Structural Characterization of the Major Adducts Obtained after Reaction of an Ultimate Carcinogen Aflatoxin B₁-Dichloride with Calf Thymus DNA in Vitro

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ABSTRACT

The major adduct formed on acid hydrolysis of calf thymus DNA which has been reacted with 8,9-dichloro-8,9-dihydroafatoxin B₁, a chemical model of the ultimate carcinogen 8,9-dihydro-8,9-epoxyafatoxin B₁ (AFB₁-epoxide), has been characterized by proton nuclear magnetic resonance and fast atom bombardment mass spectroscopy. This adduct has been identified as an N7-substituted guanine adduct analogous to that formed on reaction of AFB₁-8,9-epoxide with DNA in vivo and in vitro, namely trans-8,9-dihydro-8-(7-guanyl)-9-hydroxy AFB₁. This 8,9-dichloro-8,9-dihydroafatoxin B₁ adduct in DNA, like its equivalent AFB₁-epoxide adduct, is prone to quantitative imidazole ring opening of the substituted guanine in mildly alkaline conditions and to substantial depurination under mildly acidic conditions.

INTRODUCTION

AFB₁ (Fig. 1) is a potent mutagen and animal carcinogen that induces tumors mainly of the liver but also of the stomach, colon, and kidney. Epidemiological evidence collected over the last 20 years suggests that this compound is responsible for high incidences of liver cancer in certain regions of the world (1–3).

It is generally recognized that AFB₁ exerts its genotoxic effects after activation by cytochrome P-450-dependent mono-oxygenases to a species which is probably AFB₁-epoxide (4–9). Reaction of the latter compound with DNA in vivo (8, 10, 11) or in vitro (9, 10, 12) yields, upon subsequent DNA hydrolysis, predominantly AFB₁-Gua and, to a lesser extent, two guanine imidazole ring-opened adducts; FAPyr-AFB₁, form 1 and FAPyr-AFB₁, form 2 (10, 13).

Attempts to isolate AFB₁-epoxide have not been successful (5, 14, 15) and it remains to be proven whether or not this compound represents the major ultimate carcinogenic metabolite of AFB₁. However, various biological (16–21) and chemical (14, 15) studies have been carried out with a direct-acting mutagenic/carcinogenic model of AFB₁-epoxide, AFB₁-Cl₂, and these implicate the former molecule as the active derivative of AFB₁. Unfortunately, although AFB₁-Cl₂ appears to react with DNA in a manner analogous to activated AFB₁, no one has shown unequivocally that the DNA adducts so produced are similar to those obtained after reaction of AFB₁ with DNA. Therefore, to ascertain whether or not AFB₁-Cl₂ is a chemically valid model of AFB₁-epoxide, we have determined the structures of the major adducts formed on acid hydrolysis of DNA which has been reacted with AFB₁-Cl₂ in vitro. Experiments have also been carried out to determine the stability of the bound AFB₁-Cl₂-DNA adducts during incubation in either alkaline (which promotes imidazole ring opening of AFB₁-bound guanines in DNA) or mildly acidic (which promotes depurination of AFB₁-Gua from DNA) conditions.

MATERIALS AND METHODS

Materials

AFB₁ and calf thymus DNA type 1 were obtained from Sigma, Poole, Dorset, UK. [¹H]AFB₁ (30 Ci/mmol) was purchased from Moravek, City of Industry, CA. All other reagents were either standard laboratory or Anala grade.

HPLC

Analytical HPLC was carried out at a flow rate of 2 ml/min using a Technil-10 C18 (HPLC Technology Ltd., Macclesfield, Cheshire, UK) reversed-phase column (25 x 0.6 cm) by means of a PU4800 system (Pye-Unicam, Cambridge, UK). The column eluent was monitored at 364 nm. Two different water/methanol gradient systems were used: Gradient 1: 10% v/v methanol/water to 100% methanol over 40 min. Gradient 2: 20% v/v methanol/water to 65% v/v methanol/water over 20 min.

Preparative HPLC was carried out at a flow rate of 4 ml/min using a 25- x 0.9-cm column packed as above. Products were eluted isocratically with methanol/water (40:60 v/v), the column eluent being monitored at 364 nm.

Mass Spectroscopy

Fast atom bombardment mass spectra were obtained using a VG 70SE mass spectrometer. Samples were ionized in a glycerol matrix spiked with 0.1 M HCl.

NMR Spectroscopy

¹H-NMR spectra were obtained using a Bruker WH 400-MHz Fourier transform spectrometer. All resonances were measured relative to [²H₆]DMSO at δ = 2.51.

Radioactivity Measurement

Radioactivity of HPLC samples was obtained by liquid scintillation counting in a Packard Tri-Carb 300C liquid scintillation counter. 4 ml Optiphase ‘MP’ (Fisons PLC, Loughborough, Leics, UK) scintillator was added to 1-ml samples in minivials. Counts were converted to dpm by automatic external standardization using a previously prepared quench curve.

UV Spectroscopy

UV spectra were obtained using a Pye-Unicam SP8-100 scanning spectrometer.

Binding of AFB₁-Cl₂ to DNA

AFB₁-Cl₂ (4 mg) or [¹H]AFB₁-Cl₂ (4 mg, 1 μCi), prepared by the method of Swenson et al. (16), was dissolved in 1 ml anhydrous DMSO and added to 9 ml 1.5 v/v DMSO/25 mm sodium acetate buffer (pH 6.0) containing 10 mg calf thymus DNA. The reaction was stirred at room temperature for between 1 and 3 h and then extracted 5 times with an equal volume of water-saturated butan-1-ol. DNA was precipitated by the addition of 3 M sodium acetate (pH 6.0) to a final concentration of 0.3 M followed by two volumes of ice-cold ethanol,

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³ The abbreviations used are: AFB₁, aflatoxin B₁; AFB₁-Cl₂, 8,9-dichloro-8,9-dihydroafatoxin B₁; AFB₁-Gua, trans-9-chloro-8,9-dihydro-8-(7-guanayl)AFB₁; AFB₁-diol, 8,9-dihydro-8,9-dihydroxy AFB₁; AFB₁-epoxide, 8,9-dihydro-8,9-epoxy AFB₁; AFB₁-Gua, trans-9-dihydro-8-(7-guanyl)-9-hydroxy AFB₁; FAPyr-AFB₁, form 1, 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydro-pyridimid-5-y1 for- 
mamido)-9-hydroxy AFB₁; FAPyr-AFB₁, form 2, 8,9-dihydro-8-(2-amino-6-for- 
mamido-4-oxo-3,4-dihydopyrimid-5-y1 amino)-9-hydroxy AFB₁; HPLC, high-
performance liquid chromatography; DMSO, dimethyl sulfoxide; NMR, nuclear magnetic resonance.

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Fig. 1. Structure of aflatoxin B1.

dried under a stream of N2 and used in the experiments outlined below or immediately hydrolyzed in 0.1 M HCl at 70°C for 25 min for HPLC analysis of adducts (Gradient 1).

**Determination of AFB,-Clz-DNA Adduct Stability in Vitro**

Alkaline Conditions. 1 mg AFBi-C12 modified DNA was dissolved in 1 ml 25 mM Na2CO3/NaHCO3 buffer (pH 9.6) and incubated for 2 h at 37°C. The solution was adjusted to pH 1 with 1 M HCl and the DNA hydrolyzed as above prior to analysis by HPLC (Gradient 1).

Mildly Acidic Conditions. 10 mg [3H]AFB,-Cl2 modified DNA was dissolved in 10 ml 25 mM sodium acetate buffer (pH 5.5) and incubated for 24 h at 37°C. 2-× 0.5-ml aliquots were removed at fixed time intervals (0, 4, 10, and 24 h) and the DNA in each was precipitated by the addition of 3 M sodium acetate (pH 6.0) to a final concentration of 0.3 M followed by three volumes of ethanol. The DNA was removed from the supernatant, washed twice in 0.5 ml ethanol, and dried under vacuum. The ethanol washings were added to the precipitation supernatant. The DNA was hydrolyzed in 0.5 ml 0.1 M HCl at 70°C for 25 min and analyzed by HPLC (Gradient 2). The column eluent was collected in 1-ml fractions for radioactivity counting. The precipitation supernatants were evaporated in vacuo and solids were redissolved in 0.75 ml 20% (v/v) methanol/water prior to HPLC analysis in a similar manner.

**Large-Scale Purification of DNA Adducts**

Calf thymus DNA (50 mg) was reacted with 20 mg AFB,-Clz. Half of the recovered DNA was subjected to alkali treatment (see above) prior to acid hydrolysis, the other half was hydrolyzed immediately in 10 ml 0.1 M HCl for 25 min at 70°C. The hydrolysates were neutralized to pH 5–6 by addition of 5 M NaOH. Partial purification of the DNA adducts was achieved by passing the hydrolysate through a Waters 'Sep-Pak' C18 cartridge [obtained through Millipore (UK) Ltd., Harrow, Middlesex, UK] using the method of Bennett et al. (22). The adducts were eluted in the 80% v/v methanol/water wash. The solvent was then removed in vacuo and the residue resuspended in 40% v/v methanol/water for isocratic HPLC purification. The adducts were collected after repeated injection and concentrated to dryness by rotary evaporation and lyophilization before being redissolved in [2H6]DMSO for 1H-NMR analysis.

**RESULTS**

Binding of AFB,-Clz to DNA in Vitro and Characterization of Adducts. HPLC analysis of the acid hydrolysate of calf thymus DNA, which had been reacted with AFB,-Clz for 1 h in pH 6 buffer, yielded one major UV-absorbing peak (Fig. 2A, peak A) with a retention time of 19.6 min and two minor peaks (Fig. 2A, peaks B and C) with retention times of 17.1 and 18.0 min, respectively. Peak A is responsible for >95% of the total peak area of the chromatogram. Analysis of acid hydrolysates of DNA which had undergone reaction with AFB,-Clz for longer periods of time (up to 3 h) showed that the relative size of peak A decreased with respect to peaks B and C over time (results not shown). Furthermore, HPLC analysis also shows that the acid hydrolysate of AFB,-Clz modified DNA which had been incubated in aqueous base (pH 9.6) at 37°C prior to hydrolysis, results in the almost quantitative removal of peak A, but a concomitant increase in the size of peaks B and C (Fig. 2B). This phenomenon, which is also observed after incubation of AFB1-bound DNA in similar conditions (10, 23), is consistent with the base-assisted hydrolysis of a N7-guanine-substituted AFB1-Clz-DNA adduct to imidazole ring-opened derivatives.

The UV spectra of the compounds responsible for peaks A–C (compounds A–C) were obtained in methanol. The spectrum of A possessed absorption maxima at 264 and 364 nm and is almost identical to the spectrum recorded for AFB1-Gua (10, 12). Similarly, the spectra of B and C, which were the same as each other, were almost identical to the spectra of both FAPyr-AFB1 forms 1 and 2 (10, 13) with absorption maxima at 266, 340 (shoulder), and 366 nm. When the spectra of the three compounds were run in basic conditions, no shift was observed in the absorption maxima at 364 (A) and 366 nm (B and C), indicating that substitution had occurred at the C8-position of

Fig. 2. HPLC chromatograms of acid hydrolysates of DNA after reaction with AFB,-Clz. A was obtained after injection of a DNA hydrolysate directly after reaction with AFB,-Clz. B was obtained as A but AFB1-Clz modified DNA was incubated for 2 h in alkaline conditions (pH 9.6) prior to acid hydrolysis. The structures of compounds giving rise to peaks A–C are given in the text.
AFBi-Cl₂. The two major adducts, A and C, were purified on a large scale and their ¹H-NMR spectra obtained. The spectra of A and C are similar to those of AFBi-Gua (12) and its minor imidazole ring-opened derivative, FAPyr-AFBi form 1, respectively (13) (Tables 1 and 2) and are consistent with initial reaction occurring between the N7-position of guanine and the C8-position of AFBi-Cl₂ with retention of one chlorine atom at the C9-position of the AFBi moiety.

In compound A (Table 1), the chlorine atom deshields protons H₄, H₅, H₆, and H₇ by up to 0.7 ppm with respect to the equivalent protons in AFBi-Gua in which there is a hydroxyl group at the C9-position. The absence of spin-spin coupling between proton-pair H₄ and H₅ and protein-pair H₆ and H₇ and the presence of a small coupling between the proton-pair H₆ and H₇ (J = 5.5 Hz) in this compound indicates an identical stereochemistry to AFBi-Gua, i.e., protons H₆, H₇, and H₈ are mutually trans to each other, whereas H₄ and H₅ are in a cis configuration. Spin-spin coupling between H₄ and H₅ was established with homonuclear decoupling experiments using irradiation at δ = 4.645. This resulted in a singlet resonance for the signal assigned to H₄.

The second adduct, C, shows two separate signals for almost all the assigned protons (Table 2). A similar behaviour was observed for the imidazole ring-opened derivatives of AFBi-Gua (13). The formamide group at position 5 of the guanyl moiety of the molecule is responsible for this phenomenon. This group exists as two distinct rotamers because of restricted rotation about the guanyl C5-N4 bond. In C, the two singlet absorptions at δ = 8.38 and 7.645, in relative proportions of 1:1.8, respectively, which are ascribed to proton H₆m are close to the values obtained for the two equivalent formamide protons in FAPyr-AFBi form 1 (δ = 8.30 and 7.60 in relative proportion of 1:2) (Table 2). The combined area under these two absorptions is equivalent to one proton. Rotamer peak doubling is also observed for protons H₅, H₆, H₇, H₈, H₉, and H₁₀. Even the singlet absorption of proton H₉m showed broadening which is probably due to this phenomenon. The relative size of the peaks in each pair is the same as that for H₉m and the difference in chemical shifts is dependent on the proximity of the proton to the formamide group. Saturation transfer experiments were performed to identify the rotamer pairs for H₉, H₁₀, and H₆m by irradiation at δ = 5.515, 6.185, and 8.38, respectively. This adduct, like compound A, has an identical trans stereochemistry. This was shown by the small spin-spin coupling between protons H₅ and H₆ (J = 5.5 Hz) and the absence of coupling between proton-pair H₅ and H₆ and protein-pair H₇ and H₈. Irradiation at δ = 4.46 resulted in singlet resonances at δ = 6.64 and 6.55 which are assigned to H₇ and H₈.

Both adducts exhibit absorptions which disappear after exchange with D₂O (i.e., δ = 6.20, Adduct A), which are attributed to the amino protons in the guanine moiety of the molecule. The lack of resonances associated with the amino protons of H₇ (both compounds) and H₈ (Adduct C) is probably due to exchange broadening with the water in the [²H₆]DMSO solvent.

We attempted to obtain further evidence for the structure of these two adducts by fast atom bombardment mass spectrometry. Although the spectra of both compounds were weak, ions corresponding to [M(CCl₃) + H]⁺ and [M(Cl) + H]⁺ were observed for both compounds (m/z = 498 and 500, Adduct A, and m/z = 516 and 518, Adduct C) in approximate ratios of 3:1. Further peaks corresponding to [M + glycerol]⁺ and [M + Na]⁺ could also be detected in each of the spectra.

The results show that AFBi-Cl₂ reacts with DNA to produce, on acid hydrolysis, trans-9-chloro-8,9-dihydro-8-(7-guanyl) AFBi (compound A, AFBi-Cl₂-Gua). Incubation of this DNA adduct with alkali, prior to acid hydrolysis, causes cleavage of the 8,9-bond in the reacted guanine base. Acid hydrolysis then liberates trans-9-chloro-8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid 5-y1 formamido) AFBi (compound C). The minor product obtained after base treatment and acid hydrolysis of AFBi-Cl₂-DNA (peak B, Fig. 2) is probably equivalent to the minor imidazole ring-opened AFBi-guanine adduct, FAPyr-AFBi form 2, namely trans-9-chloro-8,9-dihydro-8-(2-amino-
6-formamide-4-oxo-3,4-dihydropyrimid-5-yl amino) AFB₁ (Fig. 3).

Stability of the Major AFB₁-Cl₂-DNA Adducts in Mildly Acidic Conditions. The data in the section above show that the primary ring-closed AFB₁-Cl₂-DNA adduct undergoes imidazole ring opening at alkaline pH in a similar manner to the analogous AFB₁-DNA adducts. However, AFB₁-Gua when bound to DNA, is also prone to other forms of decomposition, particularly acid-catalyzed depurination to release free AFB₁-Gua and produce an apurinic site in the DNA and also by hydrolysis of the AFB₁-guanine band, so yielding AFB₁-diol and leaving an unmodified guanine residue in the DNA (23–26). The stability of AFB₁-Cl₂-reacted DNA under mildly acidic conditions was, therefore, assessed. This was achieved by incubation of [³H] AFB₁-Cl₂-DNA at pH 5.5. During the incubation, aliquots of the DNA solution were removed at 0, 4, 10, and 24 h and the DNA was precipitated and acid hydrolyzed. Both the supernatant following the DNA precipitation and the acid hydrolysate were subjected to HPLC analysis and the column eluent was collected in half-minute fractions for radioactivity counting. Over the 24-h period there is a gradual loss of radioactivity from the DNA acid hydrolysate and a matched increase in radioactivity in the supernatant. The majority of the radioactivity elutes with the retention time of AFB₁-Cl-Gua (Fig. 4). A small increase in the amount of radioactivity eluting from the column prior to AFB₁-Cl-Gua was also noted in the analysis of the supernatant (Fig. 4). This may be due to the breakdown of AFB₁-Cl₂-bound DNA to 9-chloro-8,9-dihydro-8-hydroxy aflatoxin B₁ (AFB₁-chlorohydrin), AFB₁-diol and related compounds, which are similarly released from AFB₁ and AFB₁-Cl₂ aducted DNA on incubation in vitro (16, 23, 24, 26).

The results shown in Fig. 4 indicate that over the 24-h period, 60% of the radioactivity associated with the DNA is lost. Approximately 80% of this loss arises from depurination of the ring-closed adduct (AFB₁-Cl-Gua) from the DNA and the remaining 20% is presumably lost through the release of AFB₁-chlorohydrin, AFB₁-diol, etc. As expected, there is no significant increase or decrease in the radioactivity associated with the small amount of imidazole ring-opened adducts in the DNA over the 24-h period (Fig. 4). At the low pH at which this experiment was conducted, ring opening of the primary DNA adduct is unlikely to occur and the ring-opened adducts themselves are stable to depurination and are not lost from the DNA.

DISCUSSION

The NMR spectra of the two AFB₁-Cl₂-guanine adducts (Tables 1 and 2) confirm that, like AFB₁-epoxide, AFB₁-Cl₂ reacts in vitro at the N7-position of guanine in DNA. One aspect of the NMR spectra which is pointed out in “Results,” is that many of the individual proton resonances in the AFB₁-Cl₂-guanine adducts are shifted to lower field with respect to the equivalent protons in AFB₁-Gua and FA-Pyr-AFB₁, form 1. The chlorine atom at the C9-position of the AFB₁ moiety in the AFB₁-Cl₂-derived adducts evidently has a greater NMR-de-shielding effect than the hydroxyl group it replaces in the AFB₁-epoxide-guanine adducts. This phenomenon is not unexpected as a similar effect is observed when a comparison is made of the proton NMR resonances in various other AFB₁-related compounds (14, 15, 27). For instance, the hydrogen atoms in the fused bis-furan moiety of trans-AFB₁-Cl₁ possess NMR signals at distinctly higher frequencies (up to 0.86 ppm) than their counterparts in AFB₁-diol (15, 27). Analogous data are observed with the ¹H-NMR resonances of the trans-2,3-dichloro-2,3-dihydro- and trans-3-chloro-2,3-dihydro-2-hydroxy-derivatives of 3a,8a-dihydrofuro[2,3-b]benzofuran (15). It is worth noting that substitution of either the chlorine atoms or hydroxyl groups with bromine atoms in these AFB₁-derived molecules generally leads to even greater downfield shifts of these hydrogen atoms in the ¹H-NMR spectra (15).

It is known that chlorination of AFB₁ with chlorine is not stereospecific (14, 15) and affords both cis- and trans-dichloride isomers [chlorination of AFB₁ with N-chloropyridinium chloride yields 80% of the cis isomer and 20% of the trans isomer (15)]. It would, therefore, have been expected to obtain AFB₁-

Fig. 4. Time-course study to show the stability of the major AFB₁-Cl₂-DNA adducts when modified calf thymus DNA is incubated in mildly acidic (pH 5.5) buffer for 24 h. 2- x 5.0-ml aliquots of the DNA were removed at the time intervals shown and the DNA precipitated and acid hydrolyzed. Both the supernatant of the DNA precipitation and DNA acid hydrolysate were analyzed for radioactivity after HPLC. The following data are shown: (a) The amount of radioactivity in the DNA acid hydrolysate at the retention time of AFB₁-Cl-Gua on HPLC (— — —); (b) The amount of radioactivity in the supernatant obtained after precipitation of the DNA eluting at the retention time of AFB₁-Cl-Gua on HPLC (— — —); (c) same as b but the amount of radioactivity eluting from the column in the 10-min period prior to the retention time of AFB₁-Cl-Gua (— — — —); (d) same as a but the amount of radioactivity eluting from the column at the retention time of imidazole ring-opened AFB₁-Cl₂-guanine adducts (— — — — —).
Cl₂-guanine adducts with mixed stereochemistry at the 8,9-bond of the AFB₁ moity. As AFB₁-epoxide reacts with guanine in DNA to yield only trans adducts and since it cannot be ruled out that the cis- and trans-AFB₁-Cl₂-DNA adducts may induce greatly different biological effects, then it is possible that the formation of even a tiny amount of cis-AFB₁-Cl₂-guanine adducts in DNA could potentially invalidate the use of AFB₁-Cl₂ as a model for AFB₁-epoxide. However, HPLC analysis (Figs. 1 and 2) of the adducts resolves only three peaks which are attributable to AFB₁-Cl₂-Gua and the two imidazole ring-opened species. The use of slower HPLC gradient systems (e.g., Gradient 2, “Materials and Methods”), or an isocratic elution system (40:60 v/w methanol/water) did not provide any evidence for other products (data not shown). Also, if both cis and trans adducts coelute on HPLC then the presence of cis adducts would have been observed in the NMR spectra. No evidence for the formation of cis adducts was obtained from any of the NMR spectra produced during these studies. It is still conceivable that a very low percentage of the cis adducts is produced and is not detected by either HPLC or NMR. However, if this is so, their presence in DNA does not appear to cause significant qualitative or quantitative changes in the biological effects exerted by the adducts derived from reaction of AFB₁-epoxide and DNA. Humayun and colleagues have shown that both AFB₁-Cl₂ and AFB₁-epoxide guanine adducts behave almost identically as DNA replication blocks in vitro (19, 20) and that they also induce a highly similar frameshift mutation spectra in a phage M13mp8 DNA-mediated mutagenesis assay after infection of Escherichia coli cells with either AFB₁-Cl₂ or AFB₁-epoxide modified vectors (21). We propose two possible explanations for the apparent sole formation of trans-AFB₁-Cl₂-guanine adducts: (a) Both cis and trans isomers of AFB₁-Cl₂ react with guanine bases in DNA by an Sₗ₁ reaction pathway. If so, then both isomers will produce a common carbonium ion intermediate which could attack guanine selectively to yield the trans-adduct. (b) Only the cis-dichloride reacts with DNA. If this is so, then reaction must take place by an Sₗ₂ mechanism, so that the product has the observed trans configuration.

Studies have been carried out to determine the stability of the AFB₁-Cl₂-DNA adducts upon incubation of the modified DNA at either alkaline (pH 9.6) or mildly acidic (pH 5.5) pH. As expected from the known base lability of DNA-bound AFB₁-Chlorohydrin and AFB₁-diol, from the DNA during the time-course study. From the data displayed in Fig. 4, it is estimated that under these conditions the half-life of the primary AFB₁-Cl₂-DNA adduct is approximately 16 h. This value is in agreement with those calculated by other workers studying the stability of AFB₁-DNA adducts in vitro [half-lives ranging from 60 to 60 h have been published (23, 24, 26)]. However, further experiments need to be performed to determine whether the data presented here are thoroughly consistent with results from these other studies since the relative rates of depurination of AFB₁-Gua and release of compounds like AFB₁-diol from DNA in solution is known to be dependent on both pH and the level of initial AFB₁-DNA binding (23, 24, 26).

In summary, the experiments described in this paper confirm that AFB₁-Cl₂ is a good model of AFB₁-epoxide. Structural determination of the two major DNA adducts has shown that AFB₁-Cl₂ like AFB₁-epoxide, binds to DNA at the N7-position of guanine to produce adducts with a trans configuration about the 8,9-bond of the AFB₁ moiety. These adducts appear to behave comparably to their equivalent AFB₁-epoxide-derived DNA adducts during incubation of the modified DNA under either alkaline or mildly acidic conditions.

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