Pergamon

Tetrahedron Letters, Vol. 35, No. 52, pp. 9669-9672, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)02146-5

FR66979 Requires Reductive Activation to Cross-Link DNA Efficiently

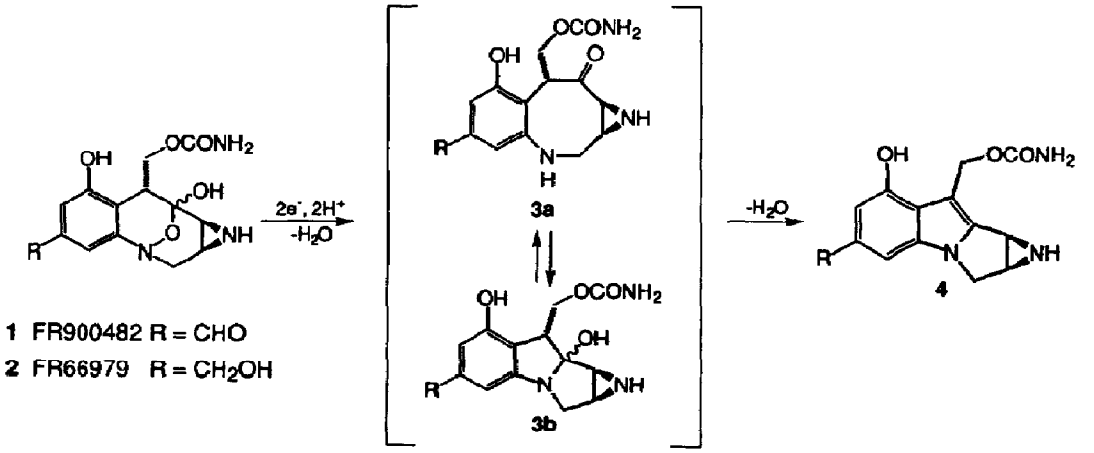
Huifang Huang[a], Scott R. Rajski[b], Robert M. Williams[b]*, and Paul B. Hopkins[a]*

[a] *Department of Chemistry, University of Washington, Seattle WA 98195*
[b] *Department of Chemistry, Colorado State University, Fort Collins, CO 80523*

Abstract: The activity of FR66979 as a DNA-DNA interstrand cross-linking agent in the absence of exogenous reducing agents was re-examined. Samples of FR66979 prepared by hydrogenation of FR900482 (H_2/Pd-C) were quite active whereas reduction of FR900482 with sodium borohydride afforded inactive or very weakly active FR66979 samples. Rigorous purification of FR66979 from either source abolished the observed cross-linking activity thus indicating that FR66979 does not efficiently cross-link DNA in the absence of exogenous reducing agents. LC-MS analysis of active samples indicated the possible presence of the over-reduced impurity **3**, below. The DNA-DNA cross-link arising from the same sample was identical in structure to that formed by reductively activated FR66979.

Our two laboratories have independently studied *in vitro* the DNA-DNA interstrand cross-linking reactions of the antitumor antibiotics FR900482 (**1**) and FR66979 (**2**).[1-4] Goto and Fukuyama[5] have proposed that FR900482 experiences reductive activation *in vivo*[6] (Scheme 1). This proposal holds that reduction of the N-O bond initiates a reaction cascade which ultimately yields a mitosene-like nucleus (**4**) presumed to cross-link DNA in subsequent reactions analogous to those of reductively activated mitomycin C.[7] Indeed, both laboratories have observed DNA-DNA interstrand cross-linking by FR900482 only upon addition of exogenous reducing agents such as thiol[1] or dithionite.[2] In contrast, divergent conclusions were reached concerning FR66979: one laboratory found highly efficient interstrand cross-linking in the *absence* of an exogenous reducing agent,[1,3] while the other found reductive activation to be *essential*.[2,4] We have now traced the source of this discrepancy to the different methods of preparation of FR66979 employed by the two groups.

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



Due to its low abundance from fermentation, FR66979 was prepared by reduction of the more available FR900482 with sodium borohydride or H_2/Pd-C.[1] By chance, prior to this study, the two laboratories had selected different reductants, and had each relied solely upon the one source. Each group has now repeated, in part, the work of the other, confirming the observations previously described: FR66979 samples derived from hydrogenation efficiently cross-linked DNA *without an exogenous reductant*; samples prepared by $NaBH_4$ reduction were either inactive or only very weakly active without an exogenous reductant.

The differing activity levels of these samples did not reside in the structures of the most abundant components as evidenced by TLC, HPLC, UV, and ^1H NMR analyses[8] of the crude reaction products and materials purified by PTLC.[9] However, HPLC and LC-MS analyses did reveal the presence of other minor contaminants in the crude products from both sources. Several observations suggested that one or more of these contaminants was responsible for the cross-linking activity of FR66979 samples in the absence of reducing agents. Firstly, reduction of FR900482 with H_2/Pd-C at 90 psi rather than 1 atm afforded significantly more active cross-linking agent, despite the fact that both reactions produced predominantly FR66979 (Figure 1). Secondly, allowing FR66979 prepared by hydrogenation to stand for several days in aqueous solution greatly reduced or eliminated the cross-linking activity, despite the persistence (NMR, TLC, HPLC analyses) of FR66979 (data not shown). Finally, exposure to methanolic silica completely abolished cross-linking activity while leaving FR66979 intact (data not shown). Given that a 200:1 ratio of drug to DNA duplex was employed in cross-linking experiments, a contaminant of only 0.5 mole-%, an amount easily overlooked by standard analytical methods, could fully account for the observed cross-linking activity. We have thus conducted a side-by-side comparison of variously derived and processed FR66979 samples.

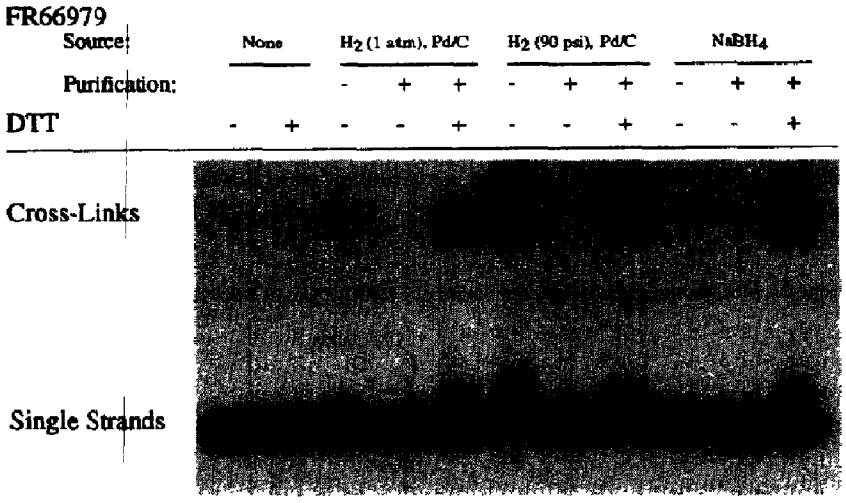
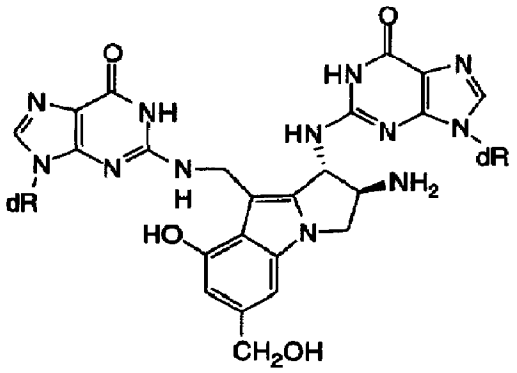


Figure 1. Effect of FR66979 source, purification status, and addition of reducing agent on DNA-DNA interstrand cross-linking. DPAGE of cross-linking reaction of 5'-^{32}P-labeled 5'-d(TGTTGAATACTGATACGTCTCTTGCTGAGGG) to its complement by FR66979. Purification refers to PTLC. The two band pattern of interstrand cross-links possibly reflects the presence of orientation isomers, but this has not been experimentally demonstrated.

FR66979 samples were assayed for interstrand cross-linking activity in a 31-mer duplex using denaturing polyacrylamide gel electrophoresis (DPAGE) (Figure 1). Crude and PTLC purified FR66979 prepared by $NaBH_4$ reduction of FR900482 did not appreciably interstrand cross-link the DNA studied, whereas crude material from hydrogenation was highly active. Purification of this material by PTLC abolished cross-linking activity.[9,10] Thus, FR66979 could not have been the source of cross-linking activity. This finding, together with the previously established inactivity of the starting material FR900482, suggested that the activity of the crude samples must have resided in some by-product of the hydrogenation which was inactivated by PTLC, either by separation or degradation of the active species. It was unlikely that the samples which were inactive were contaminated with some unspecified inhibitor of cross-linking, because FR66979 from *all* sources was active in the presence of the exogenous reductant dithiothreitol.[2,4]

The conclusion that it was some as yet unidentified substance rather than FR66979 which was responsible for the high cross-linking activity of the hydrogenation product was supported by several additional findings. Firstly, fractionation of this material by HPLC followed by assay for cross-linking activity traced the majority of the activity to a peak corresponding to the minor diastereoisomer of FR66979. The peak containing the major diastereoisomer of FR66979 was much less active. Because these two diastereoisomers rapidly equilibrated to a mixture of the two, some impurity which coeluted with the minor diastereoisomer was implicated as the active component. In fact, more thorough HPLC analysis of the active fraction revealed the presence of substances other than FR66979. LC-MS analysis of the hydrogenation reaction mixture afforded ions of m/e 307 (potentially **3a/3b**) from eluant close to that containing *both* of the two diastereoisomers of FR66979. No evidence has been obtained for the presence of a mitosene-like substance such as **4** in FR66979 samples from any source. Efforts to further purify and characterize the active substances are underway.



5 (dR= β-D-2-deoxyribofuranosyl)

Secondly, we have characterized the lesion responsible for the cross-link and found it to be identical to that derived from *reductively activated* FR66979. The self-complementary 14-mer 5'-d(ATAATACGTATTAT) was interstrand cross-linked with crude FR66979 from hydrogenation (14 psi). The interstrand cross-linked product was isolated from DPAGE and digested as previously described.[2,4] The resulting mixture was compared with the corresponding hydrolysate from the same DNA cross-linked using dithionite-activated FR66979 prepared by sodium borohydride reduction. Rigorous structural characterization had previously

proven the latter to contain substance **5**, fully consistent with the reductive activation hypothesis in Scheme 1.[4] The lesions from these two sources possessed identical HPLC retention times, and gave essentially identical UV spectra and electrospray ionization mass spectra, indicating the identity of the covalent structures of the cross-links formed by these two preparations. It is difficult to imagine how FR66979 itself could yield this lesion, which is instead most compatible with some product of over-reduction such as **3** or **4** being responsible.

The simplest explanation of all these data is that FR66979 itself is *not* an efficient DNA interstrand cross-linking agent in the absence of an exogenous reductant and that the activity of FR66979 prepared by hydrogenation results from some by-product of that reaction. The structure of this highly active agent is not yet established, but is in all likelihood reduced relative to FR66979 given the structure of the cross-link. Whether this substance is produced from FR66979 in the hydrogenation medium or by some other pathway[11] remains unclear. As this substance appears to exhibit at least modest stability, its structure may emerge in due course.

Acknowledgement. This work was supported by the National Institutes of Health (Grants CA51875 to RMW and GM32681 to PBH). We are indebted to the Fujisawa Pharmaceutical Co., Ltd. for the generous gift of FR900482.

References and Endnotes

1. Williams, R. M.; Rajski, S. R. *Tetrahedron Lett.* **1992**, *33*, 2929.
2. Woo, J.; Sigurdsson, S. Th.; Hopkins, P. B. *J. Am. Chem. Soc.* **1993**, *115*, 1199.
3. Williams, R. M.; Rajski, S. R. *Tetrahedron Lett.* **1993**, *34*, 7023.
4. Huang, H.; Pratum, T. K.; Hopkins, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 2703.
5. Fukuyama, T.; Goto, S. *Tetrahedron Lett.* **1989**, *30*, 6491.
6. Masuda, K.; Nakamura, T.; Shimomura, K.; Shibata, T.; Terano, H.; Kohsaka, M. *J. Antibiot.* **1988**, *41*, 1497.
7. Tomasz, M.; Lipman, R.; Chowdary, D.; Pawlak, J.; Verdine, G.; Nakanishi, K. *Science* **1987**, *235*, 1204.
8. Terano, H.; Takase, S.; Hosoda, J.; Kohsaka, M. *J. Antibiot.* **1989**, *42*, 145.
9. For the PTLC purifications, only the major, less mobile component of the diastereoisomeric mixture was removed from the TLC plate. This material rapidly equilibrated to reform the original pair of diastereoisomers.
10. Both laboratories have occasionally encountered minute residual activity in samples of PTLC purified FR66979 from hydrogenation. We attribute this to incomplete removal or incomplete decomposition of the active contaminant.
11. Two of us (SRR and RMW) have demonstrated that addition of Pd-C to previously inactive (PTLC and HPLC purified) FR66979 in 200 mM Tris buffer under aerobic conditions affords a highly active DNA-DNA interstrand cross-linking agent. The mechanism for this activation is currently being investigated.

(*Received in USA* 6 *October* 1994; *accepted* 27 *October* 1994)