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Depurinating Acylfulvene−DNA Adducts: Characterizing Cellular Chemical Reactions of a Selective Antitumor Agent

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Abstract: Acylfulvenes (AFs) are a class of semisynthetic agents with high toxicity toward certain tumor cells, and for one analogue, hydroxymethylacylfulvene (HMAF), clinical trials are in progress. DNA alkylation by AFs, mediated by bioreductive activation, is believed to contribute to cytotoxicity, but the structures and chemical properties of corresponding DNA adducts are unknown. This study provides the first structural characterization of AF-specific DNA adducts. In the presence of a reductive enzyme, alkenal/one oxidoreductase (AOR), AF selectively alkylates dAdo and dGuo in reactions with a monomeric nucleoside, as well as in reactions with naked or cellular DNA, with 3-alkyl-dAdo as the apparently most abundant AF−DNA adduct. Characterization of this adduct was facilitated by independent chemical synthesis of the corresponding 3-alkyl-Ade adduct. In addition, in naked or cellular DNA, evidence was obtained for the formation of an additional type of adduct resulting from direct conjugate addition of Ade to AF followed by hydrolytic cyclopropane ring-opening, indicating the potential for a competing reaction pathway involving direct DNA alkylation. The major AF-dAdo and AF-dGuo adducts are unstable under physiologically relevant conditions and depurinate to release an alkylated nucleobase in a process that has a half-life of 8.5 h for 3-alkyladenine and less than approximately 2 h for dGuo adducts. DNA alkylation further leads to single-stranded DNA cleavage, occurring exclusively at dGuo and dAdo sites, in a nonsequence-specific manner. In AF-treated cells that were transfected with either AOR or control vectors, the DNA adducts identified match those from in vitro studies. Moreover, a positive correlation was observed between DNA adduct levels and cell sensitivity to AF. The potential contributing roles of AOR-mediated bioactivation and adduct stability to the cytotoxicity of AF are discussed.

Introduction

DNA-alkylating agents have contributed to significant improvements in cancer therapy in the past five decades and remain at the front line of efforts to develop improved antitumor agents.[1−5] Alkylation of cellular DNA bases can result in various biological consequences, including blockage of DNA replication and transcription, inhibition of gene expression, and DNA strand cleavage.[3,6−8] These processes lead to cytotoxic and cytostatic effects on cancer cells,[2,4] but dose-limiting toxicities and poor selectivities are major barriers for success for alkylating agents. Natural product-derived alkylating agents can exhibit unique selectivity profiles, and understanding relevant chemical mechanisms can reveal new strategies for selectively targeting tumor cells.[9]

Acylfulvenes (AFs) are a class of antitumor agents derivatized from mushroom-derived sesquiterpene illudins.[10] Illudin S and M (**1** and **2**, Chart 1) possess potent antitumor activities (IC_{50} = 4 nM in MV 522 cell lines) but low therapeutic indices as high toxicities prevent effective clinical applications.[11,12] AFs have improved selectivity profiles for cancer cells and are notably nonapoptotic against normal cells.[13−18] One analogue,

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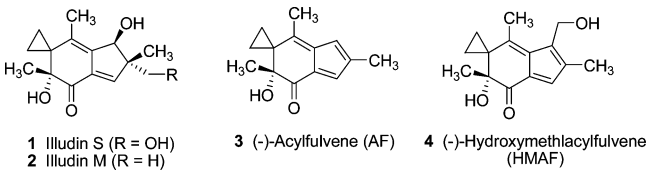
Chart 1. Structures of Natural Illudins and Semisynthetic Derivatives, AFs



(−)-hydroxymethylacylfulvene (**4**, (−)-HMAF; Chart 1) is currently in clinical trials and is being evaluated in the treatment of various human cancers including prostate and advanced solid and ovarian tumors.[19−23] Emerging concerns regarding dose-limiting toxicities, however, indicate that an improved understanding of the detailed mechanism of action is required to elucidate the novel mechanism of AFs for selective toxicity as well as for identifying strategies for effectively targeting sensitive cancers and for developing improved combination therapies.[24,25]

Like many well-characterized alkylating antitumor agents,[26−29] the cytotoxicities of AFs are thought to originate primarily from covalent binding to DNA[16,17,30,31] DNA damage induction by AFs has been established by incorporation of radioactivity into DNA isolated from cells treated with [^{14}C]AFs.[16,30] Furthermore, recent studies indicate that the toxicities of AFs are influenced by deficiencies in certain DNA repair pathways, such as transcription-coupled nucleotide excision repair (TC-NER) enzymes.[32,33]

AFs are less chemically reactive than the parent illudins and have generally higher IC_{50}'s for cancer cell lines.[12,34] On the basis of the structures of AF metabolites, it has been proposed

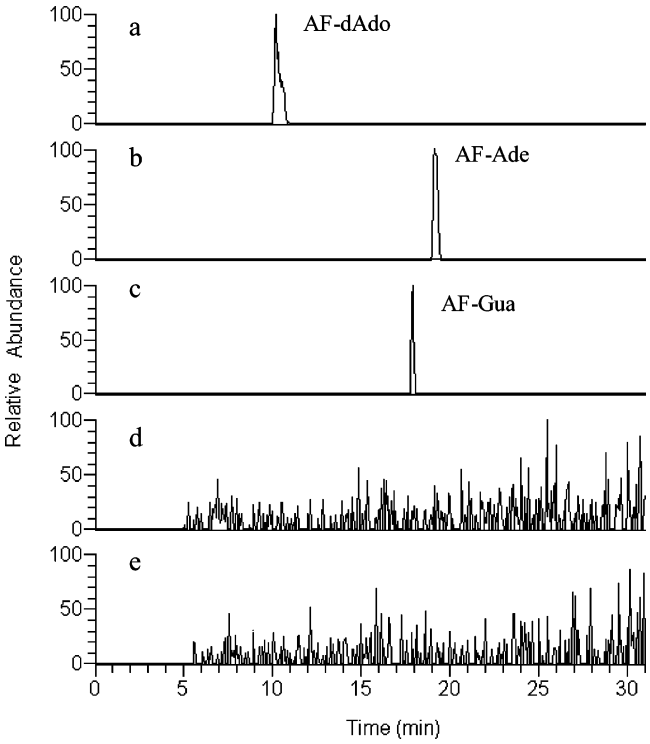


Figure 1. Extracted ion chromatographs from HPLC-ESI-MS/MS analysis of reactions of AF with monomeric nucleosides in the presence of AOR/NADPH. (a) **AF-dAdo**, transition of *m/z* 452 $[M + 1]^+$ to *m/z* 336 $[M -$ deoxyribose $+ 1]^+$; (b) **AF-Ade**, transition of *m/z* 336 $[M + 1]^+$ to *m/z* 201 $[M - Ade]^+$; (c) **AF-Gua**, transition of *m/z* 352 $[M + 1]^+$ to *m/z* 201 $[M - Gua]^+$; (d) **AF-dCyd**, transition of *m/z* 428 $[M + 1]^+$ to *m/z* 201 $[M - dCyd]^+$; (e) **AF-Thd**, transition of *m/z* 443 $[M + 1]^+$ to *m/z* 201 $[M - Thd]^+$.

that reductive bioactivation, by cytosolic enzymes[14,35,36] via reduction of the carbon−carbon double bond of the α, β-unsaturated ketone, leads to the formation of the postulated reactive cyclohexadiene intermediate **5** (Scheme 1). We have obtained evidence suggesting the biological role of a specific enzyme, alkenal/one oxidoreductase (AOR),[37−40] in the activation of AFs,[41−43] including an association between elevated AOR levels and increased cellular sensitivity to AFs.[41−43]

Putative chemical transformations of AFs are illustrated in Scheme 1. Hydrolysis of **5** gives rise to the major observed metabolite **8**. Intermediate **5** may also lead to monofunctional DNA adducts, via attack of the cyclopropyl group by a DNA base, though such monoadducts generally are considered less lethal than cross-links common to DNA-targeting anticancer agents, such as nitrogen mustards.[44] Alternatively, AFs could be envisioned to be activated chemically if cellular nucleophiles, such as glutathione, were to attack the carbon−carbon double

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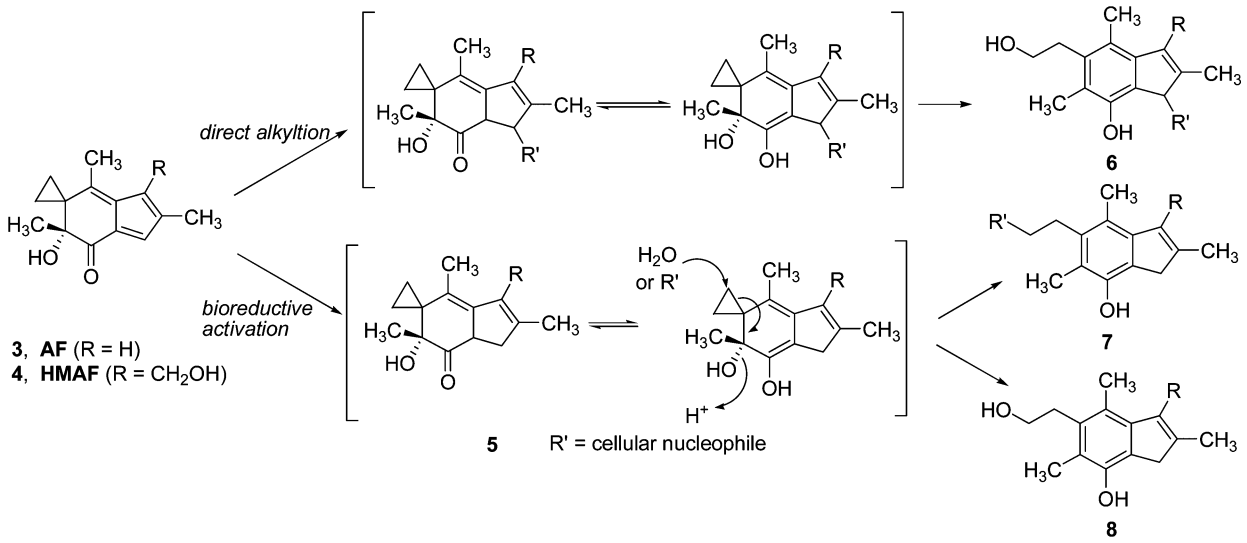
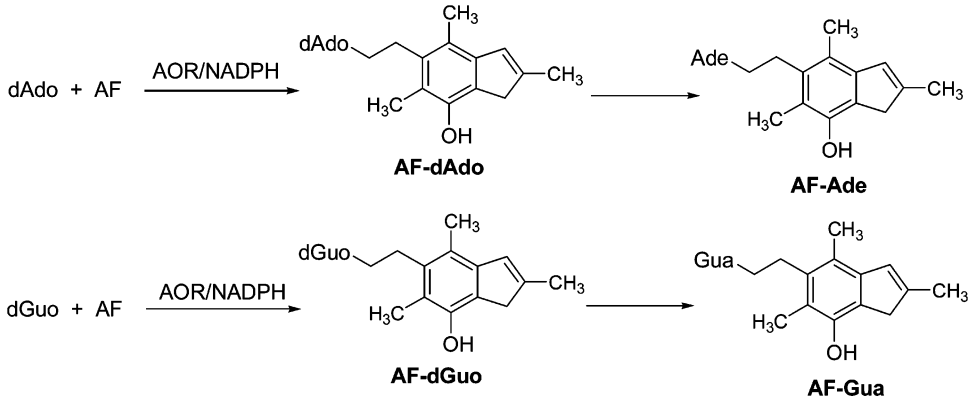
Scheme 1. Proposed Mechanism for AF Metabolism and Formation of the Major Metabolite **8** or DNA Adducts



Scheme 2. AOR-Mediated Reactions of AF with dAdo and dGuo

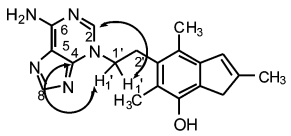


bond to form **5**. Elucidating which chemical transformations may underlie the biological activities of AFs depends on the characterization of AF−DNA adducts and assessing their biological significance. The structures and chemical properties of DNA adducts can influence cellular responses including DNA repair, transcription, and cell death. Herein, we report the first characterization of reactions between AF (**3**, Chart 1) and nucleosides, on the level of individual nucleosides, in naked DNA and in cells. These results show that AF alkylates purine bases, giving rise to chemically labile adducts. AF−DNA adducts observed in cells treated with AF match those identified from in vitro reactions, and relative levels correlate with cell sensitivity and reductase expression.

Results

Reactions of AF with Nucleosides in the Presence or Absence of AOR. As an initial step toward characterizing DNA damage induced by AF, reactions with monomeric deoxynucleosides were performed as simple and easily analyzed model systems. Additionally, these studies were used to establish analytical methods required to probe adduct formation in double-stranded DNA and in cells. On the basis of proposed mechanisms of DNA alkylation by AF (Scheme 1), DNA adducts formed from competing direct vs bioactivation-coupled pathways are predicted to contain isomeric indene moieties with characteristic MS/MS fragmentation patterns. Therefore, HPLC-MS/MS is an ideal tool to distinguish products resulting from different reaction pathways. Monomeric deoxynucleosides were reacted individually with AF in the presence or absence of AOR/NADPH, at 37 °C for 24 h in a phosphate-buffered solution (pH 7.4). After solid-phase extraction, reaction mixtures were analyzed by HPLC-ESI-MS/MS. Both deoxyadenosine and deoxyguanosine (dAdo and dGuo, respectively) were readily alkylated by AF in the presence of AOR/NADPH (Figure 1a−c), but under the same reaction conditions, no adducts were observed in reaction mixtures from deoxycytidine or thymidine (dCyd or Thd, respectively) reactions (Figure 1d, e). In the absence of AOR/NADPH, we examined the direct reaction of AF with monomeric deoxynucleosides (Scheme 1). No nucleoside adducts were detected by HPLC-MS/MS, and AF remained unchanged. To examine whether AOR or NADPH alone activates AF, control experiments were carried out in which dAdo was reacted with AF in the presence of AOR or NADPH; no adducts were observed in either case.

HPLC-MS/MS analysis of the dAdo reaction mixture displays a minor peak eluting at 13.5 min with *m*/*z* 452 and a major peak eluting at 19.3 min with *m*/*z* 336 (Figure 1a, b). MS/MS analysis of the peak at 13.5 min yields the fragments *m*/*z* 336 (M − deoxyribose + 1) and *m*/*z* 201 (M − dAdo), consistent with **AF-dAdo** (Scheme 2). The peak eluting at 19.3 min displays a major fragment ion *m*/*z* 201, consistent with depurination of **AF-dAdo**. The positively charged **AF-dAdo** is



20

Proton	Chemical Shift (ppm)	Carbon	Chemical Shift (ppm)
$H_{1'}$	4.3	C_2	143
$H_{2'}$	3.2	C_4	150
H_2	7.8	C_5	122
H_8	8.1	C_6	156

Figure 2. Selected proton−carbon correlations observed in the $^1H-^{13}C$ gHMBC NMR spectrum, and chemical shifts of selected protons and carbons of **20**.

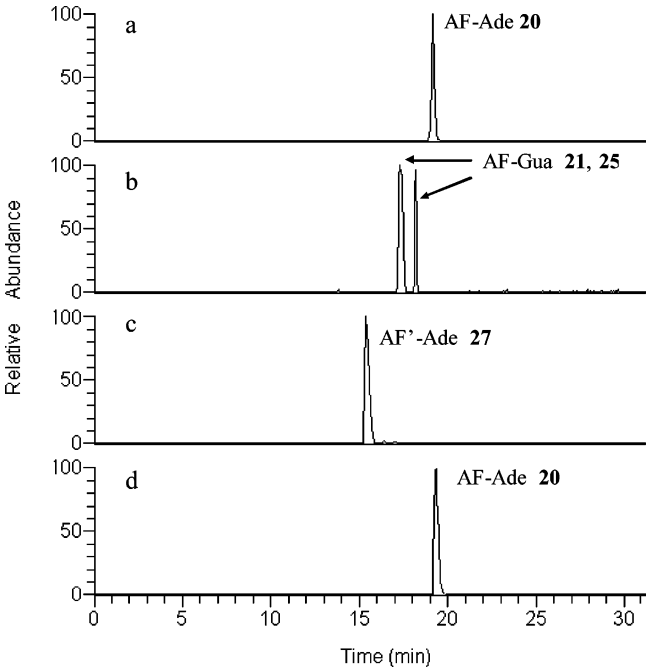


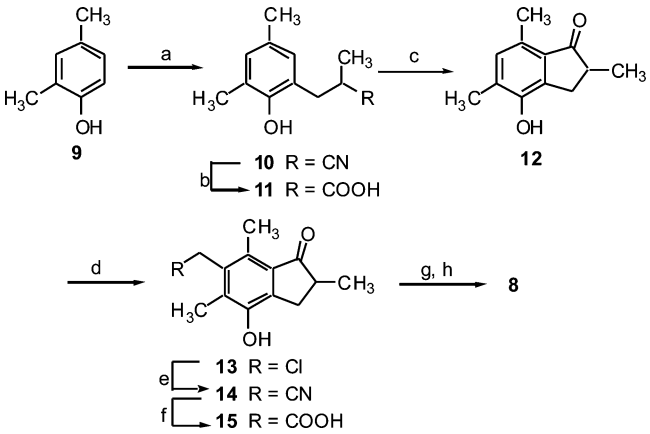
Figure 3. Extracted ion chromatographs from HPLC-ESI-MS/MS analysis of hydrolyates of DNA samples treated with AF in the presence of AOR/NADPH. (a) **20**, transition of *m*/*z* 336 $[M + 1]^+$ to *m*/*z* 201 $[M - Ade]^+$; (b) **21** and **25**, transition of *m*/*z* 352 $[M + 1]^+$ to *m*/*z* 201 $[M - Gua]^+$; (c) **27**, transition of *m*/*z* 352 $[M + 1]^+$ to *m*/*z* 217 $[M - Ade]^+$; (d) Co-injection of synthetic **20** with DNA sample, transition of *m*/*z* 336 $[M + 1]^+$ to *m*/*z* 201 $[M - Ade]^+$.

thermally unstable: heating the reaction mixture (90 °C, 30 min) results in the disappearance of the peak corresponding to **AF-dAdo** and produces a chromatogram in which **AF-Ade** is the only detected product, consistent with the conversion of **AF-dAdo** to **AF-Ade**.

HPLC-MS/MS analysis of a dGuo reaction mixture displays a peak eluting at 18.2 min with *m*/*z* 352 and a major MS/MS fragment ion *m*/*z* 201 (Figure 1c), consistent with **AF-Gua** (Scheme 2). These data are consistent with a similar pathway as that indicated for **AF-Ade** adduct, i.e., formation of a positively charged dGuo adduct which readily depurinates resulting in an AF-base adduct. The difference between the reactions with dAdo and dGuo is that the postulated intermediate **AF-dGuo** (Scheme 2) was not detected by HPLC-MS/MS. The absence of **AF-dGuo** may reflect decreased relative stability.

Identification of the AF-dAdo and AF-Gua Adduct. Levels of adduct derived from the AF-treated nucleosides described above were insufficient for definitive structural characterization by NMR analysis. Therefore we sought a synthetic route for preparing the depurination product **AF-Ade** and **AF-Gua**. Alkylation at both 3- and 7-positions of dAdo or dGuo labilizes the modified bases,[45−47] and both would be consistent with the

Scheme 3. Chemical Synthesis of the Major AF Metabolite **8**[a]



[a] Reagents and conditions: (a) $HCl/AlCl_3$, methacronitrile; (b) 10% NaOH, reflux; (c) $AlCl_3$, 200 °C; (d) $SnCl_4$, MOMCl; (e) KCN, DMF; (f) 10% NaOH, reflux; (g) BH_3/THF; (h) MeOH, H_3O^+.

MS/MS data. In this regard, two-dimensional heteronuclear NMR experiments are useful for distinguishing potential isomers.[48] A common structural feature of AF-DNA adducts resulting from AOR-mediated reactions is the indene moiety. We proposed that a useful synthetic intermediate for probing the identity of bioactivation-derived AF adducts would be AF metabolite **8**, with the primary alcohol converted into a leaving group. Metabolite **8** can be obtained by enzyme-mediated reduction of AF followed by hydrolysis or by treating AF with zinc in acid. These approaches are inefficient because they involve the synthesis of the tricyclic AF core structure, only to destroy the three-membered ring and aromatize the six-membered ring. We therefore desired an electrophilic precursor that could be prepared in abundant quantities from readily available reagents and would be amenable to structural modifications that would be useful in the subsequent studies of related AF derivatives.

The synthesis of **8** was accomplished in eight steps starting from 2,4-dimethylphenol **9** (Scheme 3). Thus, **9** was treated with methacronitrile in the presence of anhydrous $AlCl_3$ and dry HCl, affording **10** in 60% yield. The cyano group in **10** was hydrolyzed in aqueous NaOH to yield acid **11** in 90% yield. Cyclization of **11** was accomplished in 85% yield by treating with $AlCl_3$/NaCl at 200 °C. The desired chloromethylation of **12** was problematic because common chloromethylation conditions involving formaldehyde in concentrated HCl resulted in the formation of an acid-catalyzed aldol reaction product, in which the α-carbon of the ketone was substituted. Using more vigorous conditions (MOM−Cl, $SnCl_4$) however, **12** is con-

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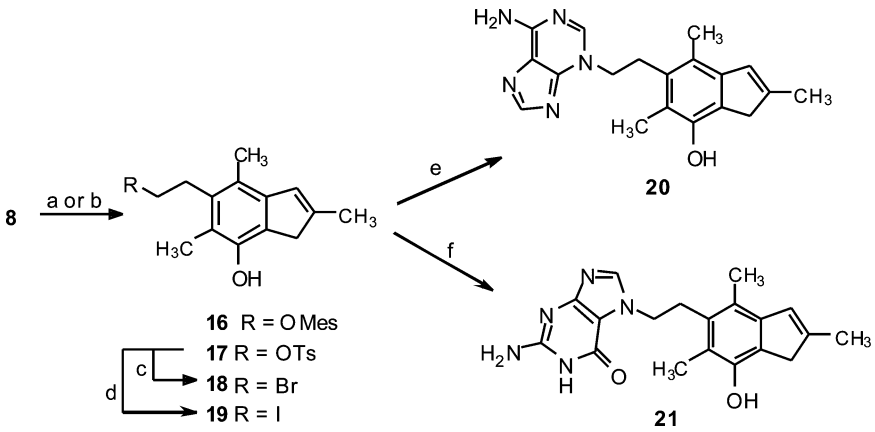
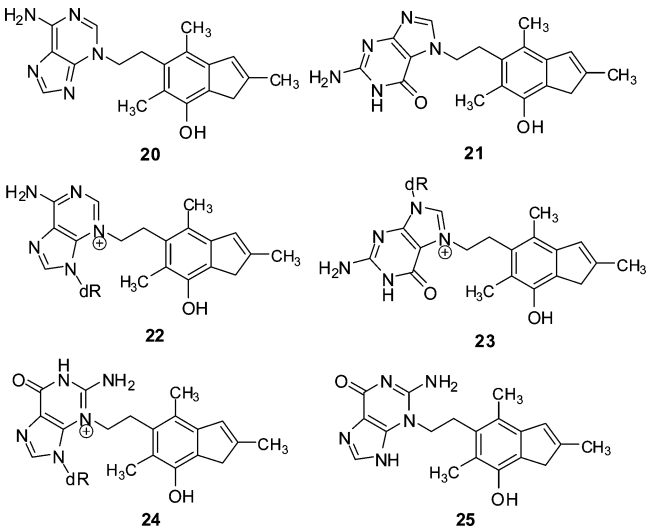
Scheme 4. Chemical Synthesis of Adduct **20** and **21**[a]



[a] Reagents and conditions: (a) 2-mesitylenesulfonyl chloride/pyridine; (b) *p*-toluenesulfonyl chloride/pyridine; (c)NaBr, DMF; (d) NaI, acetone, reflux; (e) Ade, DMA, 80 °C; (f) dGuo, DMA, 80 °C.

Chart 2. Structures of AF−DNA Adducts Proposed on the Basis of MS and MS/MS Data



verted into **13** in 70% yield. Chloride **13** was treated with KCN followed by base-catalyzed hydrolysis to generate acid **15**. Both carboxyl and ketone groups of **15** were reduced in one step using excess BH_3. Acidic workup resulted in metabolite **8** in 65% yield.

The primary alcohol of **8** was converted into various leaving groups, and their reactivities toward Ade were tested (Scheme 4). Neither a *p*-toluenesulfonyl or a 2-mesitylenesulfonyl nor an iodide derivative of **8** reacted with Ade, up to 80 °C for 48 h. On the other hand, under the same conditions, when Ade was reacted with bromide derivatives **18**, which was obtained in 50% yield by treating **17** with NaBr, product **20** was genereated with chromatographic properties consistent with those of **AF-Ade**. This material was purified crudely by normal-phase flash chromatography, followed by a Biotage Sp1 high performance flash chromatography system equipped with a reversed-phase flash column, yielding 1−2% of the product. The yield could not be improved by adding AgOTf or by varying the reaction temperature, time, and relative equivalents of reactants, but the amount of product generated was sufficient for structural characterization. The synthetic material had the same HPLC retention time, *m*/*z*, and MS/MS fragmentation patterns as those observed from **AF-Ade** produced in the AOR-mediated reaction of dAdo with AF, indicating that they are identical. ^{1}H and ^{1}H−^{13}C gHMQC NMR analysis (Supporting Information) resulted in a spectrum consistent with the formulation of an adduct that contains both indene and Ade moieties. To unambiguously assign the substitution site of Ade, a 2-D ^{1}H−^{13}C gHMBC NMR spectrum was obtained (Supporting Information). These data indicate correlations of methylene protons of indene H_1' (4.3 ppm) with C-2 (143 ppm) and C-4 (150 ppm) of Ade (Figure 2), demonstrating that the final product of the reaction of AF with dAdo is the 3-substituted Ade adduct.

In contrast to the reaction of Ade, reaction of bromide **18** with dGuo did not result in any products consistent with an **AF-Gua** adduct. However, the iodide **19** readily reacts with dGuo at 80 °C in DMA to afford a product that coelutes with the **AF-Gua** adduct that results from the reaction between dGuo and AF in the presence of AOR (Scheme 4). This compound (**21**) was isolated in 5% yield in a similar manner to that for **20**. Further analysis by HPLC-MS/MS indicates that **21** is identical to the **AF-Gua** adduct, and NMR analysis (Supporting Information), including a 2-D ^{1}H−^{13}C gHMBC NMR, demonstrated that compound **21** is a 7-substituted guanine adduct.

To enable the comparison of the relative abundances of Ade adduct **20** and Gua adduct **21** on the basis of HPLC-MS/MS peak areas, a sample containing equal concentrations of **20** and **21** (0.1 μM) were analyzed by HPLC-MS/MS and relative sensitivities for ESI-MS detection were determined. The relative response factor of adduct **20** is 2.5 times higher than that of **21**, and the HPLC-MS/MS peak areas of **20** generated from the AOR-mediated reaction of AF with dAdo is 250 times higher than that of **21** generated from the AOR-mediated reaction of AF with dGuo. This indicates that, under the same conditions, the reaction between dAdo and AF in the presence of AOR is about 100 times more efficient than that of dGuo.

Reaction of AF with Calf Thymus DNA. The chemical reactivities of nucleotides in DNA are influenced by the structural conformation of the double-stranded helix. Furthermore, cellular DNA is confined to the nucleus, forming a complex with histone proteins with highly ordered structures.[49] Despite the differences between the chemical environments of naked vs cellular DNA, naked DNA can serve as an effective

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Table 1. Extracted Ion Chromatographs of Reactions of Calf Thymus DNA with AF

adducts	AOR	MS peaks	MS/MS
AF-dAdo	+	452	336/201
AF-Ade	+	336	201
AF-Gua (two peaks)	+	352	201

model, often yielding similar patterns of adduct formation.[50−53] We therefore investigated the reaction of AF with naked DNA as a model to characterize potential cellular reactions of AF.

Calf thymus DNA was allowed to react with AF under the same conditions as those described for monomeric nucleoside reactions. Alkylated DNA was precipitated, isolated, and hydrolyzed enzymatically with DNase I, phosphodiesterase I, and alkaline phosphatase. Both the supernatant of DNA reaction mixtures and hydrolyzed DNA samples were analyzed by HPLC-MS/MS. Results indicate that, in the presence of AOR/NADPH, AF reacts with DNA giving rise to four main products (Figure 3). In both supernatant and hydrolyzed DNA samples, **20** appears to be the most abundant peak accounting for 98% of the total peak areas for adducts either in the hydrolysate or in the supernatant. The presence of **20** in the supernatant indicates that AF-dAdo adduct **22** (Chart 2) is chemically labile in double-stranded DNA and depurinates at 37 °C and pH 7.4, releasing the modified nucleobase and generating an abasic site in the DNA strand.[46,47] A peak corresponding to **22** also was observed in hydrolyzed DNA samples but slowly converted to **20** in neutral aqueous solution at 25 °C. Consistent with results from nucleoside studies discussed above, dGuo adducts are minor DNA alkylation products in both supernatant and DNA hydrolysate. In addition to the adduct **21** that elutes at 18.2 min, another peak corresponding to a mass consistent with AF-modified Gua was observed to elute at 17.1 min (Figure 3b). The identical MS and MS/MS spectra (Table 1) of both Gua adducts indicate that they are isomeric and result from alkylation at different sites on dGuo, inducing depurination of modified Gua from double-stranded DNA. Potential reactions at the 7- and 3-position of dGuo are consistent with this type of reactivity, such that the potential structure for the new guanine adduct may be **25** (Chart 2).

To test whether DNA reacts with AF in the absence of a metabolic enzyme, DNA was allowed to react with AF in a phosphate buffer (pH 7.4) at 37 °C for 24 h. DNA was isolated and enzymatically hydrolyzed as described above. HPLC-MS/MS analysis of DNA hydrolysate displays a peak that eluted at 15.1 min with *m*/*z* 352 (M + 1) (Figure 3c) and MS/MS of 217 (M-dAdo), consistent with an adduct resulting from conjugate addition to the α,*β*-unsaturated ketone of AF, followed by hydrolytic cyclopropyl ring-opening illustrated by **27** (Scheme 5). An adduct with this *m*/*z* and MS/MS pattern was not detected in the direct reaction of AF with dAdo or in the reaction of AF with DNA in the presence of AOR/NADPH. The relative abundance of **27**, on the basis of the resulting HPLC-MS/MS spectrum, is estimated to be about 0.1% that of **20** resulting from the reaction between AF and DNA, indicating the diminished direct reactivity of AF (Scheme 5, pathway A) compared to that of the intermediate generated in the bioactivation-mediated process (Scheme 5, pathway B). In the same reaction mixture, DNA adducts that may have been formed by direct chemical reaction of AF with other bases in DNA were not observed. Peaks corresponding to **AF-dGuo** (transition of *m*/*z* 484 to *m*/*z* 217), **AF-dCyd** (transition of *m*/*z* 444 to *m*/*z* 217), and **AF-Thd** (transition of *m*/*z* 459 to *m*/*z* 217) were not detected in HPLC-MS/MS analysis. DNA cross-links such as **Ade-AF-Ade** (*m*/*z* 471) and **Ade-AF-Gua** (*m*/*z* 487), that may have been formed through the nucleopilic attack of a DNA base to putative intermediate **5**, which would result from the direct reaction of AF with DNA, were not observed.

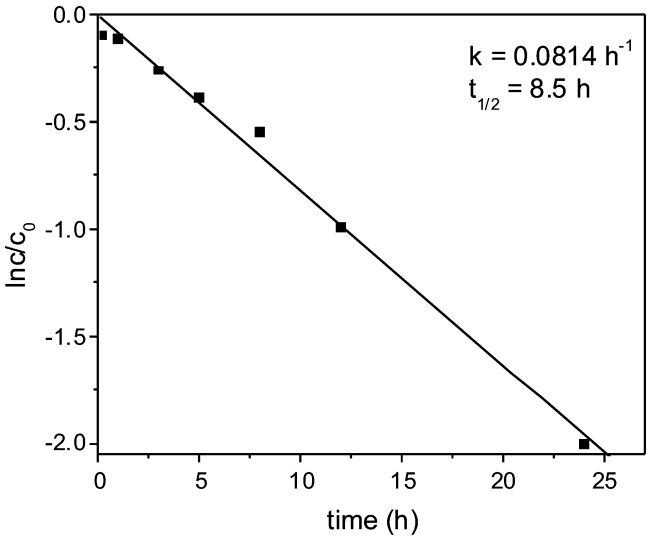

Figure 4. Neutral thermal hydrolysis of **22** from double-stranded DNA.

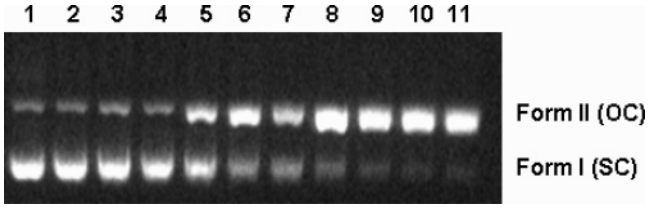

Figure 5. Cleavage of pBR322 DNA (300 ng) by AF in the presence of AOR (10 ng)/NADPH (200 μM) in TBE at 37 °C. Lane 1: Control pBR322 DNA (300 ng); lane 2: DNA (300 ng) + AF (250 μM); lane 3: DNA + AOR (10 ng); lane 4: DNA + AOR (10 ng) + NADPH (200 μM); lane 5−11: DNA + AOR (10 ng) + NADPH (200 μM) + AF (10, 20, 30, 40, 50, 60, 70 μM, respectively).

Stabilities of AF−DNA Adducts in Double-Stranded DNA. The release of AF adducts from double-stranded DNA creates abasic sites in DNA, which raises the question of whether DNA-interacting proteins encounter DNA−AF adducts or the resulting abasic sites. Because the chemical stabilities of adducts may be useful for elucidating their potential biological relevance, we determined the $t_{1/2}$ of **22** in double-stranded DNA. Calf-thymus DNA was treated with AF in the presence of AOR/NADPH. Alkylated DNA was isolated and redissolved in phosphate-buffered solution (pH 7.4). The resulting solution was heated at 37 °C, and the release of **20** was monitored by removing aliquots and analyzing by HPLC-MS/MS. An estimate of the initial concentration of **22** was established by heating the alkylated DNA at 90 °C for 1 h. Further heating of DNA

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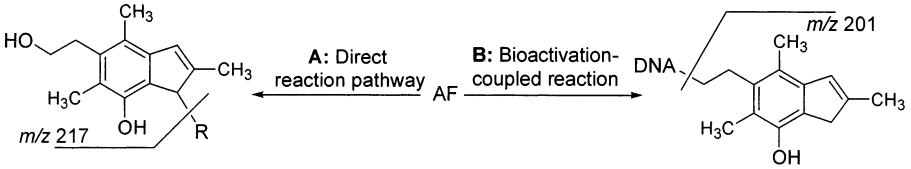
Scheme 5. Putative Competing DNA Alkylation Pathways



6 R = DNA
26 R = dAdo
27 R = Ade

7

does not give increased levels of adducts, indicating complete release of adducts from DNA. The analysis of modified DNA was carried out until over 90% of adduct underwent depurination (ca. 24 h). For MS analysis, N^2-Bn-dG[54] was used as an analytical injection standard and the peak area of **20** in each sample was normalized according to this standard. Data obtained from HPLC-MS/MS analysis yielded the rate constant $k = 0.081$ h^{-1} (Figure 4) for depurination of AF-dAdo **22** in DNA; $t_{1/2} =$ 8.5 h. Using the same analytical method, which was optimized for detection and resolution of adduct **20**, the peaks corresponding to the two dGuo adducts were not resolved. After heating at 37 °C for 8 h, however, the unresolved peak corresponding to **21** and **25** on HPLC-MS/MS spectra saturated such that the Gua adduct levels were the same as that from a sample heated at 90 °C for 1 h. Complete depurination within 8 h, assuming approximately four to five-half-lives, provides a crude estimate of the stabilities of **23** and **24** at under 2 h.

DNA Relaxation Assay. There is a substantial precedent for DNA-alkylating agents to result in depurinating adducts.[47] The formation of labile adducts can induce single-strand breaks in double-stranded DNA under basic conditions.[6] To probe whether labile AF adducts result in DNA strand cleavage, relaxation assays of supercoiled plasmid DNA were carried out. Single-stranded cleavage of circular pBR322 DNA (form I) can lead to the formation of open-circular DNA (form II), whereas double-stranded cleavage results in linear DNA (form III). Supercoiled plasmid DNA (form I) was reacted with AF at 37 °C for 48 h under alkaline conditions (pH 8.3) in the presence or absence of AOR (10 ng) and NADPH (200 μM). In the resulting gel electrophoretogram, no cleavage was observed for the DNA treated with AF (250 μM), AOR, or NADPH alone (Lanes 1−4, Figure 5). Addition of decreasing amounts of AF (10−70 μM) in the presence of AOR (10 ng) and NADPH (200 μM), however, resulted in cleavage of DNA from form I to form II (Lanes 5−11). A linear form of DNA, resulting from double-stranded cleavage, was not observed. A similar experiment was carried out in phosphate buffer (pH 7.4) in order to analyze whether AF can induce DNA cleavage in the presence of AOR and NADPH under neutral conditions, but the cleavage efficiency in neutral conditions is less than that in alkaline conditions (Figure S1, Supporting Information).

To probe whether DNA sequence influences the sites targeted by AF, we examined the cleavage of a 377 bp sequence (pBR322) of DNA using a standard Maxam−Gilbert[55] sequencing approach. pBR322 DNA was reacted with either 20 or 40 μM AF in the presence of AOR/NADPH, or 40 μM AF alone (Figure S2, Supporting Information). As comparison standards, reactions were carried out using dimethyl sulfate/piperidine or formic acid/piperidine to induce cleavage at all Gua sites or at all Gua and Ade sites, respectively. For DNA treated with AF alone, faint bands were observed consistent with cleavage at Gua and Ade sites but lacked any evidence for sequence specificity; i.e., the relative intensity of the bands was the same as that for the dimethyl sulfate/piperidine control. Likewise, in the presence of AOR, cleavage did not appear to be sequence specific (Figure S3, Supporting Information), but AOR greatly enhanced the efficiency of Gua + Ade cleavage, consistent with increases in adduct levels observed in reactions of AF/AOR with nucleosides and calf thymus DNA (Scheme 5).

Relative Levels of Adducts in Cells Transfected with pCEP and AOR Vectors. In previous studies, we observed a positive correlation between sensitivity to AFs and levels of AOR expression in a large panel of tumor cell lines. To determine whether this relationship is consistent with increases in AF-specific adducts and to probe whether the reaction products characterized in cell-free systems are consistent with cellular processes, we examined adduct formation in cells treated with AF and compared adduct levels in AF-sensitive and resistant cells. Human embryonic kidney cells were transfected with AOR vectors or blank vectors, as described previously.[41] Each cell line was treated with AF (500 nM) for 3 h, cells were lysed, and DNA was isolated. The DNA was hydrolyzed and analyzed as described for calf thymus DNA reactions. Consistent with the results from naked DNA experiments, Ade adduct **20** and Gua adducts **21** and **25** were observed in hydrolysates of DNA samples isolated from cells (Figure S4, Supporting Information). Adduct **20** is the predominant DNA adduct in AF-treated cells, accounting for 98% of the total peak area attributable to **20**, **21**, and **25**. While rigorous comparisons between absolute levels of different adducts cannot be made by HPLC-MS/MS analysis without suitable analytical standards, the data obtained allowed us to compare relative levels of a given adduct in AOR-transfected vs control cells. It is therefore significant that 5−10 times higher levels of each adduct were observed in cells that overexpressed AOR vs those in control cells (Figure 6). A peak corresponding to adduct **27**, resulting from the direct conjugate reaction of DNA with AF, was also observed. However, because of the near-detection-limit levels of this adduct in all samples, a comparison of levels between cell types could not be made (Figure S4, Supporting Information). To verify that differences in adduct levels reflected those present in cellular DNA, rather than were influenced by loss during isolation and hydrolysis, cell culture media and DNA wash solutions were also analyzed for the presence of depurination adducts, and no adducts were detected in these samples.

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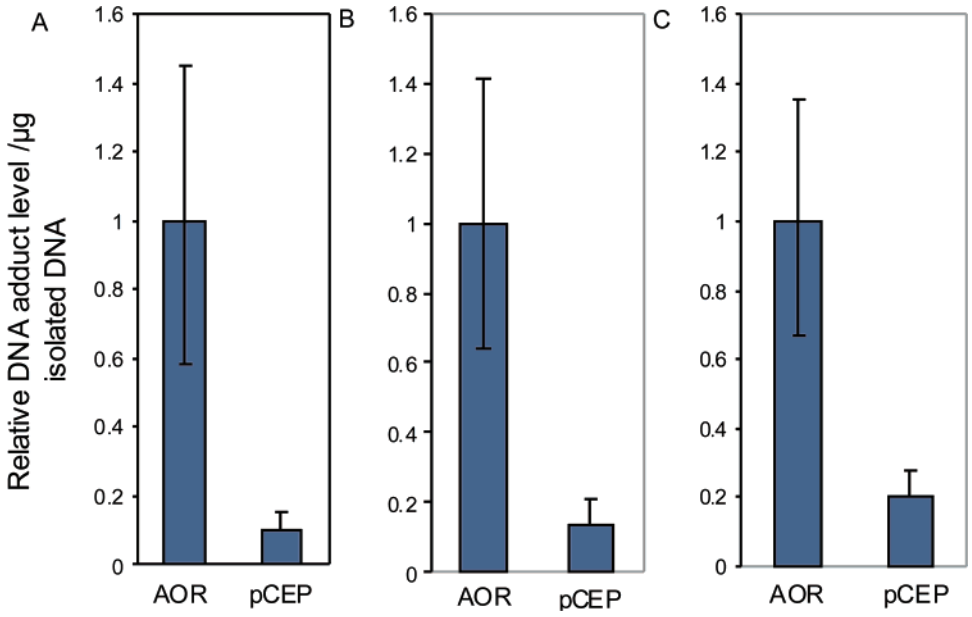

Figure 6. Relative levels of DNA adducts in cells transfected with an AOR vector or a pCEP vector. (A) Ade adduct **20**; (B) Gua adduct **21**; (C) Gua adduct **25**. Error bars indicate standard deviations from average values shown for three independent analytical runs.

Discussion

This study describes the first chemical characterization of DNA adducts induced by AF and the first evidence that the associated chemical transformations are relevant in cells. In the current study, we focus on reactions of AF as the parent structure in a class that includes the clinically relevant HMAF in which the core AF structure appears to be a key factor for selectivity. AF readily alkylates monomeric nucleosides and DNA in the presence of a bioreductive enzyme, AOR. The formation of two dAdo adducts **20** and **22** and one dGuo adduct **21** was observed in reactions of AF with monomeric nucleosides in the presence of AOR/NADPH, with **20** as the predominant species. These adducts have a common major MS fragment of *m*/*z* 201 indicative of an indene moiety and consistent with previous results suggesting that AF is activated by reduction of the carbon−carbon double bond of the α,*β*-unsaturated ketone (Scheme 1). Reactions between AF and naked or cellular DNA resulted in the formation of adducts common to those from monomeric nucleoside reactions. Additionally, a Gua adduct not present in monomeric nucleoside reactions was observed in the calf thymus and cellular DNA reaction mixtures. We observed the same pattern of adducts in AF-treated cells as those in reactions between AF and naked DNA.

On the basis of previous studies, AF alone is unreactive toward common nitrogen-based nucleophiles[34] but covalently associates with isolated proteins and DNA.[30] Using a sensitive HPLC-MS/MS analytical method, we observed the formation of a directly formed Ade adduct. On the basis of its MS/MS fragmentation, the direct adduct is structurally distinct from that formed in the presence of AOR and is consistent with a structure resulting from the conjugate addition of adenine to the α,*β*-unsaturated ketone of AF, followed by hydrolytic cyclopropane cleavage (**6**, Scheme 5). The postulated intermediate could lead to DNA cross-links, i.e., **29**, if a DNA base instead of H_2O were to act as the nucleophile. Cross-linking is a common mode of action for antitumor agents, but such adducts could not be detected in our analyses. While this observation is consistent with previous studies, it is possible that cross links are formed at presently undetected levels. An alternative reaction pathway consistent with the MS/MS data would involve addition of H_2O at the C8 position of AF, followed by homoallylic substitution by DNA. However, there is no supporting evidence to suggest that H_2O can react directly with AF.[34] The direct reaction between AF and DNA, but not between AF and nucleosides, as well as differences in products formed in reactions with monomeric nucleosides vs DNA, may indicate the role of noncovalent interactions between AF and DNA that activate AF for nucleophilic attack.[26] Furthermore, alkylation at two dGuo sites in DNA compared to one in the isolated dGuo reaction may indicate that AF or its reactive intermediate may also result from noncovalent interactions within DNA.[56,57]

Structural characterization of the major AF adduct and a Gua adduct was achieved by independent chemical synthesis of standards for **20** and **21**, revealing that when bioactivated by AOR, AF mainly alkylates dAdo at the 3-position with only a small portion of activated AF alkylating dGuo at either the 3- or 7-position. The preferential alkylation of DNA at the 3-position of dAdo is observed with other antitumor agents including CC-1065 and duocarmycins,[26,58,59] as well as the closely related toxin ptaquiloside.[60] These agents share an allylic cyclopropane as a key structural feature, and the postulated activated intermediate of AF (**5**) is most structurally similar to the toxin ptaquiloside. Adducts from ptaquiloside, however, are formed indiscriminately at both the 7-position of dGuo and the 3-position of dAdo.[60] While sequence context does not appear to influence the reactivity of AF, discrepancies observed in reactions of nucleosides vs naked/cellular DNA and between

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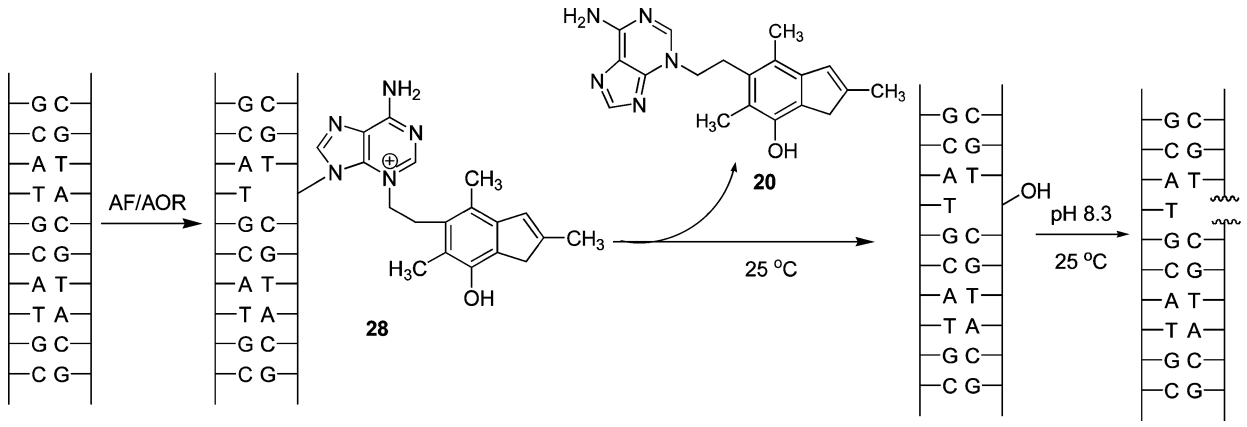
Scheme 6. Schematic Representation of AOR-Mediated Reaction of AF with Double-Stranded DNA, Illustrated for a Random DNA Sequence



AF and structurally similar agents may indicate differences in corresponding DNA binding modes.[56,57]

Purine alkylation labilizes the modified base in DNA, and the chemical stability depends on the structure.[46,47] We observed facile depurination of AF−DNA adducts from double-stranded DNA (Scheme 6). In the case of dGuo, only depurinated AF-Gua adducts were detected, reflecting the instability of the corresponding nucleoside adduct. The half-life of **22** under physiological pH was determined to be 8.5 h, and the corresponding dGuo adducts, **23** and **24**, have half-lives under 2 h in DNA. Depurination of AF adducts results in abasic DNA sites and single-stranded DNA cleavage under weakly basic conditions (pH 8.3), as evidenced by supercoiled DNA relaxation assays. The approximate degree of DNA cleavage correlates with adduct levels: treatment with AF alone leads to minimal DNA cleavage, while addition of AOR/NADPH results in high levels of DNA adducts and substantial DNA cleavage. The bioactivation-mediated process of strand cleavage results in increases in reactivity but does not impact the pattern of cleavage with respect to DNA sequence. Both processes appear to have no sequence-based selectivities, at least in the case of sequences presented by the pBR322 fragment. Finally, reactions of AF with individual nucleosides, calf thymus DNA, and a 377 bp DNA sequence demonstrate that AF alkylates dGuo and dAdo but not dCyd and Thd and that the efficiency of this process is increased dramatically by AOR-mediated bioactivation.

Cell-based studies provide direct evidence for the generation of AF-specific DNA damage and further support the role of AOR in the bioactivation of AF and sensitization of cells. AOR is a cytosolic enzyme, and it has therefore been debated whether the reactive metabolite of AF is stable enough, in the presence of cellular nucleophiles, to reach the nucleus and alkylate chromosomal DNA.[43,61] In the current studies, we observed a positive correlation between cytosolic levels of AOR and DNA adducts induced by AF. Cellular DNA samples were obtained using a nuclear extract method indicating that the activated intermediate of AF appears sufficiently stable to reach DNA targets.[62] The positive correlation between specific adduct levels and cell sensitivities to AF may indicate a contributing role of these adducts to the cytotoxicity of AF, which is consistent with previous results.[41,43] Furthermore, AF−DNA adducts may serve as useful chemical markers of the sensitivity of cells to AF.

Structural elucidation of AF−DNA adducts provides a chemical tool for probing their biological significance. Bulky DNA adducts, such as the initial nucleoside adducts induced by AF, can be cytotoxic, blocking both DNA replication and transcription. Quickly generated abasic sites, through depurination of labile alkylated purine bases, may also contribute to cytotoxicity; however, abasic sites typically are well tolerated by cells and rapidly repaired mainly through the process of base excision repair (BER).[63] It is therefore important to understand the degree to which AF−DNA adducts or abasic lesions are the major inducer of cell responses. Despite the chemical lability observed in cell free studies, these adducts appear to persist in the presence of cellular proteins. Furthermore, previous work has demonstrated that AF-induced DNA damage is exclusively repaired by transcription-coupled NER enzymes, which are anticipated to process bulky adducts but not be significantly influenced by BER pathways.[32,33] The results of this study, combined with data from cell-based experiments, indicate that the initially formed chemically labile AF−DNA adducts contribute to the cytotoxicity of AF, but further studies are required to correlate the specific structures of AF adducts, i.e., **22** vs **23**, **24**, with their interactions with biological systems, such as NER proteins.

In summary, these studies provide the first direct chemical evidence for DNA adducts specific to AF using nucleosides and naked DNA as well as in cells. A bioactivation-generated 3-substituted dAdo adduct is estimated to be the most abundant adduct in reactions with cellular DNA as well as with nucleosides or calf thymus DNA in the presence of AOR. Furthermore, AF is capable of directly damaging DNA without enzymatic bioactivation. These adducts appear to be formed at comparatively low levels in both cell-free and cell-based systems, but their impacts on biological processes are unknown. A positive correlation between cell sensitivity and levels of structurally specific bioactivation-generated DNA adducts was observed, which indicates that these DNA adducts may have medicinal significance.

Experimental Section

Chemicals and Enzymes. All chemicals were purchased from the Sigma-Aldrich chemical company (Milwaukee, WI) and used without further purification. AOR was purified according to the procedure

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previously described.[42] All other enzymes were purchased from Sigma (St. Louis, MO). The synthesis of AF was described previously.[41] N^2-Bn-dGuo was synthesized according to the published method.[54]

Apparatus and Assay Conditions. 1-D ^{1}H and ^{13}C NMR spectra were acquired on a 500 MHz Varian NMR spectrometer, and chemical shifts were reported relative to the residual nondeuterated solvent signals. 2-D gHMQC and gHMBC were recorded on a 600 MHz Varian NMR spectrometer. HPLC analysis was carried out on an Agilent 1100 series instrument with a diode array detector. Compounds were eluted using a solvent gradient from 10 to 75% acetonitrile in water over a course of 30 min at a flow rate of 0.8 mL/min. The HPLC column used was a Phenomenex Luna 5 μm C18(2) 100 A 250 mm × 4.60 mm (Phenomenex, Torrance, CA). HRMS were recorded on a Bruker BioTOF II mass spectrometer with an Electron-spray ionization source. Polypropethylene glycol (PPG) was used as matrix in all HRMS experiments. Flash chromatography (silica gel 200−400 mesh) was used for product purification.

Qualitative HPLC-ESI-MS/MS analyses were carried out with a Finnigan LCQ Deca instrument (Thermo Finnigan LC/MS Division, San Jose, CA) interfaced with a Waters Alliance 2690 HPLC multisolvent delivery system and equipped with an SPD-10A UV detector (Shimadzu Scientific Instruments, Columbia, MD), using the HPLC solvent elution system and column as described above. The ESI source was set in positive ion mode as follows: voltage, 4 kV; current, 10 μA; and capillary temperature, 275 °C. MS data were collected for a mass range of 100−1000 amu, and MS/MS data were acquired with the following parameters: isolation width, 1.5 amu; normalized collision energy, 40%; activation Q, 0.25; and activation time, 30 ms. MS samples were purified by solid-phase extraction prior to analysis as follows: A solid-phase extraction cartridge (Phenomenex, Strata-X 33 μm cartridge) was activated by eluting with 1 mL of MeOH followed by 1 mL of H_2O. The sample was loaded on the cartridge and was washed successively with 1 mL of H_2O, 1 mL of 10% aqueous MeOH, and 2 mL of 70% aqueous MeOH. The MeOH-containing portions were combined and concentrated to a volume of approximately 1 mL.

Semiquantitative HPLC-ESI-MS/MS analysis was carried out with an Agilent 1100 capillary flow HPLC (Agilent Technologies, Palo Alto, CA) interfaced with a Finnigan Discovery Max triple quadrupole mass spectrometer. Chromatographic separation was achieved with a 150 mm × 0.5 mm 5 μm particle size C18 column (Agilent Zorbax SB-C18), using the HPLC solvent elution system described in each experiment. The ESI source was set in the positive ion mode as follows: voltage, 3.7 kV; current, 3 μA; and heated ion transfer tube, 275 °C. Samples were purified by solid-phase extraction as described above but with a final sample volume of 50 μL.

General DNA Hydrolysis Procedure. Modified DNA (1.0 mg) was dissolved in 1 mL of 10 mM Tris-HCl/5 mM $MgCl_2$ buffer (pH 7.0). DNA was first digested with 1500 units of DNase I (type II, from bovine pancreas) at 37 °C for 10 min. To the resulting mixture were added 0.1 unit of phosphodiesterase I (type II, from *Crotalus adamanteus* venom) and 750 units of alkaline phosphatase. The mixture was incubated at 37 °C for an additional 60 min.

Reactions of AF with Nucleosides in the Presence of AOR/NADPH. To a 1.5 mL eppendorf tube were added 0.87 mL of water, phosphate buffer (100 μL, 1.0 M, pH 7.4); dGuo, dAdo, dCyd, or Thd (2.0 μmol); AF (0.5 μmol, 10 μL of 50 mM solution in DMSO); $NADP^+$ (5.0 μmol); glucose-6-phosphate (10.0 μmol); glucose-6-phosphate dehydrogenase (5 units); and AOR (0.2 nmol). The mixture was allowed to react at 37 °C for 24 h. The reaction mixture was analyzed using the general qualitative method.

Reactions of AF with Nucleosides in the Absence of AOR/NADPH. To a 1.5 mL eppendorf tube were added 0.99 mL of water; phosphate buffer (100 μL, 1.0 M, pH 7.4); dGuo, dAdo, dCyd, or Thd (2.0 μmol); and AF (0.5 μmol, 10 μL of 50 mM solution in DMSO). The mixture was allowed to react at 37 °C for 24 h. The reaction mixture was analyzed using the general qualitative method.

Reaction of AF with Calf Thymus DNA in the Presence of AOR/NADPH. To a 1.5 mL eppendorf tube were added 0.87 mL water, phosphate buffer (100 μL, 1.0 M, pH 7.4), calf thymus DNA (2 mg), AF (0.5 μmol, 10 μL of 50 mM solution in DMSO), $NADP^+$ (5.0 μmol), glucose-6-phosphate (10.0 μmol), glucose-6-phosphate dehydrogenase (5 units), and AOR (0.2 nmol). The mixture was allowed to react at 37 °C for 24 h. Modified DNA was precipitated by adding cold ethanol. It was washed with ethanol, dried under nitrogen for 15 min, and enzymatically hydrolyzed using the general DNA hydrolysis method described above. The supernatant and hydrolyzed DNA samples were analyzed using the general qualitative method.

Reaction of AF with Calf Thymus DNA in the Absence of AOR/NADPH. To a 1.5 mL eppendorf tube were added 0.99 mL water, phosphate buffer (100 μL, 1.0 M, pH 7.4), calf thymus DNA (2 mg), and AF (0.5 μmol, 10 μL of 50 mM solution in DMSO). The mixture was allowed to react at 37 °C for 24 h. Modified DNA was precipitated by addition of cold ethanol, washed with ethanol, dried under nitrogen for 15 min, and enzymatically hydrolyzed using the general DNA hydrolysis method. The supernatant and hydrolyzed DNA samples were isolated and analyzed using the general qualitative method.

Kinetics of Depurination of DNA−AF Adducts in Double-Stranded DNA. To a 1.5 mL eppendorf tube were added 0.87 mL water, phosphate buffer (100 μL, 1.0 M, pH 7.4), calf thymus DNA (2 mg), AF (10 μL, 50 mM in DMSO), $NADP^+$ (50 μL, 100 mM), glucose-6-phosphate (50 μL, 200 mM), and glucose-6-phosphate dehydrogenase (10 μL, 500 units/mL) and AOR (10 μL, 0.5 mg/mL). The mixture was allowed to react at 37 °C for 24 h. The modified DNA was precipitated by addition of cold ethanol and then washed with ethanol. After drying under nitrogen for 15 min, DNA was redissolved in 1.0 mL of 10 mM Tris-HCl/5 mM $MgCl_2$ buffer (pH 7.0) and was heated to 37 °C. Aliquots (50 μL) were removed following incubation for 0.2, 1, 3, 5, 8, 12, and 24 h. The aliquot was immediately treated with cold ethanol to precipitate DNA, and the supernatant was isolated and analyzed using the general semiquantitative method. To estimate the total concentration of adduct in the initial sample, an aliquot (50 μL) was heated at 90 °C for 1 h to completely depurinate. HPLC analysis was performed using a gradient system consisting of 100% solvent A (0.1% acetic acid in water) for 2 min, followed by a 25 min linear gradient to 20% A, 80% B (33% ACN in isopropanol) that was held for 10 min. The flow rate was 10 μL/min. An aq solution of N^2-Bn-dGuo (50 μL, 0.5 μM) was added to each sample as an injection standard. AF adducts and the injection standard were monitored in the selected reaction monitoring (SRM) mode with the following ion transitions: N^2-Bn-dGuo, m/z 458.2 $[M + 1]^+$ to m/z 242.2 [M − deoxyribose + 1]$^+$; **20**, m/z 336.2 $[M + 1]^+$ to m/z 201.2 [M − Ade]$^+$; AF-Gua, m/z 352.2 $[M + 1]^+$ to m/z 201.2 [M − Gua]$^+$. The peak area of each adduct was normalized to that of the injection standard. Half-life time was calculated from the first-order rate constant ($t_{1/2} = 0.693/k$), which was obtained from the slope of the plot of $\ln[c/c_0] = -kt$, where t is the reaction time, c is the concentration of adducts remaining in DNA, and c_0 is the initial concentration of adducts.

Cell Culture and Transfection. Human embryonic kidney cells (293 cells) were obtained from ATCC (American Type Culture Collection, Manassas, VA) and maintained in Eagle's medium (high glucose, Invitrogen, Inc., Carlsbad, CA), supplemented with 10% heat-deactivated fetal bovine serum (Invitrogen, Inc.). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Plasmid pCEP4-rAOR was cloned as previously described (1) and transfected into 293 cells with Lipofectamine 2000 reagents (Invitrogen, Inc.).

Genomic DNA Extraction. pCEP4- or pCEP4-AOR-transfected 293 cells were seeded onto two 10-cm culture dishes at 6 million cells/dish. After incubating for 16 h, cells were treated with fresh media containing 500 nM acylfulvene for 3 h under growth conditions described above. After washing with phosphate-buffered saline, cells were lysed in TES buffer (10 mM Tris pH 8.0, 100 mM EDTA, 0.5% SDS) and incubated at 55 °C for 30 min with the addition of protease

K at 100 μg/mL. Digested lysates were extracted with an equal volume of phenol/chloroform and centrifuged at 10 000 $\times$ *g* for 15 min. The resulting aqueous layer was saved and extracted twice with equal volumes of ether to remove residual phenol/chloroform. DNA was precipitated with the addition of 0.8 volume of isopropanol before centrifugation at 12 000 $\times$ *g* for 15 min. After washing twice with 70% ethanol, DNA pellets were dried under nitrogen for 15 min. DNA was hydrolyzed using the general DNA hydrolysis method and analyzed using the general semiquantitative method. HPLC analysis was performed using a gradient system consisting of 90% MeOH in H_2O for 2 min, followed by a 20 min linear gradient to 50% MeOH in H_2O. A 5 min linear gradient to 5% MeOH in H_2O followed, and 5% MeOH in H_2O was held for 20 min. The flow rate was 10 μL/min. AF adducts were monitored by the selected reaction monitoring (SRM) mode with the ion transitions as follows: **20**, *m*/*z* 336.2 $[M + 1]^+$ to *m*/*z* 201.2 $[M - Ade]^+$; AF-Gua, *m*/*z* 352.2 $[M + 1]^+$ to *m*/*z* 201.2 $[M - Gua]^+$; **27**, *m*/*z* 352.2 $[M + 1]^+$ to *m*/*z* 217.2 $[M - Ade]^+$.

Supercoiled DNA Relaxation. Supercoiled pBR322 DNA (25 μM bp) was incubated with AF (10−70 μM) in the absence and presence of AOR (10 ng) and NADPH (200 μM) in TBE (80 mM) (pH 8.3). The samples were incubated for 48 h at 37 °C, and the reaction was stopped by adding loading dye containing 0.1% bromophenol blue, 0.1% Xylene cyanol, and 80% (v/v) formamide solution. The gel was electrophoresed at 100 V for 3 h in TBE buffer using 0.8% agarose gel containing 0.5 μg/mL ethidium bromide. The bands were illuminated under UV light and photographed using Biorad Gel Doc 1000.

DNA Cleavage. Plasmid pBR322 DNA was digested with EcoRI followed by treatment with alkaline phosphatase, and 5′-end labeling of linearized DNA was performed using T4 polynucleotide kinase with γ-^{32}P-ATP at 37 °C for 30 min according to the manufacturer's protocol. The reaction was stopped by heating at 65 °C for 10 min. Labeled DNA was purified by Amersham G-50 spin columns by following the manufacturer's protocol. The 5′-end labeled linearized DNA was further digested with restriction enzyme BamH1 according to the previously reported procedure. The resulting 377 bp DNA was purified on 5% nondenaturing polyacrylamide gel. AF (20 and 40 μM) was reacted with 5′-end labeled DNA (2000 cpm) in Hepes buffer (pH 7.4, 10 mM) in the absence or presence of AOR (10 ng), NADPH (200 μM), and calf thymus DNA (0.25 mM) as a carrier DNA for 48 h. The reaction was stopped by addition of absolute ethanol and stored at −20 °C. DNA was precipitated by cooling the sample in dry ice and centrifugation at 12 000 rpm. The supernatant was removed, and the DNA pellet was washed with 70% ethanol (0.5 mL) and dried under a vacuum. The DNA sample was treated with piperidine (50 μL of 0.1 M) and heated at 90 °C for 30 min. G and G + A sequencing was carried out as per Maxam−Gilbert workup.[55] The sample was lyophilized using speedVac and redissolved in 20 μL of water and lyophilized again. The dried fragmented DNA was dissolved in formamide loading buffer, heated at 90 °C for 5 min, and loaded in 11% denaturing polyacrylamide gel. The gel was electrophoresed at 1500 V for 4 h, and the resolved fragments were analyzed using a phosphorimager.

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Supporting Information Available: Synthesis and characterization of **9**−**21**, gHMQC and gHMBC spectra of **20** and **21**, Figure S1−S4. This material is available free of charge via the Internet at http:/pubs.acs.org.

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