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DNA-DNA Interstrand Cross-Linking by FR66979 and FR900482: Requirement of Metal Ions During Reductive Activation

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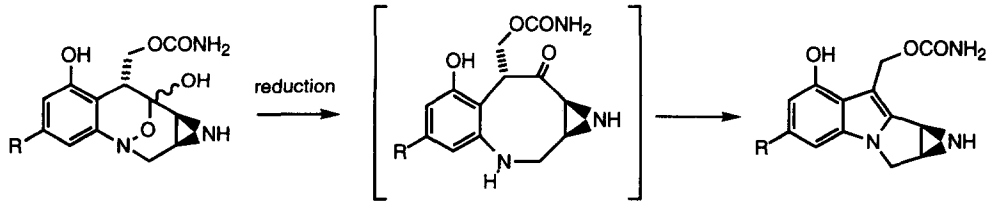
Abstract: The antitumor antibiotics FR66979 and FR900482 are believed to undergo bioreductive activation in vivo, enabling them to alkylate DNA. Reduction has previously been accomplished in vitro using sodium dithionite or dithiothreitol. We find that metal ions are a previously unrecognized, critical component of these reactions, which are suppressed by addition of EDTA and greatly stimulated by addition of catalytic quantities of iron(II) salts. This finding should greatly facilitate the elucidation of the details of the reaction cascades which follow reductive activation. Copyright © 1996 Elsevier Science Ltd

We and others have studied the in vitro DNA-DNA interstrand cross-linking reactions of the antitumor antibiotics FR66979 (**1**) and FR900482 (**2**).[1-5] These studies provided strong evidence in vitro for the proposal of Goto and Fukuyama[6] that FR900482 (and by extension FR66979) experiences reductive activation in vivo (Scheme 1) to yield mitosene-like intermediates (e.g., **3**) responsible for their DNA damaging activity.[7] Both FR66979 and FR900482 were found to cross-link DNA only in the presence of an exogenous reducing agent such as dithiothreitol or sodium dithionite.[5] The isolation from an interstrand cross-linked DNA of lesion **4** derived from deoxyguanosine and FR66979 in a 2:1 molar ratio provided further evidence for the chemical competence of such a scheme.[4,5]



1 FR66979 R = CH_2OH
2 FR900482 R = CHO

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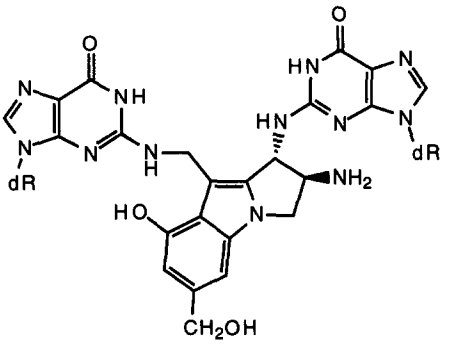
Scheme 1. Proposed Mechanism for the Reductive Activation of FR900482 and FR66979.



4 (dR = β–2'-deoxyribofuranosyl)

In a continuation of this line of inquiry, we sought to study the reductive activation cascade of these substances in the absence of DNA. Williams and Rajski have previously observed that FR900482 is not efficiently reduced by mercaptoethanol, DTT, or glutathione, although these substances activate it for DNA cross-linking.[1] Our experience was similar: FR66979 admixed with dithiothreitol, thiosalicylic acid, or sodium dithionite, all of which are effective as activating agents in DNA interstrand cross-linking assays, gave almost no reaction as indicated by ^{1}H NMR or TLC. We thus sought a new reductive activation protocol, using efficiency of DNA interstrand cross-linking activity monitored by denaturing polyacrylamide electrophoresis (DPAGE) as the assay. Endo *et al.* have reported the efficient reduction of the N-O bonds in hydroxylamine derivatives using dithiols and catalytic iron(II) in aqueous buffer,[8] prompting us to examine the impact of iron(II) in this system. The previously studied, self-complementary oligonucleotide duplex 5'-[d(TATAATACGTATTATA)]$_2$, known to efficiently form interstrand crosslinks with reductively activated FR66979,[2] was incubated with FR66979, with and without a trace of iron(II), along with either DTT, dihydrolipoamide, mercaptoethanol or thiosalicylic acid at pH 7.6, 25 °C for 16 h. The DNA was precipitated and analyzed by DPAGE (Figure 1A). Relatively efficient cross-linking (55-62%) occurred exclusively in the presence of added iron(II). In fact, only DTT showed appreciable cross-linking (5%) in the absence of added iron(II). That this was most likely due to the presence of metal ion contamination of commercial DTT was demonstrated by the inclusion of EDTA, which eliminated cross-linking. This experiment clearly demonstrated that DTT-activation of FR66979 occurs *only* in the presence of metal ions. However, catalytic iron(II) in the absence of thiol produced only a small amount of cross-link (ca. 1%), while stoichometric iron(II) resulted in a smear on the gel (data not shown). The ability of other divalent metal ions to substitute for iron(II) was tested; cobalt(II), copper(II), nickel(II), or zinc(II) were all shown to be far inferior to iron(II) in promoting cross-linking of DNA by mercaptoethanol and FR66979 (data not shown).

Sodium dithionite activation of FR66979 was also found to be strongly enhanced by the addition of iron(II) (Figure 1B). As with DTT, cross-linking by sodium dithionite was greatly enhanced by addition of iron(II) (48% vs. 18%). The involvement of metal ions was again implicated even in the reaction mixture to

which no iron(II) was explicitly added, by the inhibitory effect of EDTA (4%). Because aqueous solutions of dithionite ion decompose rapidly to generate bisulphite and thiosulfate, we tested the possibility that the latter might participate in the activation process. Bisulfite was quite active, but only in the presence of iron(II) (39%), while thiosulfate was of lower activity, even in the presence of iron(II) (9%). These findings indicate that both bisulphite and thiosulfate could participate in the apparent activity of aqueous sodium dithionite in these reactions, but likewise require metal ion assistance.

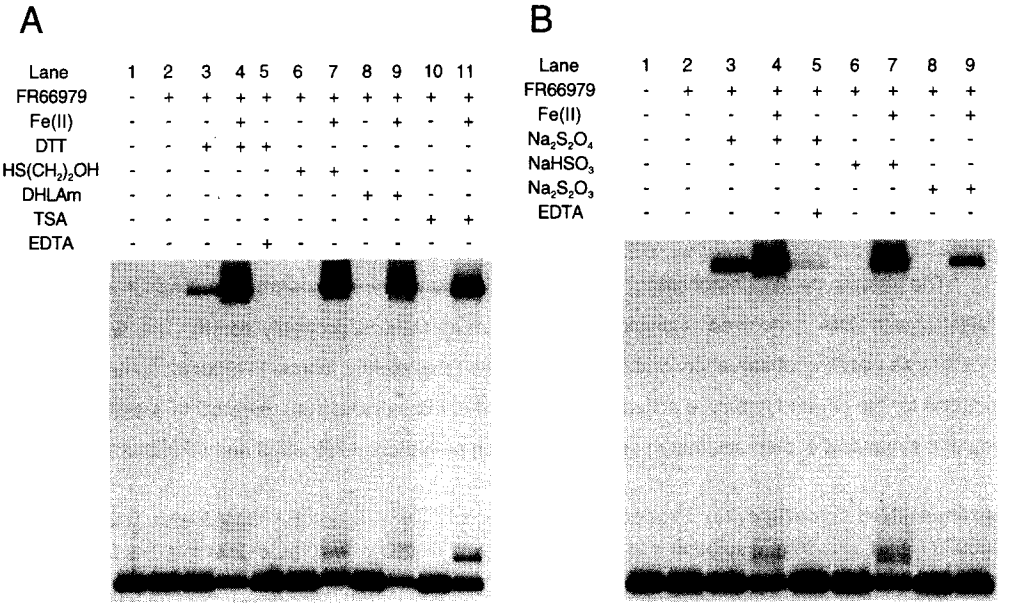
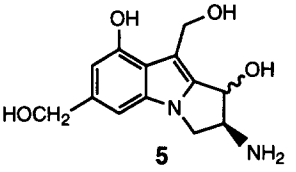


Figure 1. (A) Impact of thiols on iron(II)-catalyzed reductive activation of FR66979 monitored by DNA interstrand cross-linking. Single strand DNAs appear at the bottom of the DPAGE gel, interstrand cross-linked at the top. Abbreviations: DHLAm: dihydrolipoamide; TSA: thiosalicylic acid. (B) Impact of inorganic, sulfur-containing salts on iron(II)-catalyzed reductive activation of FR66979 monitored by DNA interstrand cross-linking.

The combination of thiols and iron(II) is also effective for the activation of FR900482 (**2**).[2] The activation of **2** with thiols and iron(II) afforded DNA cross-links in 20-30% yield, less than for **1**, but the best yields observed to date (data not shown).

As noted above, FR66979 is virtually inert to thiols in the absence of iron (II). In contrast, HPLC analysis of a mixture of FR66979 with DTT and iron(II) at 25 °C revealed the complete consumption of FR66979 over a 48 h period and the transient production of two major products in a roughly 1:2 ratio whose yield was maximal at about 5 h. The UV absorbance spectra of these two compounds, recorded from solutions freshly collected from the HPLC, were essentially identical to one another, with maximum absorbance at 232 nm, indicative of an indole substructure.[4] The mass spectra of these two substances, obtained by electrospray LCMS on a mixture of FR66979 activated with DTT/Fe(II) for 2 h, were likewise

identical to one another. We tentatively interpret the observed abundant ions at m/e 303, 287 and 247 as the $M+K^+$, $M+Na^+$, and $M+H^+-H_2O$ ions for the diastereoisomeric pair **5**. Whether the isomers **5** are in fact present in the reaction mixture, or these ions are derived from solvolysis reactions of some precursor during HPLC analysis or the electrospray ionization process is currently unknown, but this point is under investigation. The activation of FR900482 under these conditions gave similar results. The HPLC analysis of a mixture of FR900482, DTT and catalytic Fe(II) revealed the formation of two major compounds in a 2:1 ratio and LCMS afforded the same mass spectrum for both compounds. The fragmentation pattern was equivalent to that obtained for FR66979, except each ion was lower in mass by 2 units.



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The results herein demonstrate that the previously reported *in vitro* reactions of FR66979 and FR900482 with DNA[1-5] involved catalysis by adventitious metal ions, probably iron(II). This finding explains the otherwise puzzling observation of the failure of thiols to efficiently reduce FR900482.[1] The discovery of conditions for the efficient reductive activation of this family of compounds is expected to greatly facilitate the study of the manifold of products that result from reduction activation of FR66979 and FR900482.

Representative Experimental Procedure. Activation of FR66979 and FR900482 with Thiols and Fe(II). FR66979 (40 μL of a 30 mM solution in H_2O, 1.2 μmol) was diluted with 40 μL of 200 mM Tris buffer (pH 8.9) and the mixture was deaerated by bubbling N_2 through the solution for 10 min. DTT (11 μL of a 225 mM solution in deaerated H_2O, 2.4 μmol), and $(NH_4)_2Fe(SO_4)_2$ (25 μL of a 2.5 mM solution in deaerated H_2O, 60 nmol) were then added. The mixture was stirred at 25 °C. Aliquots of the mixture were withdrawn *via* syringe and analyzed by HPLC.

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