persist under thermodynamic control (Scheme 1).^[2–4] Conversion of these products from



Scheme 1. Generation and regeneration of a quinone methide.

their kinetic to thermodynamic distribution has recently been shown to involve competition between repeated capture and release of the intermediate QM in a process that is sensitive to the electronics of its π system and the strength of the departing leaving group and/or incoming nucleophile.^[5] This covalent, yet dynamic, process has also been essential for QMs to participate in target-promoted alkylation and efficient cross-linking of DNA.^[6,7] Continual capture and release of QM should also nearly immortalize this fleeting intermediate by extending its effective lifetime from milliseconds^[8] to hours or even days. The consequence of this is now illustrated below with a bis-functionalized QM–acridine conjugate (bisQMP)^[6] that retains its potential for cross-linking DNA under aqueous conditions when nucleophiles are present to preserve rather than quench its activity.

The efficiency of DNA cross-linking is often compromised by the irreversibility of the reagent's chemistry. Under these circumstances, the first link to DNA permanently anchors the reagent to sites that are not necessarily susceptible to a second link needed for cross-linking. In contrast, reagents that act

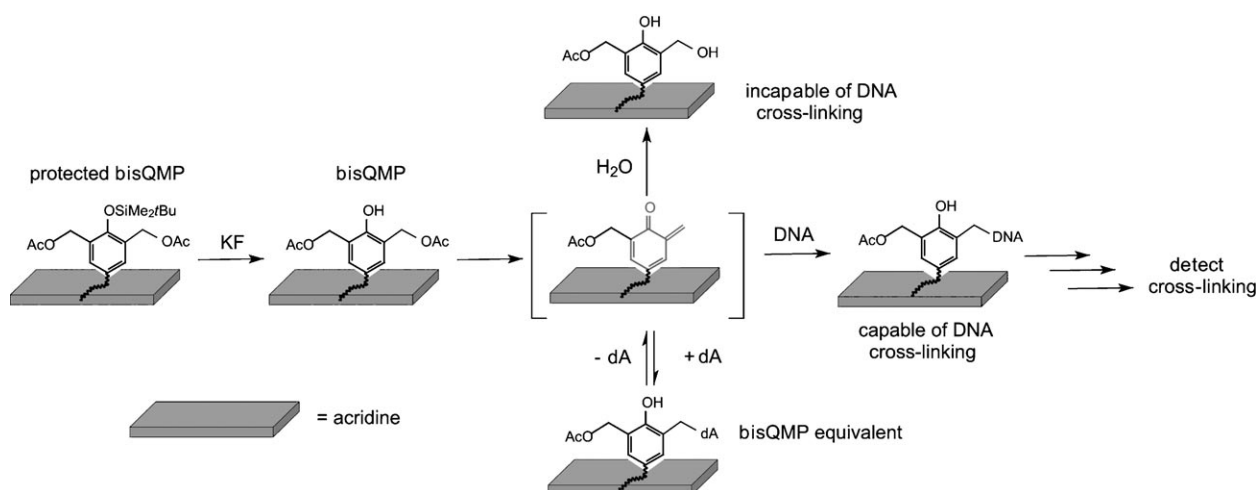
reversibly may overcome such trapping at nonproductive sites by regenerating their reactive intermediate for subsequent reaction at other sites, ultimately leading to cross-linking. This feature most likely explains the higher ratio of DNA cross-linking vs. monoalkylation generated by a bisQMP–acridine conjugate relative to that generated by an equivalent aniline mustard conjugate.^[6,9] Both conjugates use acridine for their association to DNA and both favor alkylation of dG N7, but only the QM product remains reversible. Examining the cross-linking by this bisQMP conjugate serves here as the most stringent test for its persistent activity since both sites of QM formation are required for cross-linking.

In the absence of nucleophiles that act reversibly, QM intermediates formed under aqueous conditions are vulnerable to irreversible quenching by water. For example, the bisQMP–acridine conjugate eliminates acetate spontaneously after deprotection of the phenolic oxygen to yield its QM intermediate and then a final benzyl alcohol derivative (Scheme 2). This alcohol is unable to regenerate the QM and hence no longer has the capacity to cross-link DNA even though its remaining QM equivalent may still alkylate DNA. Time-dependent loss of cross-linking then provides a measure for the effective longevity of the QM equivalents under aqueous conditions. As expected, DNA cross-linking by the bisQMP conjugate is maximum (25%) when irreversible trapping is minimized by initiating reaction in the presence of duplex DNA formed in this experiment by 5'-d(CAGAT-TACGCGCAGAAAAAAGGATCTCAAG) (**OD1**) and 5'-d(CTTGAGATCCTTTTTTCTGCGCGTAA) (**OD2**) (Figure 1 A, lane 1). However, the yield of DNA cross-linking dissipates quickly when the QM is allowed to react with water prior to addition of duplex DNA. The half-life for this quenching is ≈ 0.5 h, and cross-linking is no longer detected after a pre-incubation of 2 hours (Figure 1 A, lanes 1–6 and Figure S2 in the Supporting Information).

The cross-linking activity of the bisQMP–acridine conjugate can nonetheless persist within an aqueous environment if strong nucleophiles are present to capture and release the QM reversibly and prevent its quenching by water. Although dA (20 mM) competes with DNA for reaction with the QM intermediate and lowers the maximum yield of DNA cross-linking to 17% (Figure 1 A, lane 7), the resulting dA adduct also provides a continual source of the QM intermediate (Scheme 2). The cross-linking activity of the bisQMP–acridine conjugate is preserved beyond at least 49 hours under aqueous conditions in the presence of dA (Figure 1 A, lane 12). The effective lifetime of QM and its equivalents is consequently increased by almost 100-fold by the ability of dA to forestall irreversible trapping by water (Figure 1 B). This observation is consistent with the efficient and reversible reaction of dA N1.^[2–4] The expected symmetric dA N1 bis-adduct was confirmed by two-dimensional NMR spectroscopy

[*] H. Wang, M. S. Wahi, Prof. S. E. Rokita
Department of Chemistry and Biochemistry
University of Maryland
College Park, MD 20742 (USA)
Fax: (+1) 301-405-9376
E-mail: rokita@umd.edu
Homepage: <http://www.chem.umd.edu/faculty/rokita/>

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Scheme 2. Extending the lifetime of a DNA cross-linking agent depends on its partitioning between reversible and irreversible reactions.

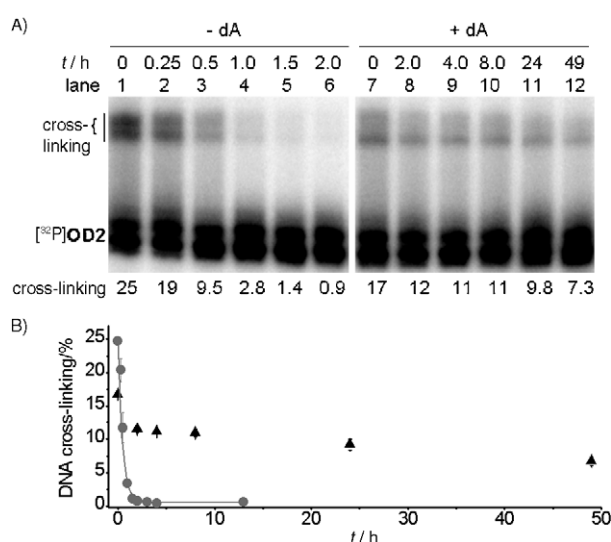


Figure 1. The presence of dA extends the effective lifetime of a quinone methide for cross-linking DNA under aqueous conditions. A) Reaction of the bisQMP-acridine conjugate (100 μ M) was initiated by addition of KF (100 mM) in 10 mM β -morpholinoethanesulfonic acid (MES, pH 7) and 20% acetonitrile under ambient conditions for the indicated time in the absence (lanes 1–6) and presence of 20 mM dA (lanes 7–12). The persistent ability of these samples to cross-link DNA was measured by subsequent addition of OD1/5'-[³²P]OD2 (3.0 μ M, 30 nCi annealed with 10% excess OD1). Samples were then further incubated for 48 hours under ambient conditions. Each solution was then frozen, lyophilized, and analyzed by denaturing 20% polyacrylamide gel electrophoresis. Cross-linked products were quantified by phosphoimage analysis and reported (%) relative to total DNA. B) The average of these data and an independent repetition is summarized graphically as well for reaction in the absence (●) and presence of dA (▲). The range of data is indicated by the error bars, and the line represents a fit to a first order rate of decomposition in the absence of dA (see also, Figure S2 in the Supporting Information).

py using an aprotic solvent that suppresses its decomposition and a model bisQMP derivative lacking the acridine linker for simplicity (see Figure S1 in the Supporting Information). Previous studies suggest that the half-life of the reversible dA N1 adduct is only approximately 2 hours under aqueous

conditions,^[2] and thus dA N1 likely captures and releases the bisQMP-acridine conjugate repeatedly in order to maintain cross-linking activity throughout incubations of 49 hours prior to addition of duplex DNA (Figure 1 B).

An adenine derivative was selected in these experiments as a mediator of QM activity since its N1 position is both accessible and abundant in vivo within ATP, NAD(P)(H), mRNA etc. Concentrations of ATP in human cells typically range between 1 mM and 5 mM but can rise as high as 9 mM.^[10] ATP levels also tend to be greater in tumor than in normal cells,^[10] and thus it may selectively prolong the biological lifetime of electrophiles acting reversibly in these cells. In our model above, persistence of the cross-linking activity depended on dA concentration and saturated at approximately 5 mM (see Figure S3 in the Supporting Information). Trapping the QM with an alternative nucleophile such as 2-mercaptoethanol that acts irreversibly has the opposite effect of dA and suppresses cross-linking. When the reaction of the bisQMP conjugate is initiated in the presence of dA, 2-mercaptoethanol, and OD1/[³²P]OD2, DNA cross-linking decreases by 90 %, and no cross-linking is observed if the DNA is added 4 hours after initial generation of the QM intermediate (see Figure S4 in the Supporting Information).

Single-stranded DNA is also capable of extending the effective lifetime of QM equivalents. Most interestingly, the resulting products formed between the bisQMP-acridine conjugate and single-stranded DNA do not prevent strand hybridization to form duplex DNA nor block interstrand transfer of the QM to form DNA cross-linking (Figure 2 A). This is evident from the cross-linking observed after OD2 (3 μ M) was added in place of dA (20 mM) to trap the bisQMP conjugate reversibly during a pre-incubation of 0–72 hours prior to addition of OD1 (Figure 2 B, lanes 1–5). The same cross-linked species was detected regardless of which strand (OD1 or OD2) was labeled with a 5'-[³²P] (see Figure S5 in the Supporting Information).

The persistence of cross-linking activity again suggests that QM intermediates are trapped and released multiple times during the pre-incubation period. While dA might appear to extend the longevity of cross-linking to a greater

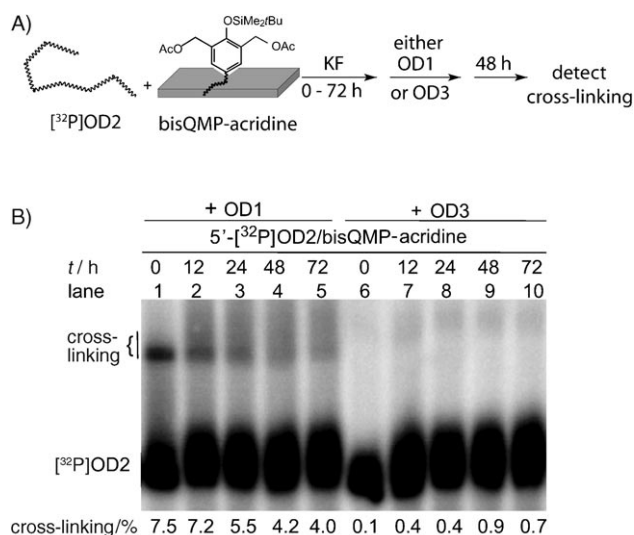


Figure 2. Single-stranded DNA extends the lifetime of a quinone methide and promotes its selective interstrand transfer for cross-linking DNA under aqueous conditions. A) Reaction of the bisQMP-acridine conjugate (30 μ M) was initiated by addition of KF (10 mM) in 10 mM MES (pH 7), 20% acetonitrile, and 5'-[³²P]OD2 (3.0 μ M, 30 nCi) under ambient conditions. After the indicated time, either the complementary strand OD1 (3.3 μ M) (B, lanes 1–5) or a noncomplementary strand OD3 (3.3 μ M) (B, lanes 6–10) was added, and the samples were further incubated for an additional 48 hours under ambient conditions. B) DNA cross-linking was determined by gel electrophoresis and phosphorimager analysis as described in Figure 1.

extent than OD2, this phenomenon is concentration dependent and the effect of OD2 is still remarkable. A low concentration of dA (0.5 mM) had a rather weak effect and helped to maintain a DNA cross-linking yield of only $\approx$ 6% after a pre-incubation for 8 hours (see FigureS3 in the Supporting Information). However, this concentration of dA is still ten times greater than the equivalents used in Figure 2. An OD2 concentration of 3 μ M corresponds to a 45 μ M solution of nucleophiles (dA N1, dC N3, dG N7) that can act reversibly.^[4]

The efficiency of intrastrand trapping of the bisQMP-acridine conjugate by OD2 may in part be driven by nonspecific association between the attached acridine and the compact structure of single-stranded DNA.^[11] However, subsequent transfer of QM to form interstrand cross-linking requires specific association between complementary strands of DNA. QM adducts formed between OD2 and the bisQMP conjugate during the pre-incubation period of 0–72 hours did not subsequently produce more than background levels of cross-linking to 5'-d(GGTACACATAGAGATAGAGAGATACACACAC) (OD3), which has the same deoxynucleotide composition, but not sequence, as OD1 (Figure 2B, lanes 6–10). Initial formation of adducts between the bisQMP conjugate and OD2 also revealed a sensitivity to the presence of 2-mercaptoethanol similar to that described previously with dA (see Figure S6 in the Supporting Information). Interstrand transfer of QM to form DNA cross-links, however, was surprisingly insensitive to nucleophilic competition by the same thiol. Cross-linking was suppressed by only

50% when the thiol was added along with OD1 after 24 hours of pre-incubation of OD2 and the bisQMP-acridine conjugate (see Figure S7 in the Supporting Information).

The reversibility of QM reactivity significantly expands the potential biological activity of this intermediate based on its repeated capture and release to forestall irreversible trapping. The dynamics of QM adduct formation also allows for covalent reorganization in response to changing constituents in solution as demonstrated by QM transfer from intra- to interstrand positions for generating the observed DNA cross-links. An equivalent activity had previously been described for an oligodeoxynucleotide self-adduct formed by a monofunctional QM extended from a 5'-hexamethyleneamino linker,^[7] but obviously such a highly defined system is not necessary for supporting selective cross-linking that can distinguish between complementary and noncomplementary DNA (Figure 2). A role for a reversible adduct now also deserves consideration in the therapeutic activity of a recent and highly promising anticancer drug that releases simple QMs in vivo.^[12]

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- [1] a) M. Tomasz, *Chem. Biol.* **1995**, 2, 575–579; b) P. W. Fan, F. Zhang, J. L. Bolton, *Chem. Res. Toxicol.* **2000**, 13, 45–52; c) D. Cabaret, S. A. Adediran, M. J. Garcia Gonzalez, R. F. Pratt, M. Wakselman, *J. Org. Chem.* **1999**, 64, 713–720; d) D. Shabat, M. Shamis, *Chem. Eur. J.* **2007**, 13, 4523–4528.
- [2] W. F. Veldhuyzen, A. J. Shalloo, R. A. Jones, S. E. Rokita, *J. Am. Chem. Soc.* **2001**, 123, 11126–11132.
- [3] a) M. Freccero, C. Di Valentin, M. Sarzi-Amadè, *J. Am. Chem. Soc.* **2003**, 125, 3544–3553; b) M. Freccero, R. Gandolfi, M. Sarzi-Amadè, *J. Org. Chem.* **2003**, 68, 6411–6423.
- [4] E. E. Weinert, K. N. Frankenfield, S. E. Rokita, *Chem. Res. Toxicol.* **2005**, 18, 1364–1370.
- [5] E. E. Weinert, R. Dondi, S. Colloredo-Melz, K. N. Frankenfield, C. H. Mitchell, M. Freccero, S. E. Rokita, *J. Am. Chem. Soc.* **2006**, 128, 11940–11947.
- [6] W. F. Veldhuyzen, P. Pande, S. E. Rokita, *J. Am. Chem. Soc.* **2003**, 125, 14005–14013.
- [7] Q. Zhou, S. E. Rokita, *Proc. Natl. Acad. Sci. USA* **2003**, 100, 15452–15457.
- [8] a) E. Modica, R. Zanaletti, M. Freccero, M. Mella, *J. Org. Chem.* **2001**, 66, 41–52; b) Y. Chiang, A. J. Kresge, Y. Zhu, *J. Am. Chem. Soc.* **2001**, 123, 8089–8094.
- [9] A. S. Prakash, W. A. Denny, T. A. Gourdie, K. K. Valu, P. D. Woodgate, L. P. G. Wakelin, *Biochemistry* **1990**, 29, 9799–9807.
- [10] T. W. Traut, *Mol. Cell. Biochem.* **1994**, 140, 1–22.
- [11] a) G. M. Clore, A. M. Gronenborn, *Eur. Biophys. J.* **1984**, 11, 95–102; b) G. Vesnaver, K. J. Breslauer, *Proc. Natl. Acad. Sci. USA* **1991**, 88, 3569–3573; c) J. Isaksson, S. Acharya, J. Barman, P. Cheruku, J. Chattopadhyaya, *Biochemistry* **2004**, 43, 15996–16010.
- [12] a) K. Kashfi, B. Rigas, *Biochem. Biophys. Res. Commun.* **2007**, 358, 1096–1101; b) N. Hulsman, J. P. Medema, C. Bos, A. Jongejan, R. Luers, M. J. Smit, I. J. P. de Esch, D. Richel, M. Witjans, *J. Med. Chem.* **2007**, 50, 2424–2431.