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# Semiquinone Footprinting

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In the long march toward understanding modulation of gene expression by small molecules the technique of footprinting has proved indispensable as a means of investigating sequence-specific binding of numerous ligands to DNA, including drugs,[1−4] antitumor antibiotics,[2,5,6] synthetic peptides,[2,7−9] and proteins.[10] Several methodologies are frequently employed for quantitating small-ligand−DNA interactions, namely DNase I footprinting,[1−6,9] hydroxyl radical footprinting,[10] and MPE-Fe(II) footprinting.[8] Among these techniques, the most popular method is certainly DNase I footprinting since it has the advantages of a simple protocol, commercial availability, and reproducible quantitative results. However, DNase I does not digest DNA indiscriminately, often resulting in a rather uneven ladder of bands after electrophoresis which causes difficulty in the integration of band areas. Hydroxyl radical footprinting, on the other hand, affords indiscriminate cleavage of DNA but demands skilful attention to the concentration of several reagents and reaction time, resulting in a rather involved protocol. In this contribution we report the application of a designed dipeptide conjugate as a convenient footprinting reagent capable of operating via a novel semiquinone mechanism.

The new reagent DMQ-MA-$\beta$-Ala-Arg-OMe (DBRO) (Figure 1) employed here is a dipeptide conjugate of 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA).[11,12] The dipeptide $\beta$-Ala-Arg-OMe was readily coupled to DMQ-MA via its pentafluorophenyl ester. In the presence of ferrous sulfate and sodium periodate in phosphate-buffered saline, and in the absence of UV irradiation, DBRO shows five strong ESR (electron spin resonance) hyperfine signals centered at $g = 2.0050$, much stronger than those of the parent compound DMQ-MA (Figure 2). We envisage that these five hyperfine ESR lines correspond to radical structure II (Figure 1), in resonance stabilization and equilibrium as previously proposed by us for DMQ-MA derivatives.[12] The semiquinone radical (structure I), being stabilized by four resonance forms,[12] is likely to be responsible for the DNA cleavage process. DVRO, an analogue of DBRO, in which the $\beta$-Ala residue is replaced by a Val residue also shows identical strong ESR hyperfine signals and DNA cleavage properties (not shown).

Using polyacrylamide gel electrophoresis we monitored the concentration dependence for the cleavage of the 162-mer *tyr*T DNA restriction fragment[6] induced by DBRO (Figure 3a and b). Digestion of the DNA is complete within 5 min and can be extended to over 30 min at 37 °C (kinetic data not shown). The effective concentration range of the DBRO is wide (1−10 $\mu$M, Figure 3a). Thus, the handling of drug concentrations and reaction time requires much less skilful manipulation than that demanded for hydroxyl radical footprinting.

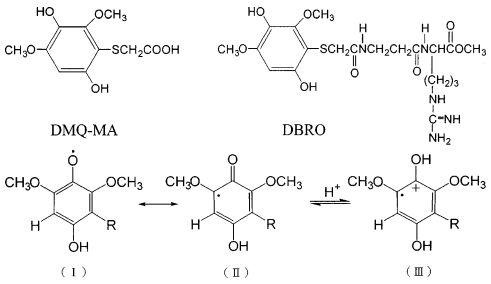


***Figure 1.*** (Upper panel) Structures of DMQ-MA and DBRO. (Lower panel) Proposed resonance and equilibrium forms of DBRO or DMQ-MA in aqueous solution (for details, see ref 12).

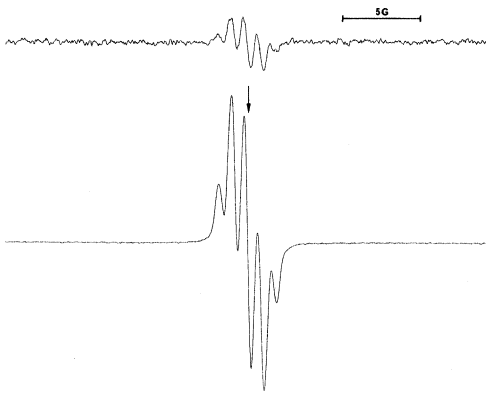


***Figure 2.*** ESR spectra obtained by reacting 89 mM DMQ-MA (upper trace) or 44 mM DBRO (lower spectrum) with 2.5 $\mu$M ferrous sulfate and 0.1 mM sodium periodate in 7.5 mM phosphate buffer saline (pH 7.5) at room temperature. Microwave power 20 mW; modulation frequency 100 kHz; sweep time 3.6 G/min; receiver gain (DMQ-MA, $8.93 \times 10^5$; DBRO, $1.78 \times 10^5$); time constant 2.62 s; $g = 2.0050$.

***Table 1.*** $C_{50}$ Values[a] (nM) of SP-30 on Various Binding Sites of the 5′-$^{32}$P-Labeled *tyr*T DNA Obtained by Semiquinone Footprinting (induced by DBRO) or DNase I Footprinting

| binding site | semiquinone footprinting | DNase I footprinting |
|---|---|---|
| 5′-TTA-3′ | 85 | 179 |
| 5′-GGA-3′ | 82 | 238 |
| 5′-GAA-3′ | 94 | 218 |
| 5′-TTA-3′ | 50 | − |
| 5′-TTCA-3′ | 74 | 220 |
| 5′-TTTTCTC-3′ | 65 | − |

[a] $C_{50}$ value is defined as the peptide concentration that produces half of the maximal reduction in electrophoretic band intensity.[5]

Relative cleavage plots (Figure 4a) can be used to compare the profile of cleavage sites produced by DNase I cutting and by semiquinone attack induced by DBRO in the absence of binding ligands. It is clear that DNase I has many weak cleavage sites on the DNA, namely, 5′-TTT-3′, 5′-CAG-3′, 5′-TTA-3′, 5′-TCA′, 5′-

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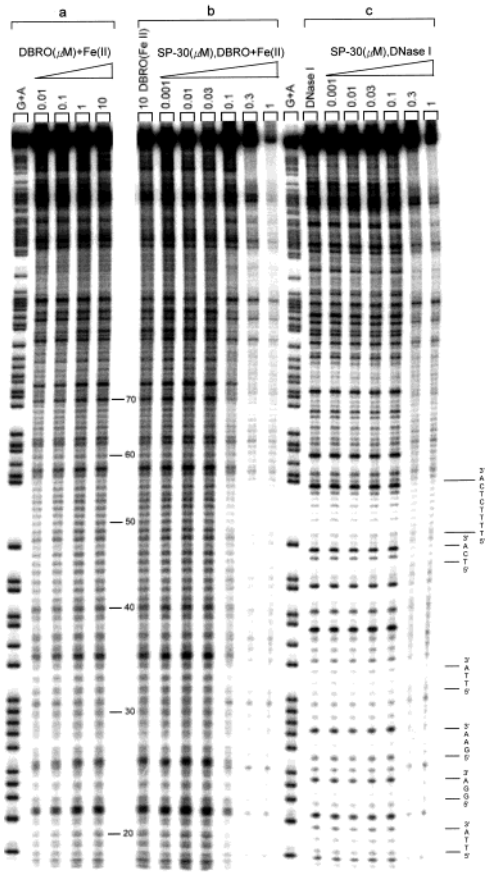


***Figure 3.*** (a) Autoradiograph showing cleavage of 5′-$^{32}$P-labeled *tyr*T DNA by incubation with various concentrations of DBRO and 20 $\mu$M of ferrous sulfate at 37 °C for 30 min. (b) Autoradiograph showing footprinting of *tyr*T DNA by polypeptide SP-30 using 10 $\mu$M DBRO and 20 $\mu$M of ferrous sulfate or (c) DNase I (normal procedure).

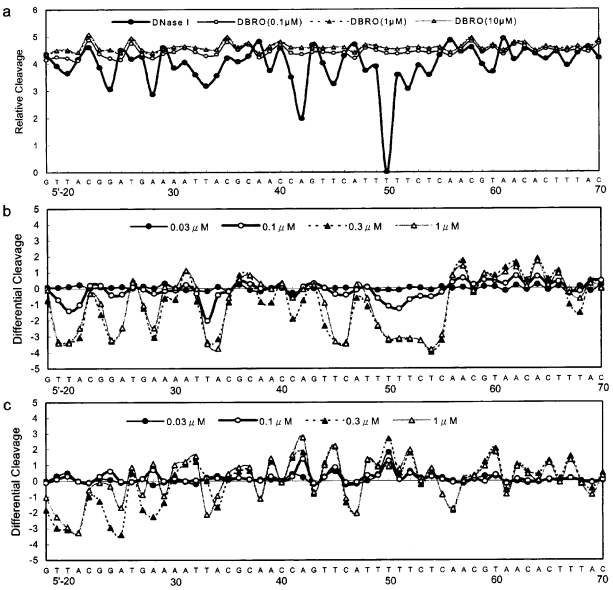


***Figure 4.*** Plots comparing the susceptibility of *tyr*T DNA to cleavage under the following conditions: (a) after incubating DNA with DBRO and 20 $\mu$M ferrous sulfate at 37 °C for 30 min or with DNase I (normal procedure) in the absence of SP-30, (b) after incubating with various concentrations of SP-30 and then reacting with 10 $\mu$M DBRO and 20 $\mu$M ferrous sulfate at 37 °C for 30 min, (c) after incubating DNA with various concentrations of SP-30 and then reacting with DNase I at room temperature.

TTC-3′, 5′-GAA-3′, and 5′-GGA-3′ (Figure 4a, where troughs represent the poorly cleaved sites in the absence of SP-30). Quantitative difficulty and inaccuracy will arise when the binding sites of a test ligand happen to fall within these weak DNase I cleavage sites. On the other hand, the cleavage sites induced by DBRO (Figures 3a, 4a) in the presence of 20 $\mu$M ferrous sulfate are less sequence dependent, yielding a more even cleavage ladder than that seen with DNase I.

To compare the accuracy and effectiveness of semiquinone footprinting with those of DNase I footprinting, a 30-mer polypeptide (SP-30)[9] which is known to bind to DNA in a sequence-selective fashion was used as a test ligand. Since DNase I has several weak cleavage sites on *tyr*T DNA between positions 41 and 54 (Figure 4a), the DNase I blockages by SP-30 around this locus are vague (Figures 3c, 4c), rendering quantitation by the integration of electrophoretic bands very difficult.

On the other hand, the major binding sites for SP-30 on the *tyr*T DNA shown by semiquinone footprinting appear more conspicuous (Figures 3b, 4b). Notably, semiquinone footprinting using DBRO also reveals strong blockage around position 48−55, corresponding to the binding site 5′-TTTTTCTC-3′ which is hardly detected at all by the DNase I procedure. In addition, the $C_{50}$ values[5] computed for SP-30 by semiquinone footprinting are often lower than those given by DNase I footprinting (Table 1), indicating that the former method can yield useful quantitative binding data especially in loci where the latter method gives weak cleavage.

We conclude that the strong semiquinone radicals generated by simply dissolving DBRO and ferrous salt in buffer are useful cleavage agents and offer significant advantages for quantitative footprinting. The broad effective concentration (1−10 $\mu$M) of the drug, the simple experimental protocol, the more even DNA cleavage ladder, and the high quality of the footprinting results commend this novel semiquinone footprinting approach as an attractive alternative to the popular DNase I footprinting method.

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**Note Added after ASAP Publication:** This communication was published on the Web 6/5/2004 with captions to Figures 3 and 4 transposed. The final Internet version published 6/8/2004 and the print version are correct.

**Supporting Information Available:** Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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