Human Mismatch Repair and G-T Mismatch Binding by hMutSα in Vitro Is Inhibited by Adriamycin, Actinomycin D, and Nogalamycin*

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Loss of the human DNA mismatch repair pathway confers cross-resistance to structurally unrelated anticancer drugs. Examples include cisplatin, doxorubicin (adriamycin), and specific alkylating agents. We focused on defining the molecular events that link adriamycin to mismatch repair-dependent drug resistance because adriamycin, unlike drugs that covalently modify DNA, can interact reversibly with DNA. We found that adriamycin, nogalamycin, and actinomycin D comprise a class of drugs that reversibly inhibits human mismatch repair in vitro at low micromolar concentrations. The substrate DNA was not covalently modified by adriamycin treatment in a way that prevents repair, and the inhibition was independent of the number of intercalation sites separating the mismatch and the DNA nick used to direct repair, from 10 to 808 base pairs. Over the broad concentration range tested, there was no evidence for recognition of intercalated adriamycin by MutSα as if it were an insertion mismatch. Inhibition apparently results from the ability of the intercalated drug to prevent mismatch binding, shown using a defined mobility shift assay, which occurs at drug concentrations that inhibit repair. These data suggest that adriamycin interacts with the mismatch repair pathway through a mechanism distinct from the manner by which covalent DNA lesions are processed.

Recent studies suggest that the human mismatch repair (MMR) pathway contributes to the cytotoxic effect elicited by several different anti-neoplastic agents. MMR normally functions to correct base misincorporations that arise during replication, but it may also be involved in pathways that react to specific types of DNA injury (reviewed in Refs. 1–6). Cultured cells with low level resistance to cisplatin, DNA alkylating drugs, 6-thioguanine, and doxorubicin (adriamycin (AD)) have been identified with deficiencies in human MMR proteins (reviewed in Refs. 3 and 7–9). In response to cisplatin (cis-diaminedichloroplatinum(II)) or alkylating agents such as N-methyl-N′-nitro-N-nitrosoguanidine, N-methyl-N-nitrosourea, and temozolomide, the tumor suppressor p53 becomes stabilized and processed by the MMR pathway (12). Although cisplatin and alkylators generate a range of DNA lesions, it appears that the suspected cytotoxic lesions are tightly bound by MutSα (17), consistent with a requirement for lesion recognition by MutSα for drug-induced apoptosis.

The futile repair and direct signaling models both depend on recognition of covalent DNA modifications. However, AD-dif-

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1 The abbreviations used are: MMR, DNA mismatch repair; AD, adriamycin; bp, base pair(s); kb, kilobase(s).
fers from drugs such as cisplatin and DNA alkylators because AD can reversibly bind DNA. Because this is a distinguishing feature among these anticancer drugs, we examined the effect of AD on the MMR pathway with the goal of defining the early steps in the MMR-mediated response to AD. By using an in vitro repair assay for human MMR activity (29), we have found that AD, nogalamycin, and actinomycin D inhibit the correction of DNA mismatches in vitro. We have exploited this assay to distinguish among several possible models that seek to explain how these drugs might interact with MMR to produce a cytotoxic response.

MATERIALS AND METHODS

Chemical Reagents—All reagents, including those required for the human MMR assay and band shift experiments, were purchased from the Sigma. Stock solutions of daunomycin were prepared in water, whereas AD and actinomycin D were prepared in a buffer containing 25 mM Hepes-KOH (pH 7.5) and 110 mM KCl (buffer A). Etoposide (100 mM) and m-amssacrine (5 mM) were prepared in 100% Me2SO and working concentrations were made by diluting the Me2SO stock into buffer A. Nogalamycin was dissolved in 75% Me2SO and diluted into buffer A. Whenever Me2SO was used as a solvent, the final concentration of Me2SO was 1% in the assay, and the data presented were not corrected for the effects of solvent (data not presented). Concentrations of AD, daunomycin, nogalamycin, actinomycin D, and ethidium bromide were determined spectrophotometrically using the following extinction coefficients: AD, ε245 = 13,000 M⁻¹ cm⁻¹ in methanol (30); daunomycin, ε260 = 11,500 M⁻¹ cm⁻¹ in water (31); nogalamycin, ε255 = 24,755 M⁻¹ cm⁻¹ in water (Merck Index); ethidium bromide, ε240 = 6,500 M⁻¹ cm⁻¹ in water (Sigma); actinomycin D, ε441 = 24,400 M⁻¹ cm⁻¹ in methanol (Sigma). Stock solutions were stored for short periods at −20 °C.

Mismatch Repair Substrates—GT mispaired substrates with a single-strand break placed so that 128 bp intervene reading 5' → 3' from the nick were constructed using published protocols (29, 32). To prepare A-C heteroduplexes with 10, 128, or 808 bp between the mispair and the nick were constructed using published protocols (29, 32). To prepare 3'-glex-strand break placed so that 128 bp intervene reading 5' → 3' the products of Bsp106 and Hin1I digestion. The restricted DNA substrate and AD were incubated for 20 min at 37 °C in the presence of HeLa nuclear extract as described for the MMR assay (above). In a parallel mock experiment, the HeLa proteins were omitted. After incubating the heteroduplex substrate for 20 min at 37 °C in the presence or absence of 10 μM AD, the mock treated substrates (plus or minus AD) were precipitated from the assay by adding sodium acetate to 0.3 M and 2.5 volumes of absolute ethanol. The samples were centrifuged for 30 min at 14,000 × g, and the bulk of the AD remained in the supernatant, allowing recovery of the DNA. The precipitated substrates were then washed with absolute ethanol, air dried, and assayed under standard conditions with HeLa nuclear extract to test for the presence of covalent modifications that might inhibit mismatch correction.

Mobility Shift Assays using MutSα—Band shift experiments were performed using protocols described in Ref. 17. Oligonucleotides of the sequence: 5'-GAATTCGTACAGGTTTGCTCTAGAAA-3' (30-mer oligonucleotide 1), a homoduplex complement (30-mer oligonucleotide 2) and the heteroduplex complement: 5'-TTTCTAGACTCGAAGCTTGGACTAGGATTCT-3' (30-mer oligonucleotide 3) were synthesized by GE-Novo Synthesis and purified on preparative polyacrylamide gels.

RESULTS

Our goal in this work was to establish how the anticancer agent AD interacts with the human MMR pathway at the molecular level. The biological basis for this putative interaction derives from the observation that human cells resistant to cisplatin by virtue of the loss of mismatch repair are also cross-resistant to other DNA-interacting drugs, a structurally diverse group that includes AD (10, 34). A direct connection has been established for agents such as cisplatin or specific DNA alkylating agents, because they generate DNA lesions that are recognized by MutSα, the heterodimer primarily responsible for mismatch recognition (28). However, it is less clear how a drug that reversibly intercalates into DNA might trigger a MMR-dependent cytotoxic response.

AD Blocks an Early Step in MMR—As a first step, we asked whether AD is capable of interfering with the repair of a 6.4-kb circular G-T heteroduplex by HeLa nuclear extract (Fig. 1). The

Mismatch Repair Assay—The MMR assay was performed as described (29) with the following minor modifications. Reactions (15 μl) contained 21 fmol of GT nicked heteroduplex substrate, 45 μg of HeLa extract, 5 mM MgCl2, 110 mM KCl, 1 mM glutathione, 1 mM each of dNTP, 50 μg/ml bovine serum albumin, and 1.5 mM ATP. When possible, master mixes were made of all components except the drug under consideration and HeLa extract. To score for repair, the recovered substrate was digested with Bsp106 (1 unit/100 ng of DNA) to linearize the substrate and with HindIII (1 unit/100 ng of DNA) to score for GT mispairs corrected to AT or G-XhoI digestion. The restricted DNA fragments were separated on a 1% agarose gel in 1× Tris-acetate ethylenediamine tetraacetic acid, pH 8.5 for 300 Vh, stained with ethidium bromide and the image captured using a ChemiImager 4000 cooled CCD camera (Alpha Innotech). When agarose gels are presented in figures, the information is given as an inverted image, where the fluorescence associated with stained DNA appears as a dark band on a light background. The NIH image software (version 1.62) was used to calculate the relative percentage of bands in a given lane. More specifically, Mismatch Repair Assays in the Presence of DNA Intercalators—The MMR assays were carried out exactly as described above, except that the drug of interest and the HeLa extract were added to the side of a 1.5-mL Eppendorf tube containing all the assay reagents and placed on ice. Reactions were initiated by pulse centrifugation to combine the components, briefly mixed by manual agitation, and then placed at 37 °C. Similar results were obtained when the drugs were premixed either with the extract or the assay mixture containing the heteroduplex substrate.

Testing for AD-induced Covalent Damage—Incubations were carried out in the MMR assay buffer described above using 21 fmol of GT heteroduplex with a nick 128 bp 5' of the mispair and performed in the presence or absence of 10 μM AD. Under these conditions the DNA substrate and AD were incubated for 20 min at 37 °C in the presence of HeLa nuclear extract as described for the MMR assay (above). In a parallel mock experiment, the HeLa proteins were omitted. After incubating the heteroduplex substrate for 20 min at 37 °C in the presence or absence of 10 μM AD, the mock treated substrates (plus or minus AD) were precipitated from the assay by adding sodium acetate to 0.3 M and 2.5 volumes of absolute ethanol. The samples were centrifuged for 30 min at 14,000 × g, and the bulk of the AD remained in the supernatant, allowing recovery of the DNA. The precipitated substrates were then washed with absolute ethanol, air dried, and assayed under standard conditions with HeLa nuclear extract to test for the presence of covalent modifications that might inhibit mismatch correction.

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MMR assay is based on the nick-directed correction of the G-T heteroduplex, where the substrate DNA is recovered from extracts by SDS treatment, followed by phenol extraction and precipitation (29). Mismatch correction is scored using two restriction enzymes; Bsp106 linearizes all the molecules at a site remote from the mismatch, whereas HindIII scores for nick-directed G-T correction to A-T. DNA bands at 3.1 and 3.3 kb are diagnostic for site-specific mismatch correction. In the absence of digestion with restriction enzymes, the circular substrate was recovered from the assay (lanes labeled as undigested). The upper arrow points to catenated substrate, and the lower arrow points to supercoiled substrate. Panel III, AD does not cause covalent DNA damage that affects MMR under the assay conditions in the absence of HeLa nuclear extract. In the presence of 10 \( \mu \)M AD, MMR activity is inhibited in vitro (compare lanes 1 and 2). In parallel, the G-T substrate was incubated with the same concentration of AD under mock assay conditions (omitting only HeLa nuclear extract; see “Materials and Methods”) and then recovered by ethanol precipitation. This treatment has no effect on the ability of the AD-treated substrate to participate in a subsequent assay for MMR in vitro (compare lanes 3 and 4).

Experimentally, two results strongly support the identification of the high molecular weight species as catenated DNA. First, the putative catenate yields a single, linear product upon digestion with the unique cutter Bsp106 (Fig. 1). Second, treatment of the large molecular weight species with purified type II topoisomerase from Drosophila (33) yields a relaxed circular species that comigrates with the original substrate. Third, as described below, the presence of the type II topoisomerase inhibitor adriamycin prevented formation of the high molecular weight species.

Three related phenomena were apparent when the MMR assay was performed in the presence of low micromolar concentrations of AD. First, we observed a sharp decline in repair efficiency when AD was present at low micromolar concentrations (Fig. 1, panel I). Second, in the absence of digestion by Bsp106 and HindIII, a majority of the substrate was recovered from the assay as supercoiled molecules at an AD concentration of 8 \( \mu \)M (Fig. 1, panel II). The assignment is based on the facts that the DNA molecule comigrates with supercoiled DNA and that digestion with a restriction enzyme having a unique site yields a linear product. Formation of a supercoiled species

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1. bound as mismatch
2. MutS\(_a\) binding is blocked
3. oxidative damage is generated
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DNA cross-links in vivo (39), although similar reactions were not observed in cell free extracts (39, 40). We therefore asked whether incubation of the substrate DNA with AD produced DNA damage capable of preventing correction of the G/T mispair.

Heteroduplex substrate was incubated with AD under conditions identical to the MMR assay, except that HeLa nuclear proteins were absent (see “Materials and Methods”). The DNA substrate was recovered by ethanol precipitation, which lowers the AD concentration to levels undetectable by visible absorbance, well below those having a measurable effect on MMR. Precipitation is not expected to affect covalent DNA modifications or cross-links. We found that the G-T heteroduplex, whether treated with 0 or 10 μM AD and reisolated by precipitation, was repaired with equal efficiency in each case (Fig. 1, panel III). Covalent damage that can block repair in vitro, therefore, is not inflicted on the DNA by AD under the assay conditions in the absence of HeLa proteins.

In parallel experiments, concentrated HeLa nuclear extract was incubated with 5 μM AD in the absence of G/T substrate DNA. This experiment was intended to test the possibility that the extract can reductively activate AD. The mixture was then diluted into a MMR assay containing substrate DNA to achieve an AD concentration below that found to substantially inhibit repair (1.3 μM). Under these conditions, the mismatch was corrected with efficiency similar to untreated HeLa nuclear extract (data not shown). Overall, we found no evidence for covalent modification of MMR protein components or DNA substrate that contributes to loss of repair competence. However, we could not exclude the possibility that covalent DNA modification occurs when all of the assay components are present. This is because the repair reaction absolutely depends upon the presence of a single-strand nick to direct repair, and this nick normally becomes ligated under the assay conditions. Heteroduplex DNA recovered from an assay is therefore not competent for use in subsequent assays.

Actinomycin D, Nogalamycin, and Daunomycin Inhibit MMR at Concentrations Similar to AD—To explore the importance of DNA intercalation by AD in the mechanism of inhibition of MMR, other intercalators were tested for their ability to inhibit MMR in vitro. The compounds tested can be sorted into three general classes, the first of which includes AD, nogalamycin, daunomycin, and actinomycin D. Nogalamycin and daunomycin are anthracyclines that possess anti-tumor activity and are structurally similar to AD, containing an intercalating chromophore substituted by a bulky carbohydrate. Nogalamycin contains an additional carbohydrate substitution, and intercalation depends on the passage of one bulky group through the DNA helix (41). Actinomycin D is an inhibitor of DNA-dependent RNA polymerase (42) and is composed of an intercalating chromophore with two bulky circular peptides.

Fig. 3 shows that this first class of drugs inhibits MMR at concentrations from 0 to 5 μM, with AD nogalamycin and actinomycin D showing close similarity in their ability to inhibit MMR. The relative ranking of these drugs as MMR inhibitors is also qualitatively consistent with the DNA binding affinities of these drugs. For example, daunomycin is the closest structural analog of AD, and it displays a 2-fold higher IC_{50} and a shallower concentration dependence of inhibition. This observation is consistent with the fact that daunomycin, when tested for DNA binding affinity under the same experimental conditions as AD, binds less tightly by a factor that ranged from 1.5 (43) to 4 (44, 45). This is the only case where pairwise comparisons of binding affinities can be made reliably between the drugs tested and AD. Nonetheless, the dissociation constants determined for AD (38) and actinomycin D (46) are essentially identical, as are their ability to inhibit MMR. No comparable value for nogalamycin has been reported, although its intercalation gives sequence-dependent differences in DNase I footprints when present at low micromolar concentrations (47).

Ethidium bromide is a prototype intercalator that lacks bulky substitution, and it represents the second class of inhibitors. When tested in a MMR assay (Fig. 3), two distinguishing features were observed. First, the IC_{50} for ethidium bromide was roughly 10-fold higher than the first class of drugs, and second, the slope of the inhibition through the central data points was roughly 6-fold reduced. The reduced ability of ethidium bromide to inhibit MMR, in comparison with AD, also parallels its reduced binding affinity for DNA. When compared directly, ethidium bromide bound 7-fold less well to pBR322 DNA than AD (48). Although association constants for ethidium bromide binding to DNA are variable, the measured binding affinities are consistent with a roughly 10-fold reduced affinity for DNA when compared with AD (49, 50). However, depending on the substrate DNA, conditions, and experimental approach, the measured affinity of ethidium bromide for DNA was variable (for example, see Refs. 51 and 52).

**Structurally Distinct Topoisomerase Inhibitors Do Not Inhibit Human MMR**—Because AD, actinomycin D, and nogalamycin are all known to inhibit topoisomerase activity, we asked whether other inhibitors of either type I or type II topoisomerases could inhibit DNA MMR. This is critical in light of the observation that MMR inhibition occurs concurrently with a block to substrate catenation, implicating inhibition of a type II topoisomerase activity. We tested the type II topoisomerase inhibitors etoposide and m-amsacrine for inhibition of MMR, as
well as the type I topoisomerase inhibitor camptothecin, and the prokaryotic gyrase inhibitor novobiocin. None of the topoisomerase-blocking drugs were found to inhibit MMR over the concentration range tested, up to at least 300 μM in each case (not shown). In some cases, drug solubility limited testing at higher concentrations.

Of special note in this group of drugs are etoposide and m-amsacrine. Etoposide is a nonintercalating inhibitor of type II topoisomerase (53), but we found no evidence for an effect on mismatch correction in vitro (data not shown). m-Amsacrine also represents the third class of DNA intercalating drugs, and it had no effect on MMR (data not shown). The observation that a DNA intercalating drug is without effect on MMR is less surprising in light of the fact that m-amsacrine has a relatively weak association constant for DNA ($K_a = 4 \times 10^4 \text{ M}^{-1}$) compared with AD ($K_a = 3.6 \times 10^6 \text{ M}^{-1}$, taken from Ref. 48). These data indicate that the characterized inhibition of type II topoisomerase activity by AD is likely to be distinct from its ability to inhibit MMR, because other topo II inhibitors do not inhibit MMR. However, in the case of etoposide or m-amsacrine, a block to substrate cation was not observed in vitro, as it was in the case of AD (Fig. 1).

**MMR Inhibition Is Independent of the Distance between the Nick and Mispair**—To test whether AD inhibits repair by physically restricting the movement of proteins, such as MutSα, along the DNA helix, we constructed three molecules with varying distances separating a single-strand nick and an A–C mispair. The nick serves to direct MMR to the discontinuous strand in human MMR assays (29, 54). Furthermore, Fang and Modrich (55) have demonstrated that a tract of DNA that spans the mismatch and nick is excised during mismatch correction. Mispaired substrates containing nicks positioned 10, 128, or 808 bases away from an A–C mismatch were assayed for repair in the presence or absence of AD. When the nick is placed 10 bases away from the mispair, there are few potential AD intercalation sites between the mismatch and nick, especially considering that AD intercalation saturates, on average, at once every 5–6 bp (44, 56). The number of potential intercalation sites is increased over 130-fold in the substrate with an 808-bp separation. Assuming that the base content across this span of DNA is not highly skewed against intercalation, more physical “roadblocks” will be present as the distance increases at a given drug concentration.

In the absence of AD, the absolute level of repair was remarkably similar for the three substrates tested. Approximately 50% of the A–C heteroduplex molecules were repaired in each of the three substrates as the distance separating the nick and mismatch varied from 10 to 808 bp. The relative extent of repair was then plotted as a function of AD concentration (Fig. 4). The drug concentration needed to block repair was indistinguishable among the three substrates. Although this experiment does not address the possibility that MMR inhibition results from intercalation at a specific site, such as directly at the nick or mismatch, it does not support the possibility that intercalated chromophores with bulky side groups contribute a physical block to communication between these two sites. Furthermore, intercalation directly at the nick site is an unlikely mechanism to explain the observed inhibition, because it does not prevent nick ligation by the HeLa extract at concentrations that completely block MMR (Fig. 1).

Traditionally, in vitro assays have been performed on substrates where the mismatch to nick separation ranges roughly from 125 to 1000 bp (29, 54, 55). The A–C substrate, where the nick and mismatch are separated by only 10 bp, represents a setting where little information is available about the MMR pathway. Although we have not yet shown rigorously that repair of the shorter tract substrate is dependent upon the identical set of proteins required for long patch MMR excision and resynthesis, mismatch correction is minimally dependent upon the MSH2 protein. Heteroduplexes with either a 10- or 128-bp separation between nick and mismatch were incubated with LoVo nuclear extract, which functionally lacks MSH2 protein. When the nick is 128 bp removed from the mismatch, mismatch correction is nick-dependent, strand-specific, and dependent upon the addition of purified MutSα (MSH2/MSH6; Ref. 23). When the nick and mismatch are separated by 10 bp, a repair event with similar characteristics and magnitude is obtained where at least 75% of the nick-directed mismatch correction is dependent upon MSH2 (data not shown). The fraction of repair that is MSH2-independent (up to 25%, depending upon the substrate and extract) may represent exonucleolytic activity from the extract initiated at the nearby nick or an alternative repair pathway.

In addition to the complementation studies, other hallmarks of MMR in vitro are present (29, 55, 57). For example, maximal repair efficiency of roughly 50% was reached within 15 min, and prolonged incubation had little further effect. Although MMR is classically considered a “long patch” pathway, these data are consistent with a mismatch correction activity that can access a strand signal at substantially shorter distances. We are currently exploring the physical limits of MMR where the strand discrimination signal is positioned closer to the mismatch. It is also interesting to note that Grudin et al. (58), using DNase I footprinting, showed that the MSH2/MSH6 heterodimer protects a 25-bp asymmetric footprint surrounding the mismatch. Such binding would place the nick 10 bp removed from the mismatch either within or at the edge of the initial MutSα footprint.

**Intercalated AD Is Not Recognized as a Mismatch by MutSα**—It has been shown that MutSα can recognize 1,2-platinated adducts formed between cisplatin and DNA, as well as specific alkylation damage such as O6-methylguanine (17, 22). Upon intercalation into DNA, AD might appear as a base insertion mismatch, and this adduct might be recognized by MutSα. A plausible explanation for inhibition of MMR in vitro...
might be that, in the presence of intercalated AD, the limited supply of MutS\(_a\) is titrated away from the G-T mismatch where repair is monitored. To test this possibility, we performed band shift assays with MutS\(_a\) and 30-bp A-T homoduplex and G-T heteroduplex oligonucleotides in the presence of increasing concentrations of AD (Fig. 5). These oligonucleotides are identical in sequence to a 30-bp region surrounding the G-T mismatch in the assay substrate. In these experiments, homoduplex and heteroduplex control oligonucleotides were incubated with AD prior to the addition of 100 ng of MutS\(_a\). If DNA containing intercalated AD is recognized as a mispair, a ternary complex between MutS\(_a\), AD, and oligonucleotide is expected with a mobility in native gels similar to the complex between MutS\(_a\) and G-T heteroduplex oligonucleotide.

Because AD intercalation is a stochastic process, it is not possible to introduce an intercalated base at the same site in each member of the population of oligonucleotides as it is for a G-T mismatch. The fact that the 30-mer oligonucleotide can accommodate multiple AD intercalations, up to an average of one every five base pairs near saturation (44, 56) must also be taken into account. Based on published dissociation constants determined for AD intercalation that range from 240 to 435 nM (reviewed in Ref. 38), homoduplex oligonucleotides were incubated with AD over a 10,000-fold concentration range, from 1 nM to 10 \(\mu\)M. These were chosen to ensure that MutS\(_a\) was presented with oligonucleotides that contained, on average, a range of less than one to several intercalated AD molecules. Incubations were performed in the presence of either unlabelled homoduplex competitor oligo or 21 fmol of f1MR3 viral double-stranded DNA to parallel the DNA base content and stoichiometry present in the MMR assay. As shown in Fig. 5, no evidence for a ternary complex involving MutS\(_a\), AT homoduplex, and AD was observed at any drug concentration.

As expected, MutS\(_a\) bound G-T heteroduplex oligonucleotides in the absence of AD (Fig. 5). However, when AD was present at 3.3 or 10 \(\mu\)M, a sharp decrease in the ability of MutS\(_a\) to form a stable complex with the mismatch was observed. These results were independent of the order in which the reagents were assembled; if MutS\(_a\) was added to the reaction first, to allow binding with the mismatch, and AD added last, the same results were obtained (data not shown). This suggests that AD can disrupt mispair binding by MutS\(_a\), even if MutS\(_a\) is already bound to the heteroduplex. In parallel with inhibition of MMR activity, the block to mispair binding by MutS\(_a\) in \textit{vivo} was observed over the same narrow concentration range where AD also blocks the MMR assay (compare Fig. 1 with Fig. 5).

A similar situation was observed for other intercalating MMR inhibitors tested, which include actinomycin D, nogalamycin, and ethidium bromide. In each case, binding of MutS\(_a\) to G-T heteroduplexes in the presence of the drug (Fig. 6; data not shown for ethidium bromide) was disrupted over a concentration range similar to that which inhibited MMR in \textit{vivo} (compare Figs. 3 and 6). As with AD, this bandshift disruption occurred whether MutS\(_a\) or drug was added first (data not shown). From these data, it appears likely that repair of the G-T substrate is impeded because MutS\(_a\) does not recognize mispaired DNA in the presence of the intercalating drug. The parallel with AD is not exact, however, because AD completely blocked complex formation between MutS\(_a\) and the mismatch at 10 \(\mu\)M, whereas a fraction of the bound complex remains in the presence of actinomycin D or nogalamycin at 10 \(\mu\)M (Fig. 6). MMR was not detectable above concentrations of 5 \(\mu\)M in the presence of any of the three drugs.

It is essential to note minor differences between MMR inhibition in the crude nuclear extract and the defined loss of mismatch recognition in the bandshift assay. First, despite the fact that the bandshift experiment is performed with a subset of the DNA sequence found in the MMR assay substrate flanking the G-T mismatch, the DNA content is not identical when compared with the assay substrate. The drugs tested display very different binding affinities for DNA and patterns of cooperativity, depending upon the sequence content and length (43, 44, 56). Second, in the case of the MMR assay, each drug is present throughout the assay, whereas in the bandshift, the negatively charged complex and positively charged drug must be diluted with load dye, introduced into an acrylamide gel, and placed under a constant voltage. Finally, the crude extract contains an estimated 4 \(\mu\)g of RNA in each assay (not shown), in comparison with the 80 ng of double-stranded G-T substrate. The RNA pool provides an undefined repository for intercalating drug, obscuring our ability to make rigorous comparisons between the two types of experiments.

Because the inhibition of MMR in \textit{vivo} occurred at concentrations similar to those that prevented mispair recognition, we also sought to establish that the binding affinity of AD to the oligonucleotide substrate was comparable with its affinity for bulk DNA. The fluorescence of AD is quenched upon intercalation into DNA, and this property has been used to determine DNA binding affinity (59). The homoduplex oligonucleotides used in the mobility shift assay yielded a macromolecular dissociation constant of roughly 200 nM (data not shown). This is in good agreement with published values using calf thymus and other oligomeric DNA (38). Although this strengthens the conclusion that AD intercalation disrupts mismatch recognition, our data do not allow us define the mechanism by which AD blocks the interaction between MutS\(_a\) and the G-T mispair.
Specifically, we cannot distinguish between an inhibition based on drug intercalation and a direct interaction of the drug with either MutS\(\alpha\) or the MutS\(\alpha\)/DNA complex.

**DISCUSSION**

In this work, we focused on clarifying the mechanism by which AD interacts with the human MMR pathway. AD, along with covalent DNA-modifying drugs such as cisplatin or alkylating agents, have the potential to trigger a cytotoxic response mediated by the MMR pathway. However, unlike drugs that cause genome-wide covalent DNA damage, AD has the potential to engage a MMR-dependent apoptotic response without directly modifying DNA. Although AD can be reduced in vitro to generate reactive oxygen species that can damage DNA (60), it may serve as a prototype for a less destructive drug that kills cells by a mechanism mediated by the MMR pathway. To that end, we sought to characterize how AD might affect the human MMR pathway in vitro.

We found that AD, as well as actinomycin D, nogalamycin, and daunomycin, was capable of inhibiting the correction of G-T or A-C mispairs by HeLa nuclear extracts when present at low micromolar concentrations. In comparison, AD introduced into cell culture at high nanomolar concentrations can kill tumor cells. Because AD is both a potent DNA intercalating agent and relatively hydrophobic, it rapidly equilibrates into cell nuclei in various tissues within an hour of subcutaneous injection into hamsters (61). Gigli et al. (62) have demonstrated that AD-sensitive human K562 leukemia cells in culture containing 0.1 \(\mu\)M AD rapidly concentrate it to a nuclear concentration of 11 \(\mu\)M, as measured by microspectrofluorometry. At other doses of AD, a 100-fold concentration was also observed. These data are consistent with the hypothesis that cytotoxic doses of AD reach nuclear concentrations capable of inhibiting mismatch correction in vivo.

When we found that AD interfered with mismatch correction in vitro, we initially focused on demonstrating whether the effect could be explained either by reductive activation of molecular oxygen (60) or by type II topoisomerase inhibition (38). When considered in the context of the drugs subsequently identified as MMR inhibitors, such as nogalamycin, actinomycin D, and the weaker inhibitor ethidium bromide, neither model remains attractive. First, it is unlikely that the inhibitors are all reduced enzymatically to produce reactive species, either oxygen or drug-based radicals, that generate DNA damage capable of inhibiting MMR at similar concentrations. Experimentally, we found no evidence for covalent DNA modification capable of inhibiting repair under the assay conditions. AD incubated with either heteroduplex substrate or HeLa extract alone under mock assay conditions does not result in MMR inhibition if the AD is removed or diluted prior to the assay.

If all four compounds acted via a specific type II topoisomerase, then such inhibition would have to directly or indirectly impact MMR. The only result consistent with topoisomerase inhibition is the block to DNA catenation, which occurs concurrently with MMR inhibition. However, isolation of unrepaird, supercoiled, heteroduplex molecules is also consistent with ligation of the nicked substrate to a covalently closed molecule in the presence of an intercalator (63), which is expected to yield a positively supercoiled molecule following AD removal by phenol extraction. The inhibition of MMR by AD also stands in contrast to the nonintercalating or weakly intercalating topoisomerase inhibitors such as etoposide or \(m\)-amsacrine that failed to affect MMR at any dose tested. A more attractive explanation for the observed MMR inhibition is that reversible DNA intercalation prevents mismatch recognition by human MutS\(\alpha\).

In this work, a correlation was observed between the relative association constants of the tested drugs for DNA intercalation and the concentrations that inhibited MMR. Unfortunately, no single study has determined binding affinities for all the drugs studied here under comparable experimental conditions. This prevents a simple comparison of drug concentrations that inhibit MMR with their affinity for DNA intercalation, because the association constants measured are highly dependent upon the DNA chosen, the experimental conditions, and the approach used. Perhaps the best insight comes from studies where association constants were determined for pairs of drugs in the same study. Consistent with the relative ranking of MMR inhibitors, daunomycin binds \(1.5\)–\(4\) fold less strongly than AD (43–45), whereas ethidium bromide binds DNA roughly \(7\) fold less tightly than AD (48), which has a dissociation constant of roughly 300 \(\text{nM}\). These binding data quantitatively recapitulate the drug ranking as MMR inhibitors, with the effective concentration for MMR inhibition in crude nuclear extracts roughly \(10\) fold above the measured dissociation constant for binding to calf thymus DNA.

A correlation was also observed between DNA binding affinity of the intercalating drugs and the concentration range over which inhibition occurs. The strongest DNA binders (AD, nogalamycin, and actinomycin D) inhibit MMR as the drug concentration varied over \(1–5\ \mu\)M, implicating a highly cooperative step in the inhibition. Molecules with weaker affinity for DNA, such as ethidium bromide, show inhibition profiles spread over a broader range. In both cases, the origin of this apparent cooperativity is unclear. Although AD, actinomycin D, and daunorubicin do exhibit positive cooperative binding at low drug occupancies (\(r < 0.1\) mol of drug bound per mol of base pair at 0.1 \(\text{mNaCl}\); Ref. 43), no evidence for positively cooperative binding at higher concentrations was observed. Inhibition of MMR in vitro and mismatch binding occurs at concentrations above the dissociation constant, where little cooperativity was observed.

Considering both the mutagenic potential of DNA intercalators and their ability to inhibit MMR in vitro, it is useful to revisit possible mechanisms of mutagenesis. Intercalators such as ethidium bromide have been suggested to decrease the fidelity of DNA replication by increasing the frequency of frameshift mutations and single base substitutions (64). By disrupting the ability of a replicative polymerase to select or insert the correct base, an increase in biosynthetic errors would result. Such errors must also either escape surveillance by the MMR pathway or exceed its capacity for mismatch correction. Based on this work, it is also plausible that a diminution of MMR efficiency contributes to the reduced replication fidelity in the presence of DNA intercalators. Specifically, drug intercalation proximal to a mismatch might prevent its recognition.

The limited data available concerning the effect of AD on replication fidelity suggest it to be a mutagen in Chinese hamster ovary cells (65, 66). At concentrations that kill roughly 80% of cultured Chinese hamster ovary cells, a 10-fold increase in mutation frequency at the xanthine-guanine phosphoribosyltransferase gene locus was determined, of which 35% of the characterized mutations were large deletions that removed at least one exon (66). This is not consistent with a complete loss of MMR, which might be expected to increase the mutation rate from 100- to 1000-fold (67). However, it is not yet possible to characterize the extent to which a putative interference with MMR contributes to the number or spectrum of the observed mutational events.

In attempting to dissect the mechanism by which AD reduces replication fidelity, it must be appreciated that this intercalator shows pleiotropic effects on DNA metabolic activities. For
example, AD has been shown to inhibit viral DNA polymerases (56, 68) murine DNA helicase activity (69) and mammalian type II topoisomerase (37). Adriamycin’s best characterized effect, inhibition of mammalian type II topoisomerase activity, occurs at high nanomolar concentrations (37). Furthermore, decatenation assays designed to test topoisomerase catalytic function indicate that in MCF-7 tumors, AD fully inhibits type II topoisomerase activity at 5 μM (70). At drug concentrations that inhibit MMR, DNA metabolism in general is likely to be substantially altered because of inhibition of multiple essential activities.

Another conceivable target for a drug such as AD is an interaction with one of the MutL homologs essential for mismatch correction, such as human MLH1 or PMS2. Recently, an N-terminal fragment of the Escherichia coli MutL protein was shown to have structural similarity to the ATP-binding domain of the DNA gyrase B subunit, an E. coli type II topoisomerase (71). The gyrase inhibitor novobiocin binds to and inhibits the N-terminal fragment of the interaction with one of the MutL homologs essential for mis-

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Human Mismatch Repair and G·T Mismatch Binding by hMutSα In Vitro Is Inhibited by Adriamycin, Actinomycin D, and Nogalamycin

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