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SIMPLIFIED METHOD FOR THE ISOLATION OF THERMALLY LABILE DRUG-DNA ADDUCTS: CHARACTERIZATION OF CHLORAMBUCIL AND CARZINOPHILIN/AZINOMYCIN B ALKYLATION PRODUCTS

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Summary: A simple and rapid method has been developed for the isolation of thermally labile DNA alkylation products. Use of calf thymus DNA in combination with size exclusion filtration provides good yields >40% of chlorambucil-guanine adducts. Application of this method to the isolation of alkylation products of carzinophilin/azinomycin B affords material consistent with guanine monoalkylation.

The alkylation of DNA by small molecules is a topic of intense investigation in correlation with the ever-increasing number of cytotoxic agents isolated. The outcome of chemical transformations induced by these drugs has provided insights into their mechanisms of action. Characterization of these products has been a primary method for modeling events at the molecular level. Some of the drug-base adducts which have been isolated and characterized include mitomycin C,[1] anthramycin,[2] CC-1065,[3] duocarmycin A,[4] and Kapurimycin A3.[5] Methods for formation and isolation of alkylation products vary widely in accordance to drug stability and include reaction with small duplexed synthetic oligonucleotides, reaction with DNA followed by selective precipitation of products and chromatography, and incubation with individual bases proposed as target alkylation sites. Having a limited amount of the antitumor antibiotic carzinophilin/azinomycin B (**1**)[6] led us to investigate other methods which would not rely on the unknown behavior of the alkylation products and which would require minimal material and number of transformations. Described herein is the implementation of such a strategy to the isolation of the guanine (G) adduct of the known DNA alkylator chlorambucil (**2**).[7] Application of this procedure to carzinophilin provides evidence for formation of a monoadduct with G.

An authentic sample of the monoadduct of chlorambucil was obtained by alkylation with 2'-deoxyguanine (**3**), affording the N7 adduct as the chloride derivative **4** which could be further converted to alcohol **5** (Scheme 1).

Scheme I

3 + 2 → (CF_3CH_2OH, 45 °C, 15 hr, 86%) → 4 X=Cl → (H_2O, 90°C, 90 min, 99%) → 5 X=OH

Our strategy for generation of similar alkylation products with DNA was based on the fact that these adducts are released from the oligomer by thermal and/or acid/base depurination, resulting in strand scission. For large polymers, it should be possible to differentiate strictly by molecular weight distribution the target adducts from DNA. Calf thymus DNA type 1 (100 mg) was used as an inexpensive source of bulk DNA. This material was subjected to sonication for 12 hours at 0° C (1 mL, 10 mM Tris, pH 7) followed by centrifugation (3000 rpm) in a commercial filtration device.[8] This initial filtration removes small oligomers and individual nucleosides contaminating the crude sample using a 3000 molecular weight (<10 bp approximately) cutoff filter. After rinsing with water (2 mL), the DNA was taken up into 500 uL of Tris buffer (pH 7, 10 mM), and transferred to a separate vessel containing 40 mg of chlorambucil. The reaction was allowed to proceed at 11°C for 48 hr, at which time crude material was transferred to the same filtration device. The buffer was removed by centrifugation, followed by sequential washes with deionized water (1 mL), methanol (1 mL) and acetonitrile (1 mL). The DNA was resuspended in buffer (500 uL) and heated for 20 min at 90°C. Buffer was removed by centrifugation and the solid was washed with additional buffer (1 mL) and methanol (2 mL). The combined filtrates were concentrated and separated by HPLC using a reverse phase column (Axxiom - Axxi-Chrom ODS C18 25 X 10 mm ID) with a 25 min. linear gradient: water-->30% water/70% CH_3CN.

Table I provides the results of HPLC monitoring of successive washes resulting from preincubation of chlorambucil with DNA. As shown below, after 48 hr at 11°C the major component in solution is the hydrolyzed chlorambucil derivative **6**. In addition, there is a small amount of oligomers at the cutoff range resulting from minor decomposition of the DNA. The water wash effectively produces the same profile. Upon addition of methanol, a measurable amount of unreacted chlorambucil (**2**) was obtained. This material presumably is eluted at this stage due to its limited solubility in water.[9] The final acetonitrile wash provided only trace quantities of products. Dissolution of DNA into buffer followed by heating and filtration resulted in only two peaks being observed by HPLC analysis. The first corresponded to a mixture of small oligos which result from depurination and strand scission at chlorambucil alkylation sites. This material was radiolabeled with $\gamma\,^{32}P$ ATP using T4 kinase and analyzed by polyacrylamide gel electrophoresis, confirming that it is a mixture of small oligonucleotides (< 10 bp). The second HPLC peak corresponded to the chlorambucil G adduct **5**.[10] The total weight of **5** was 9.4 mg, which is equivalent to a >40 % yield based on percent G content in 100 mg of calf thymus DNA (40% GC).[11]

We next turned our attention to the isolation of carzinophilin-guanine adducts. Previous work[12] suggested that this drug forms interstrand cross-links in duplex DNA between G and purine adducts two base-pairs removed. In single stranded DNA, CZ acts like other aziridine-containing alkylators, resulting in exclusive G alkylation as determined by the formation of a G specific cleavage pattern when treated with hot aqueous piperdine and analyzed by PAGE. Initial attempts to react 2'-deoxyguanine (3) with CZ were unsuccessful, since the decomposition of the drug occurs more rapidly than addition of G. Analysis of the reaction mixture of CZ with with 3 by ^1H NMR and mass spectrometry supports the formation of a hydrolyzed product resulting from addition of H_2O to the aziridine (Scheme II). PAGE analysis demonstrated that the hydrolyzed material produced neither G specific adducts nor intrastrand cross-links, suggesting that the azabicyclo [3.1.0.] system may be the primary site of alkylation on DNA.

Table 1

wash	6	2	nucleosides and small oligos	5
buffer	+++	0	+	trace
D.I. water	++	0	+	trace
MeOH	+	+++	+	trace
CH_3CN	trace	trace	trace	trace
↓ 90°C, 20 min				
buffer-CH_3CN	0	0	+++	+++

Using the protocol described above, 0.1 mg of carzinophilin was reacted with 20 mg of calf thymus DNA. Analysis of the wash indicated that some decomposition of the drug had ocurred, resulting in multiple peaks (by HPLC analysis) and consistent with the low stability of the drug in an aqueous solvent. Subsequent washing, heating and filtration afforded an HPLC trace containing three major components (in addition to a peak corresponding to small oligomers). The first and second components were minor constituents which eluted at 21.2 and 22.7 min respectively (25 min linear gradient: H_2O-->70% H_2O/30% CH_3CN). The identity of these components could not be determined due to the small amounts obtained. The third and major component, eluting at 23.7 min afforded a mass spectrum in a CsI/PNB matrix (HRFAB) of 907.1587 mass units. The calculated mass (M^++Cs) for a carzinophilin-guanine mono-adduct is 907.1664. Isolation of this product as the major component supports our belief that the primary reaction of carzinophilin with DNA occurs at guanine.

Scheme II

1 + 3 ⟶ 7

A protocol has been developed for the synthesis and isolation of drug-base adducts. This technique is effectively independent of the specific properties of the adducts and relies on HPLC purification for final isolation of products. This procedure has been successfully applied to the isolation of a G mono-adduct of the clinically active agent chlorambucil and the antitumor antibiotic carzinophilin/azinomycin B.

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8 The Amicon, Centricon 3 Microconcentrator was utilized as the size exclusion device due to the ease and efficiency of isolation of both DNA and the filtrate.

9 In addition, partial denaturation of the DNA might improve solubilization of unreacted drug.

10 5: ^{1}H NMR (360 MHz, DMSO) δ 7.88 (s, 1H), 6.94 (d, J = 8.42 *Hz*, 2H), 6.71 (d, J = 8.51 *Hz*, 2H), 6.32 (s, 2H, NH_2), 4.30 (t, J = 6.11 *Hz*, 2H), 3.67 (t, J = 6.14 *Hz*, 2H), 3.42 (t, J = 6.11 *Hz*, 2H), 3.25 (d, J = 5.95 *Hz*, 2H), 2.42 (t, J = 7.11 *Hz*, 2H), 2.17 (t, J = 7.33 *Hz*, 2H), 1.73 (m, 2H). ^{13}C NMR (90 MHz, DMSO) δ 174.40, 159.23, 154.70, 153.02, 145.82, 143.20, 129.14, 128.64, 111.87, 108.13, 57.87, 52.76, 51.53, 43.85, 33.15, 32.95, 26.65. HRMS 401.1918. IR (DMSO) 3460, 2250, 2124, 1663, 1638, cm^{-1}.

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