postinoculation life span of mice that died. Temporal patterns of death and observations at necropsy examination were the major criteria for assigning deaths to leukemia or drug toxicity. Inoculum response data, defining the relationship between life span and inoculum size, were used to estimate the body burdens of leukemia cells that survived treatment and, hence, the percentages of such cells that were killed.

Registry No. **4a**, 120595-77-9; **4b**, 97337-38-7; **4c**, 104291-18-1; **5a**, 124890-71-7; **5b**, 124416-80-4; **5c**, 124416-74-6; **6a**, 124890-72-8; **6b**, 124416-81-5; **6c**, 124426-69-3; **7a**, 124890-73-9; **7b**, 124416-82-6; **8**, 57873-01-5; **9**, 124416-72-4; **10**, 124416-73-5; $ClNH_2$, 10599-90-3.

Properties of the Nucleic Acid Photoaffinity Labeling Agent 3-Azidoamsacrine

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This paper reports the study of the photochemical, physical, and biological properties of 3-azidoamsacrine. The binding of 3-azidoamsacrine to DNA was studied with UV spectroscopy. The UV spectral behavior is quite similar to that of the parent amsacrine and argues that 3-azidoamsacrine is a good photoaffinity labeling agent for amsacrine. The biological properties (cytotoxicity and mutagenicity) of 3-azidoamsacrine in the mammalian mutagenesis V79 and L5178Y assay systems were measured. Light-activated 3-azidoamsacrine is toxic, but not mutagenic, to V79 cells. 3-Azidoamsacrine with and without light activation, as well as amsacrine, are toxic and mutagenic to L5178Y cells. To probe the interactions of 3-azidoamsacrine with DNA, studies of the photoreactivity of this compound were conducted. 3-Azidoamsacrine was photolyzed in the presence of the plasmid pBR322, and the effect of the photoadducts on restriction endonuclease cleavage was investigated. Amsacrine and 3-azidoamsacrine, without light activation, did not block any of the restriction endonucleases. Light-activated 3-azidoamsacrine blocked cleavage by the restriction endonucleases *Alu*I, *Hin*fI, *Nci*I, *Nae*I, *Dra*I, *Sau*96I, *Hpa*II, and *Hae*III. Photolysis experiments with mononucleosides, blocked mononucleosides, dinucleotides, and DNA all indicated that 3-azidoamsacrine formed adducts with G and A. The structures of these adducts are discussed based upon mass spectral data. Thus, it appears that 3-azidoamsacrine covalently attaches to DNA and that this covalent binding results in the production of toxic and, in some cases, mutagenic lesions in mammalian cells and the inhibition of restriction endonuclease cleavage of DNA.

Acridines are important biochemical probes and have a range of pharmacological activity. Unsubstituted 9-aminoacridine is an antibacterial agent, while substituted acridines have antitumor activity and/or antimalarial activity. In addition, acridines are frameshift mutagens in *Salmonella* strain TA1537.[1-6] Recently, there has been interest in synthesizing acridines linked to oligonucleotides as antivirial agents.[7-9]

Acridines are known to bind to DNA by two processes. One process, which is commonly thought to be intercalation, is a strong binding process. The second process is a weaker binding process and is thought to involve outside (ionic) binding.[1,10-12] Acridines also have effects on membranes in certain redox systems and induce topoisomerases and strand breaks in DNA.[13-18] The pharmacological activity of acridines may be related to all of these processes. The mutagenic activity of acridines is also related to their binding to DNA.[1,5,10,19,20]

Photoaffinity labeling offers an approach to selectively forming acridine–nucleic acid covalent bonds after intercalation has occurred. Subsequent analysis of the adduct profile could provide information on sequence specificity of acridine–DNA interactions. In addition, the biological effects of acridines covalently linked to nucleic acids via photoaffinity labeling can also provide information on the

Table I. Reactivity of 3-Azido-AMSA under Various Conditions

compound	medium	light	half-life
AMSA	water	no	>2 weeks
AMSA	water	yes	degrades rapidly (ca. 1 day)
3-azido-AMSA	water	yes	about 2 min
3-azido-AMSA	water	no	>2 weeks
3-azido-AMSA	water + DNA	yes	about 2 min
3-azido-AMSA	water + DNA	no	>2 weeks

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Table II. Mutation Induction for L5178Y Cells

compound	dose, μg/mL	relative toxicity 20-h treatment	viability	relative	mean	mutation frequency ($F \times 10^6$)	induced frequency
			Experiment 1				
control dark		100	89.0 ± 6.1	100	48.3 ± 7.6	108	0
3-azido-AMSA dark$_1$	0.005	69	66.7 ± 0.7	53	99.7 ± 4.0	427	319
3-azido-AMSA dark$_2$	0.008	55	22.3 ± 4.9	23	63.0 ± 3.0	565	457
control light		100	186.0 ± 9.5	100	143.3 ± 27.6	154	0
3-azido-AMSA light$_1$	0.005	69	57.0 ± 2.7	30	123.7 ± 12.3	434	280
3-azido-AMSA light$_2$	0.008	55	0		0		
			Experiment 2				
control dark		100	30.0 ± 4.6	100	20.7 ± 5.7	138	0
AMSA dark	0.005	62	20.0 ± 1.7	67	57.7 ± 10.4	577	439
3-azido-AMSA dark	0.005	65	37.7 ± 5.8	124	95.0 ± 5.7	504	366
control light		100	129.0 ± 17.7	100	83.0 ± 4.0	129	0
AMSA light	0.005	62	12.3 ± 5.0	10	53.0 ± 10.4	862	733
3-azido-AMSA light	0.005	65	35.7 ± 3.9	28	114.3 ± 18.6	640	411

relationship between covalent bond formation and biological activity. Coffman et al.[21] and Iketa and Dervan[22] have used aromatic azides along with restriction endonuclease cleavage to probe the sequence specific binding of ethidium and related compounds.

This paper reports the study of the interaction of 3-azidoamsacrine (3-azido-AMSA) with mononucleosides, dinucleotides, DNA, and the study of the biological properties of 3-azidoamsacrine in the V79 and L5178Y mammalian cell mutation assays in both the light and the dark. The effect of light-induced 3-azidoamsacrine binding to DNA (plasmid pBR322) on restriction endonuclease cleavage is also reported.

3-Azidoamsacrine will be toxic at some level in V79 cells; light acts to make it more toxic. 3-Azidoamsacrine, with and without light activation, and amsacrine are toxic and mutagenic to L5178Y cells. In addition, light-activated 3-azidoamsacrine inhibits cleavage of pBR322 DNA by *Alu*I, *Hinf*I, *Nci*I, *Nae*I, Dra*I*, *Sau*96I, *Hpa*II, and *Hae*III.

Results and Discussion

Figure 1 shows that in the presence of DNA the long-wavelength absorption of 3-azidoamsacrine experiences a shift to longer wavelength. This shift is similar to that observed for amsacrine in the presence of DNA and argues that 3-azidoamsacrine binds to DNA in a similar manner. This fact, along with the fact that amsacrine and 3-azidoamsacrine show a similar spectrum of antitumor activity and mutagenicity,[24] indicates that 3-azidoamsacrine is an acceptable photoaffinity labeling agent for amsacrine.[26-29]

The photochemical properties of 3-azidoamsacrine have been investigated (Figure 1). For the irradiations of 3-azidoamsacrine + DNA, the photolysis was continued until the spectrum indicative of photolytic destruction of the 3-azidoamsacrine was obtained (the spectrum represented by the dotted line). Repeated ethanol precipitation of modified DNA to constant absorbance gave spectra which show that the DNA is modified (a longer wavelength absorption is present, see Figure 1, spectrum E). This suggests that the amsacrine moiety was covalently bound to the DNA.

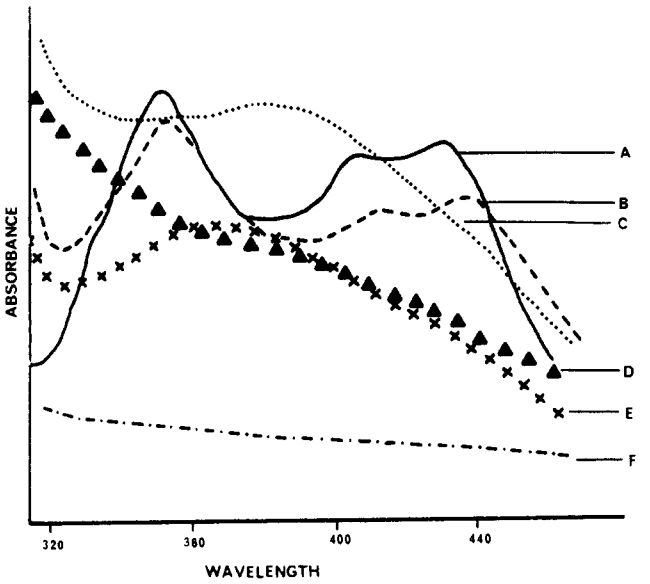

Figure 1. Shifts in the visible region of absorption spectra of 3-azidoamsacrine (3-azido-AMSA) in 5 mM KH_2PO_4, pH 5.5, 4 × 10^{-5} M: (A) (–) 3-azido-AMSA only; (B) (- -) 3-azido-AMSA + calf thymus DNA drugs/nucleotides (D/N) = 1/50; (C) (··) 3-azido-AMSA + calf thymus DNA, D/N = 1/50, after 30 min of photolysis; (D) (×) 3-azido-AMSA after 30 min of photolysis; (E) (▲) ethanol precipitation of C; (F) (-··) ethanol precipitation of (B).

Photolysis results in a significant alteration of the chromophore. This is expected for the photolysis of aryl azides since they undergo a number of light-induced reactions.[23,26-30] Table I shows that 3-azidoamsacrine is extremely unstable in light, even in water. This result may indicate that only a small fraction of the 3-azidoamsacrine molecules will react with DNA to form adducts.

3-Azidoamsacrine was not mutagenic to V79 cells either with or without light activation. Three studies testing 3-azidoamsacrine at 0.01 or 0.02 μg/mL with light activation or 0.02 μg/mL without light activation demonstrated mutation frequencies that were not significantly above the background in all groups. Interestingly, while the initial toxicity plates showed no effect, the viability plates (prepared after the five day mutant expression time)

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Table III. Summary of Effect of 3-Azido-AMSA on Restriction Nuclease Cleavage

			3-azido-AMSA						AMSA		
			no activation			light activation					
no. of sites	enzyme	cleavage site	1:8[a]	1	4	1:8	1	4[b]	1:8	1	4
16	*Alu*I	AG/CT	–	–	–	+	+	++	–	–	–
10	*Hinf*I	G/ANTC	–	–	–	+	+	+	–	–	–
10	*Nci*I	CC/$^{C}_{G}$GG	–	–	–	+	+	+	–	–	–
4	*Nae*I	GCC/GGC	–	–	–	+	+	++	–	–	–
3	*Dra*I	TTT/AAA	–	–	–	+	++	++	–	–	–
15	*Sau*961	G/GNCC	–	–	–	+	+	++	–	–	–
26	*Hpa*II	C/CGG	–	–	–	+	+	++	–	–	–
22	*Hae*III	GG/CC	–	–	–	+	+	+	–	–	–

[a] Drug/DNA. [b] A too high drug concentration causes DNA to precipitate.

showed a significant decrease in the light-activated 3-azidoamsacrine groups (viability as percent of control: 0.02 μg/mL with light, 30% (experiment 1, 24% experiment 2); 0.01 μg/mL with light, 60%, 76%; 0.02 μg/mL dark, 87%, 77%). Thus, 3-azidoamsacrine is not mutagenic, but its toxic effects are increased by photoactivation.

3-Azidoamsacrine was both toxic and mutagenic in the L5178Y cell mutation assay (Table II). Both 3-azidoamsacrine and amsacrine induced similar mutation frequencies with and without light activation. In all cases in which mutation was observed, 3-azidoamsacrine was toxic to the cells. At identical doses, 3-azidoamsacrine was more toxic, based upon cell viability after the expression time, with light activation than without light activation.

In the L5178Y assay, amsacrine and 3-azidoamsacrine can be observed to cause the development of smaller colonies, which are the dominant type of mutant observed. These smaller colonies are usually associated with agents that induce chromosomal exchange or aberrations that may not be due to single base substitution types of mutation at the TK locus.[25] Amsacrine is known to cause double-strand breaks and chromosomal damage due to the inhibition of topoisomerases.[14,15] However, whether these effects are related to the mutagenicity in the L5178Y assay or are merely related to toxicity remains to be established.

Effects of a typical restriction endonuclease (*Nci*I) treatment of the photolyzed mixture of plasmid in 3-azidoamsacrine are shown in Figure 2. It is clear from these results that 3-azidoamsacrine with light activation blocks cleavage by *Nci*I. Effects of variations of the concentration of *Nci*I and variation of the drug/DNA ratio are shown in this figure. The results generally indicate that there is a threshold for blocking of restriction nucleases. In addition, analysis of the digestion fragments using end labeling gave identical results. The results for other restriction nucleases are summarized in Table III.

The recognition sequence of the restriction nucleases studied are listed in Table III. Light-activated 3-azidoamsacrine blocks cleavage at all of these sites. These results lead to the hypothesis that 3-azidoamsacrine is relatively nonspecifically bound to all of these sites and inhibits cleavage. This is consistent with other studies which show that amsacrine and other acridines bind to a large number of sequences on double-stranded DNA.

In order to gain more information on the structure of the light-induced adducts of 3-azidoamsacrine to DNA, 3-azidoamsacrine was photolyzed in the presence of the deoxyribonucleosides (dA, dG, and dC). This led to the formation of 1:1 adducts after purification. These adducts were detected with FAB mass spectrometry. The presence of adducts was also confirmed by UV–visible spectral analysis of HPLC traces. Table IV lists the expected molecular ion ($M + H^+$) for the 1:1 adducts that were obtained, along with the exact mass of the observed molecular ion and the exact mass of the largest other peak

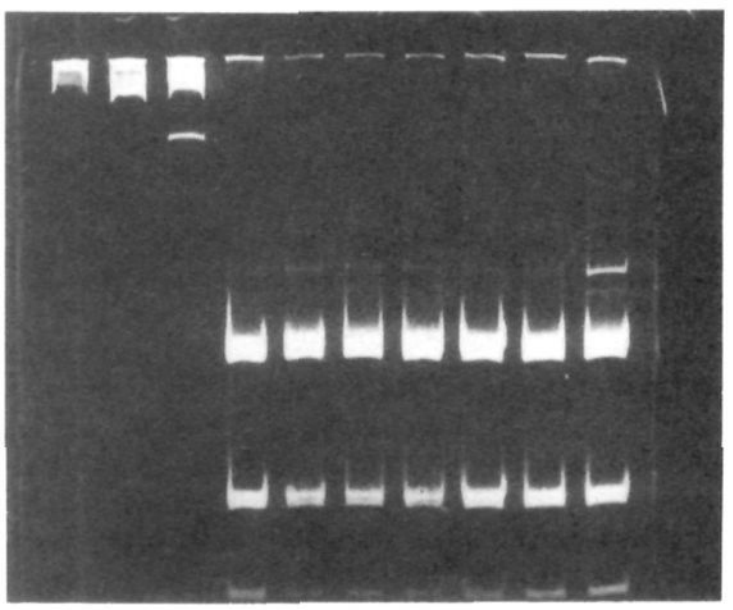


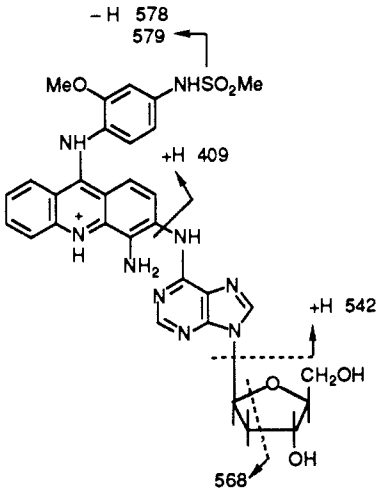
Figure 2. Polyacrylamide gel electrophoresis of *Nci*I restriction enzyme digests of drug-treated DNA at different drug concentration and different amount of enzymes as indicated. D/N ratio denotes the ratio of 3-azido-AMSA to nucleotide used in the treatment of the plasmid.

Table IV. Mass Spectral Data for Deoxyribonucleoside Adducts of 3-Azido-AMSA

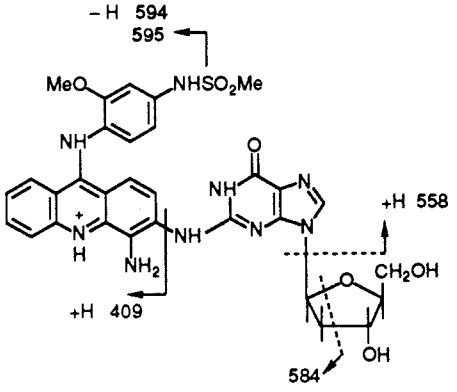
	molecular ion		mass of largest peak in MS/MS
adduct	expected	observed	
dA–3–azido-AMSA	658 ($C_{31}H_{32}O_6N_9S$)	658.2196	542.1735
dG–3–azido-AMSA	674 ($C_{31}H_{32}O_7N_9S$)	674.2150	558.1672
dC–3-azido-AMSA	634 ($C_{30}H_{32}N_7O_7S$)	634.2084	518.1608

in the MS/MS spectrum. This peak always corresponds to the loss of the deoxyribose ring from the adduct. One possible structure for these adducts is shown and results from the addition of the amino group of the deoxyribonucleotide to the 3-position of azidoamsacrine. This type of addition is expected, on the basis of the studies of Schrock and Schuster[23] of photolysis of arylazides, and results from the addition of an amine to the azarine formed by photolysis. These structural suggestions were consistent with the depurination experiments which indicated that the N^7 of guanine and adenine are not the site of addition. Attempts to form adducts of 3-azidoamsacrine with dA and dG blocked on the exocyclic amino group failed, indicating this may be the site of addition. Unfortunately, the complexity of the mixture of adducts has, so far, prevented us from isolating enough adduct to obtain an NMR spectrum.

Attempts to form photoadducts with DNA in solution produced variable results. In approximately 30–50% of the cases, photoadducts were formed. However, in the rest of the cases, no photoadducts could be detected even after mass spectral analysis. This type of behavior is consistent



dA adduct



dG adduct

with the fact that the amounts of adducts formed are at or near the limit of detection, which is in the range 10^{-8}–10^{-9} g, and that the photoreaction with DNA is extremely complex. Nevertheless, in several of these reactions, molecular ion peaks at 574 and 658 were obtained, indicating that dG and dA adducts were formed. In some cases, it was possible to obtain the MS/MS spectrum of these ions, and these spectra were consistent with those shown in Table IV, confirming the formation of these adducts.

Control experiments indicated that no unusual phenomena were occurring. Photolysis of either DNA or 3-azidoamsacrine, prior to mixing, produced no adducts. Photolysis of amsacrine in the presence of DNA also produced no detectable adducts. Mixing of 3-azidoamsacrine and DNA without photolysis produced no detectable adducts, and photolysis of 3-azidoamsacrine, prior to the addition of DNA, also produced no detectable adducts.

Experimental Section

Chemicals. 3-Azidoamsacrine [*N*-[4-[(3-azido-9-acridinyl)amino]-3-methoxyphenyl]methanesulfonamide] hydrochloride salt was prepared by following the procedure of Cain and coworkers.[24] Purity was checked by melting point (obs mp 282–284 °C; lit.[24] mp 282–283 °C) and silica gel TLC with methanol/CH_2Cl_2 (1:9, v/v). The molar extinction coefficients for 3-azidoamsacrine were measured in ethanol and water and were 10 900 (at 352 nm in EtOH), and 9510 (at 432 nm in EtOH), 9550 (at 353 nm in water), and 8760 (at 433 nm in water).

Mass Spectra. All mass spectra were measured on a Kratos MS-50 mass spectrometer. MS/MS spectra were measured with bandwidth filtering set at 10 Hz and a scan rate of 100 s/decade.

Binding of 3-Azidoamsacrine to DNA. 3-Azidoamsacrine was mixed with calf thymus DNA and irradiated with a medium-pressure mercury lamp in a Pyrex photolysis apparatus (Ace glass) using a 15% cobalt nitrate solution as a filter in the outer compartment. The DNA was analyzed for the presence of covalently bound acridine by two procedures. In the first, the DNA was repeatedly dissolved in buffer and precipitated with ethanol and the absorption spectrum was monitored. In the second procedure, the DNA was enzymatically degraded to deoxyribonucleosides with DNase, venom phosphodiesterase (*Crotalus atrox*), and alkaline phosphatase and then chromatographed on a Sephadex LH-20 column. After elution of the deoxyribonucleosides with water, the acridine–deoxyribonucleoside adducts were eluted with methanol, and the methanol-eluted material was analyzed by HPLC.

V79 Mutation Assay. Chinese hamster cell line V79-16 was obtained from Dr. Leila Diamond of the Wistar Institute of Anatomy and Biology, Philadelphia, PA. The cells were maintained by transferring 8×10^5 cells every 3 days into 75 cm^2 flasks. The medium used throughout the mutation assay was Eagle's basal medium (BME, MA Bioproducts) supplemented with 10% FCS (Reheis) and 10% L-glutamine (Gibco). Cells (3×10^6) were seeded in 75 cm^2 flasks, and, after 2 h, the acridine diluted with medium was added to the cells to give different concentrations. After 20 h of exposure, the cells were harvested and the induced toxicity was determined by placing 100 cells in 5 mL of BME per plate on 10 60-mm petri dishes and staining 7 days later. Cells (12×10^4) in 20 mL of BME were seeded in a 75 cm^2 flask for a 5-day expression time. The cells were then harvested and a second toxicity measurement (viability) was conducted in the same manner as the first measurement. The mutation frequency was measured by plating 4×10^4 cells in 5 mL of BME containing 6-thioguanine (0.1 mM) into 25 60-mm plates. The cultures were refed with fresh BME with 6-thioguanine 5 days after plating and stained with giemsa 11–14 days after plating.

L5178Y Assay. L5178Y cells were obtained from Donald Clive, Burroughs Wellcome Co., Research Triangle Park, NC.[25] The culture was maintained and the assay was carried out by using the procedures of Turner et al., Burroughs Wellcome Co., Research Triangle Park, NC, except that the activating enzyme was not used. The suspension culture was saturated with 5% CO_2 in air, and the culture was incubated in a New Brunswick shaker at 150 rpm at 37 °C. The cells were grown and selected as described previously.[25] Then, 20 mL of 3×10^5 cells/mL were treated with the amount of acridine indicated in Table II in a 25 cm^2 flask and incubated for 20 h in a shaker. The treated cells were centrifuged, the pellet was washed twice with 10 mL of Fop (Fishers media for leukemic cells of mice) and resuspended in 20 mL of Fop for incubation for an additional 24 h. Cells were then transferred to new medium in order to maintain 3×10^5 cells/mL. Under the same conditions, the solvent control culture always reached 1×10^6 cells/mL after 24 h of growth. The toxicity of the acridine was determined by the density of the treated cells vs that of the control cells after a 20-h treatment. The cell densities were measured by a Coulter counter. The viability of the treated cells was measured by plating 200 cells in three 100-mm petri dishes in soft agar cloning medium. The resistant mutants were obtained by placing 10^6 cells in three 100-mm petri dishes in cloning medium with 0.1 mg/mL TFT (trifluorothymidine, Sigma). The number of colonies on plates for viability and TFT were determined 10 days after plating by using a Bio Scan II automated colony counter.

Light-Activated Mutation Assay. Cells (6×10^6, either V79 or L5178Y) were treated for 20 h with 3-azidoamsacrine and were immediately centrifuged. The L5178Y cells were treated in suspension and irradiated for 6.5 min. The V79 cells were treated in monolayer culture, trypsinized, and resuspended in 20 mL of fresh medium and irradiated for 6.5 min. Irradiation was conducted in an Ace photolysis apparatus using a mercury medium-pressure lamp filtered by both the Pyrex immersion well and a 10% cobalt acetate filter solution. These conditions filtered off approximately 100% of the light with wavelength less than 340 nm. In order to insure that all of the 3-azidoamsacrine was photolyzed, the medium was monitored by the disappearance of the UV absorbance at 350 nm. A control experiment showed that after 6.5 min of irradiation the azido functionality of the molecule was completely destroyed.

Photolysis of 3-Azidoamsacrine Plasmid Mixtures.

Plasmid pBR322 DNA (4 μg) was equilibrated with the concentration of 3-azidoamsacrine required to achieve the concentration ratios listed in Figure 2 in ice in 5 mM potassium phosphate buffer, pH 5.5, for 30 min in the dark. The sample was then photolyzed under optimized conditions with a 450-W Ace Hanovia medium-pressure Hg-vapor lamp in a Pyrex photolysis apparatus (thus filtering light with wavelengths below 340 nm). Each irradiation was monitored by the disappearance of the UV absorption at 350 nm, which is characteristic of the 3-azidoamsacrine, to insure that the reaction was complete. The drug–plasmid complexes were precipitated by adding $^1/_{10}$ volume of 3 M sodium acetate, 0.1 M EDTA, and 2 volumes of ethanol at –20 °C and dried as a pellet in a vacuum desiccator.

Analysis of Blockage of Restriction Nucleases. The restriction endonucleases were purchased from Bethesda Research Laboratory. 3-Azidoamsacrine or amsacrine and plasmid pBR322 were mixed in three different ratios of drug to DNA in which the drug concentration was 0.015, 0.12, or 0.48 mM, the total volume was 20 μL, and the drug to DNA ratio was 1:8, 1:1, or 4:1. The drug/DNA mixture was irradiated as described above for 1 h. Another identical set of drug/DNA solutions was kept in dark as a control. For each endonuclease digestion, the sample was diluted to a total volume of 20 μL with 7 μL of DNA plus drug, 2 μL of 10× buffer, 2 μL of 10 mM DTT, and 8 μL of H_2O and digested with 10× excess enzyme overnight. Samples were loaded on 1.2% agarose gel and separated by electrophoresis using TBE buffer (75 mM).

End Labeling. In a second experiment, the restriction fragments were end labeled by using the following procedure: (1) After the modified plasmid was completely digested with restriction endonucleases, 5 μL of buffer (0.5 M Tris, 50 mM magnesium chloride, and 1 mM zinc chloride), 0.5–1.0 unit of alkaline phosphatase (calf intestine, Sigma), and water were added to give a total reaction volume of 50 μL. The mixture was incubated at 37 °C for 1 h, then 150 μL of water was added and the solution was extracted with a mixture of phenol/chloroform/amyl alcohol (25:24:1) and then with ether. It was then precipitated with ethanol and dried briefly. (2) The sample was then mixed with 1 μL of kinase buffer (100 mM glycine, 10 mM spermidine, 1 mM EDTA), 6 μL of water, 2 μL of γ-^{32}P ATP (5000 mCi/mmol, 100 μCi/100 μL) and heated to 90 °C. It was then returned to an ice bath for 5 min before adding 1 μL of T_4 polynucleotide kinase (10 units). The sample was then incubated for 1 h at 37 °C. The end-labeled fragments were analyzed with autoradiography.

Photoreactions with Mononucleosides, Protected Mononucelosides, and Dinucleotides. For these experiments, 5 mL of a 500-fold excess of each mononucleoside (2-deoxyadenosine, 2-deoxyguanosine, 2-deoxyuridine, 2-deoxycytidine, and thymidine) and N-protected nucleoside [N^6-benzoyl-5-*O*-(4,4-dimethoxytrityl)-2-deoxyadenosine and N^2-isobutyryl-5-*O*-(4,4-dimethoxytrityl)-2-deoxyadenosine] were used. For the dinucleotides ApU and GpC, 5 mL of a 0.1 M solution was used. These solutions were then mixed with 15 mL of a 1×10^{-4} M 3-azidoamsacrine solution under nitrogen in a Pyrex round-bottom flask. The solution was mixed for 5 min and then photolyzed for 30 min with the mercury lamp described previously.

The mononucleoside adducts were isolated by extraction with 3×10 mL of chloroform, chromatography of the resulting water phase on a Sep Pak C_{18} cartridge, and elution of the adducts with methanol. A methanol eluant was then concentrated and chromatographed on a reverse-phase HPLC using a 30–95% methanol/water gradient over 30 min. The FAB mass spectrum of each peak was measured. Alternatively, the methanol concentrate was used directly for FAB MS analysis prior to HPLC. A study of the photoreactivity of 3-azidoamsacrine with each of the nucleosides was also carried out. In this case, 2-deoxyadenosine, 2-deoxyguanosine, and 2-deoxycytidine (0.05 M) were prepared and reacted with 1×10^{-4} M 3-azidoamsacrine in a total volume of 15 mL by using the same procedures as described above. The reaction mixture was extracted with $CHCl_3$ and loaded on a Sep Pak C_{18}. The Sep Pak was eluted first with water and then methanol. The methanol was concentrated and submitted to FAB mass spectral analysis using standard conditions.

In another experiment, the 2-dA and 2-dG adducts, prepared as described above, were subjected to depurination by treatment of the water phase with 0.15 N HCl at 37 °C overnight. The water phase was then chromatographed on a Sep Pak C_{18} column, washed with distilled water, and eluted with methanol. A 50-μL aliquot of methanol concentrate was injected into the HPLC, and the fractions were collected and subjected to mass spectral analysis using FAB. For comparison purposes, photoreaction of 3-azidoamsacrine with adenine was also carried out. In this experiment, 5 mL of 0.5 M adenine was mixed with 15 mL of 1×10^{-4} M 3-azidoamsacrine in a Pyrex round-bottom flask. The workup was identical with that used for the nucleoside adducts.

Photoreactions with Calf Thymus DNA. Calf thymus DNA was purified by using the following procedure: Approximately 100 mg of DNA was dissolved in 10 mL of doubly distilled water, treated with 1 mg/mL proteinase K and 0.1 mg/mL RNase A, and incubated at 37 °C for 24 h. Then, an equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added to the DNA solution in a 30-mL Pyrex tube. The tube was mixed and centrifuged for 10 min at 1500 rpm. The top aqueous layer containing the DNA was removed, and 6 mL of chloroform/isoamyl alcohol was added. The tube was then mixed and centrifuged again for 10 min at 1500 rpm. The upper layer was removed and a 10-μL aliquot was analyzed with UV. The samples had an absorbance ratio of (A260/A280) greater than 1.6 units. The DNA was then precipitated with 2 volumes of 95% ethanol and $^1/_{10}$ volume of 2 M NaCl. It was transferred to a clean vial, rinsed with 100% ethanol, and dried under a stream of nitrogen.

Photoreactions with DNA. Photoreactions were carried out both in solution and in frozen solution. In both cases, 100–150 mg of purified DNA was dissolved in 10 mL of distilled water. 3-Azidoamsacrine (15 mL, 1×10^{-4} M) was then added, and the solution was mixed for 15–20 min and photolyzed for 30 min by using the same procedure as described above. The DNA was then degraded to deoxyribonucleosides by using the following procedures: 100 μL of saturated $MgCl_2 \cdot 6H_2O$ and 10 mM of Tris buffer was added, along with 100 μL of DNase I from bovine pancreas. The mixture was incubated for 24 h at 37 °C. Then, an equal volume of pH 9, 0.1 M Tris buffer was added, and 0.1 unit of phosphodiesterase from *C. atrox* venom was added. After incubation for 24 h, 0.1 unit more of the snake venom phosphodiesterase was added, and the solution was incubated for 24 h. Alkaline phosphatase from *Escherichia coli* (2.5 units) was then added and the mixture was incubated for 24 h. The digested DNA adduct mixture was then loaded onto a Sep Pak C_{18} cartridge. After 100 mL of water wash, the adducts were eluted with methanol. The methanol concentrate was then submitted for further chromatography or for FAB mass spectral analysis, using glycerol as a matrix. For the reactions in ice, the same procedure was used, except that the aqueous solution was frozen and thawed under vacuum pumping three times to degas the solution. The mixture was then frozen on the inside of a round-bottom flask and photolyzed for 30 min by constantly rotating the flask with a rotary evaporator. The adduct mixture was isolated as described above under Photoreactions with Calf Thymus DNA.

Control Experiments. Control experiments involving photolysis of the DNA prior to addition of the azide, photolysis of the acridine DNA mixture without the prescence of azide, photolysis of the 3-azidoacridine and then mixing with DNA, and mixing 3-azidoamsacrine and DNA without light activation were carried out. All samples were purified, as described above, and analyzed with FAB mass spectrometry. No adducts were detected by FAB/MS under these conditions. The limits of detection of these control experiments cannot, at present, be determined because we have not been able to isolate enough adduct to "spike" various samples and experimentally determine the limit of detection. However, generally, FAB/MS is estimated to have a detection limit in the range of 10^{-8}–10^{-9} g.

Conclusion

These data taken together indicate that 3-azidoamscarine is a suitable photoaffinity labeling agent for amsacrine and is capable of forming light-induced DNA adducts. These DNA adducts may be responsible for the increased toxicity of light-activated 3-azidoamsacrine in the mammalian cells. It was impossible to unequivocally determine the structure of the adducts; however, data indicated that they may be formed by addition of the exocyclic amino

group of the bases to the azarine formed from photolysis.

This is the most definitive data to date to suggest the aromatic azides are useful photoaffinity labeling agents for DNA. Further studies are needed to determine (1) the relative reactivity of light-activated 3-azidoamsacrine with different bases and base sequences and (2) whether the reactivity is indiscriminate enough to give a similar probability of reaction at each DNA binding site and, thereby, allow a study of the sequence specificity of binding of 3-azidoamsacrine to DNA.

Acknowledgment. This research was supported by NIH Grants GM29175, CA40228, and AI27152. We thank Constance G. Moynihan for excellent technical assistance.

Preparation of Triazolo[1,5-*c*]pyrimidines as Potential Antiasthma Agents

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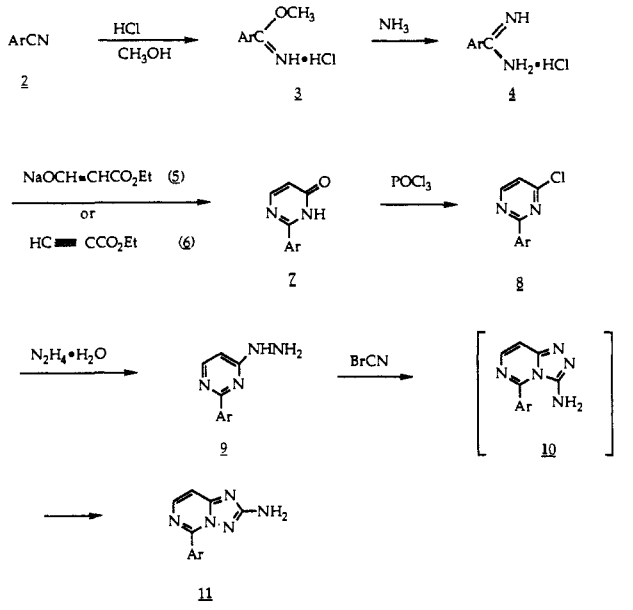
Received March 13, 1989

With the use of the human basophil histamine release assay, 5-aryl-2-amino[1,2,4]triazolo[1,5-c]pyrimidines were found to be active as mediator release inhibitors. These compounds were prepared by reacting arylamidines with sodium ethyl formylacetate or with ethyl propiolate to give pyrimidinones. Treatment with phosphorus oxychloride gave a chloropyrimidine, which was converted to a hydrazinopyrimidine with hydrazine. Cyclization, using cyanogen bromide, gave the triazolo[1,5-c]pyrimidines, after a Dimroth rearrangement. Following a structure–activity evaluation, the 5-[3-(trifluoromethyl)phenyl]-2-amino (**8-10**), 5-(3-bromophenyl)-2-amino (**8-13**), 5-[3-(difluoromethoxy)-phenyl]-2-amino (**8-11**), and 5-(4-pyridinyl)-2-amino (**6-7**) compounds were found to have the best activity. They were chosen for further pharmacological and toxicological study.

It has been pointed out by Reed[1] that in the past 10 years there was a 4-fold increase in the number of prescriptions written for obstructive lung diseases, while the rate of hospitalization for asthma has increased at almost the same rate. In addition, the death rate for asthma has not decreased. The implication of these results is that current methods of asthma treatment are inadequate. Most therapies treat the symptoms of the disease and it would be an improvement to treat asthma prophylactically. One of the few prophylactic drugs currently available is disodium cromoglycate[2] (DSCG) which must be taken by inhalation. However, the method of taking DSCG may result in lack of patient compliance and limits its usefulness. One approach to the treatment of asthma would be to prevent the release of mediators of anaphylaxis from mast cells and basophils[3] by an oral medication, since it is believed that the release of mediators, such as histamine, leukotrienes, PAF, and others, precipitate the bronchoconstruction of asthma and the inflammation of allergic attacks.

In searching for antiasthmatic compounds, the rat mast cell has been used as a screen[4] and also as an evaluation model.[5] It has been concluded that rat mast cells differ in their pharmacology from human mast cells,[6] thus rat mast cells are not ideal models for asthma. Obviously the best method to study mediator release with would be the human mast cell, since it is believed that the reaction of an antigen with IgE on the mast cell surface triggers the release of mediators. However, since human mast cells are not available in quantities for screening, a good substitute is the readily available human basophil. Like the mast cell, the basophil has on its surface IgE, which reacts with antigens. Release of mediators from this cell has been used to confirm active compounds found by the rat passive cutaneous anaphylaxis (PCA) test,[7] but to the best of our

Scheme I



2 3 4 7 8 9 10 11

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0022-2623/90/1833-1230$02.50/0