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PROTEIN DAMAGE CAUSED BY A SYNTHETIC ENEDIYNE CORE

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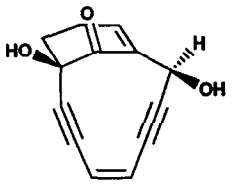
Abstract: *The simple enediyne core molecule* ***1*** *is shown to cause protein damage to several cellular protein isolates in vitro. The aromatization of* ***1*** *to generate a benzene diradical under simulated physiological conditions is also described.*

Calicheamicin γ_1^I,[1] esperamicin A_1,[2] neocarzinostatin chromophore[3] kedarcidin chromophore[4] and dynemicin[5] are among the most potent antitumor antibiotic agents isolated. These natural enediynes are presumed to exert their biological activity via formation of an aromatic diradical which subsequently cleaves DNA[3,6,7,8]. Numerous experiments describing the in vitro interactions of these natural products[3,6,7,8] and synthetic enediynes[9] with DNA have been reported. During the course of our program to synthesize enediyne compounds for evaluation as antitumor agents, we have studied the interactions of these compounds with an additional class of cellular macromolecules, proteins.

In this communication we present evidence that *in vitro*, **1** extensively damages proteins, a target hitherto not usually discussed for this class of synthetic compounds. The simple enediyne **1** exhibits considerable *in vitro* cytotoxicity[10] and *in vivo* antitumor activity[11] in spite of displaying considerably reduced affinity for DNA when compared to the natural products.

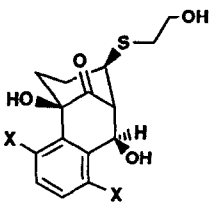


1

Enone **1**, a simple core mimic of the natural product esperamicin, was synthesized from a previously published intermediate.[12,13]. *In vitro* studies using the human colon carcinoma cell line HCT116, showed the compound to possess potent cytotoxicity (IC_{50} of $1x10^{-7}M$),[10] albeit 10^5 -

fold less than esperamicin A1 (IC_{50} of $1x10^{-12}M$)[10]. However, **1** exhibited superior antitumor activity against murine P388 leukemia models and also possessed activity against murine solid tumor and human tumor xenograft models.[11]

At the outset, it was established that **1** could generate, upon thiol activation and under physiological conditions, a diradical intermediate via a Bergman type cycloaromatization.[14] This observation can be contrasted with the considerably higher temperatures reported for the aromatization of similar bridgehead ketone enediynes in organic solvents.[15] Exposure of **1** to one equivalent of 2-mercaptoethanol in a 1:1 mixture of pH 7.4 phosphate buffer and THF for 4h at 37°C resulted in a rapid reaction to provide the aromatized ketone **2** with a 57% isolated yield . Under these conditions, no reaction was observed when thiol was omitted; other alkyl and aromatic thiols produced similar results. Carrying out the same experiment in an NMR probe at 37°C, using THF-d_8 and deuterated phosphate buffer, revealed an initial fast conjugate addition of thiol (essentially complete after 5 minutes). This was followed by a smooth conversion of the resulting enediyne intermediate to dideuterated, aromatized product **3**. $T_{1/2}$ for aromatization was 18 minutes at 37°. The reported $T_{1/2}$ for the corresponding calicheamicin dihydrothiophene is ≈ 4.5 seconds.[16]



2 X = -H

3 X = -D

The ability of a diradical arising from **1** to cleave DNA was confirmed *in vitro*. Reaction of **1** with supercoiled pM2 DNA in the presence of β-mercaptoethanol resulted in cuts observed starting at drug concentrations of $2x10^{-5}M$ levels. Experiments with 5'-end labeled restriction fragment pBR322 SalI BamHI under reducing conditions showed non-discriminatory cutting of the DNA at a drug concentration of $4.6 \times 10^{-2}M$. In the absence of thiol, the cleavage was greatly reduced. The DNA cleavage observed is consistent with the formation of a diradical intermediate. However, in contrast to the natural enediynes, the relatively high concentrations of **1** required for *in vitro* DNA activity suggested other mechanisms could possibly contribute to the observed *in vivo* activity and cytotoxicity. Consequently, in the search for other targets, one area of study was proteins, ubiquitous macromolecules in the cell.

In a preliminary experiment, HCT116 membrane protein extract was reacted at physiological pH with various amounts of **1**. Analysis of the reaction mixtures on SDS polyacrylamide gels indicated total damage to the cell membrane proteins and the formation of high molecular weight agglomerates first observable at a drug concentration of $4.6x10^{-4}M$ (Fig. 1). The same results were

observed when reacting the compound with the lysate of cells from the Jurkat human T cell line. Experiments using either 3':5'-cyclic AMP dependent protein kinase or prostatic phosphatase acid resulted in similar observations.

An experiment using 4.6×10^{-4}M **1** in the presence of calf brain tubulin showed protein agglomeration within two hours of incubation, with the damage increasing progressively with time (Fig. 2). When **1** was incubated with all 5 groups of calf thymus histones , the relative induced damage to the individual proteins was as follows: H4>> H2A, H2B, H3> H1. Incubation of histone H4 with **1** at a drug concentration of 4.6×10^{-3}M showed damage to the protein within 1 hour (Fig. 3). Interestingly, none of the protein reactions required the addition of thiol. This implies that **1** is activated either by a protein nucleophilic site or a protein thiol. To the best of our knowledge this is the first example of

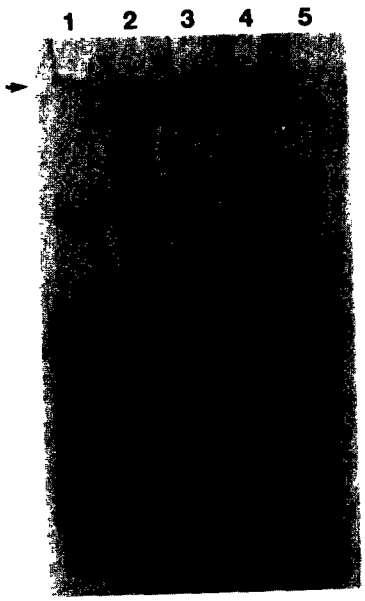


Fig.1. 8% SDS polyacrylamide gel of the reaction of HCT116 cell membrane protein extract with **1**. Conditions:90:10 of 50 mM Tris-HCl pH 7.5: DMSO, 1mg/ml extract, total volume 10 µL, overnight incubation at 37°C. Lane 1, standards from 200 to 14.3 Da; lanes 2,3,4, **1** at 0.01, 0.1 and 1mg/ml; lane 5, control.

protein damage caused by an enediyne diradical species. Protein cleavage by other radicals has been reported and results in the formation of peptide fragments of lower molecular weight.[17]

It is interesting to note that when esperamicin A_1 or calicheamicin γ_1^I were reacted with tubulin or the histones (either in the presence or absence of thiol), little damage was observed at concentrations of 10^{-3}M even after an overnight incubation.[18] DNA damage caused by these enediynes at a concentration of 10^{-6}M occurrs within minutes of incubation in the presence of thiol.[6,7] It is conceivable, that in the case of the natural enediynes the fast rate of cyclaromatization of the core [15] along with the protective role of the highly functionalized appendages to the aglycone may prevent the extensive protein damage observed with **1**. At this time the exact nature and etiology of the protein agglomerates is unknown.

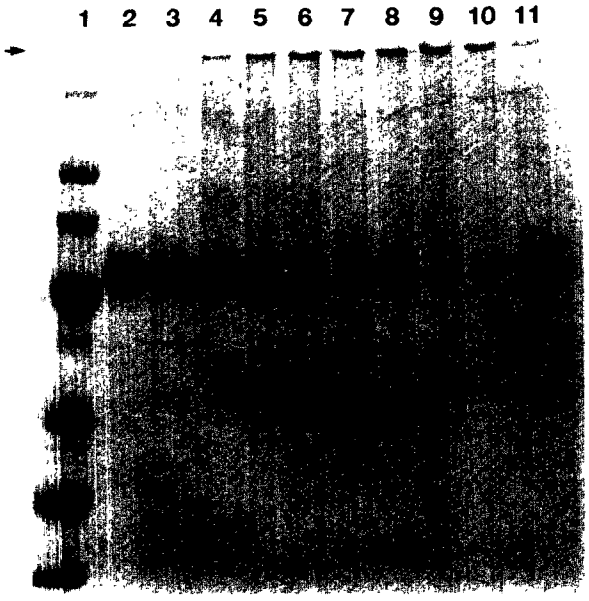


Fig.2. 8% SDS polyacrylamide gel of the reaction of calf brain thrice-cycled microtubulin with **1**.Conditions:90:10 of 50 mM Tris-HCl pH 7.5:DMSO,1 mg/mL tubulin,0.1 mg/mL drug in a total volume of 10 µL, 37°C. Lane 1, standards; lanes 2,3,4,5,6,7,8,9,10,**1** at 0.25,1,2,3,4, 5,6,8 and 24hrs; lane 11, control.

It would be tempting to speculate that *in vivo*, interaction with proteins might result in a fast deactivation of **1** prior to reaching a biological target. However, the distal tumor activity (subcutaneous tumor implant with intravenous administration of drug) observed for **1** *in vivo* establishes that total deactivation before reaching a relevant biological target is not occurring.

In conclusion, our investigation demonstrates that the synthetic enediyne **1** causes extensive protein damage *in vitro* at concentrations which would seem to be relevant to the mechanism of the drug. Studies to characterize the structural nature and mechanism of the *in vitro* protein damage and to determine the relevance of these observations to the *in vivo* setting are in progress.

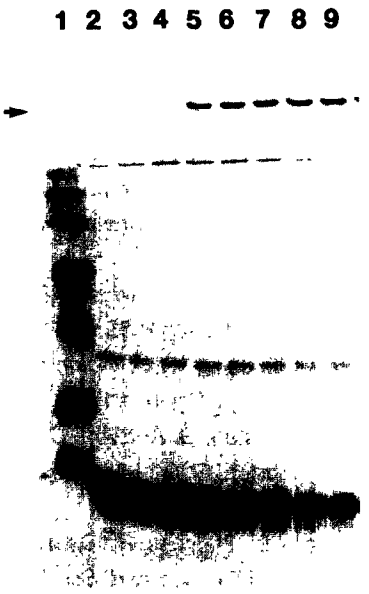


Fig.3. 17% SDS polyacrylamide gel of the reaction of H4 with **1**. Conditions 90:10 of 50 mM Tris-HCl pH 7.5:DMSO,1 mg/mL H4, 1mg/mL drug in a total volume of 10 μL. Lane 1, standards; lane 2, control; lanes 3,4, 5,6,7,8,9, **1** at 0.5,1, 2 ,3 ,4 ,5 and 7 hrs.

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