Synthesis and DNA-Sequence Selectivity of a Series of Mono- and Difunctional 9-Aminoacridine Nitrogen Mustards

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The aim of this work was to identify nitrogen mustards that would react selectively with DNA, particularly in G-rich regions. A series of mono- and difunctional nitrogen mustards was synthesized in which the (2-chloroethyl)amino functions were connected to the N^9 of 9-aminoacridine by way of a spacer chain consisting of two to six methylene units. The length of the spacer chain connecting the alkylating and putative DNA-intercalating groups was found to affect the preference for the alkylation of different guanine-N^7 positions in a DNA sequence. All of the compounds reacted preferentially at G's that are followed by G as do most other types of nitrogen mustards, but the degree of selectivity was greater. The compounds reacted at much lower concentrations than were required for comparable reaction by mechlorethamine (HN2), consistent with initial noncovalent binding to DNA prior to guanine-N^7 alkylation. The degree of DNA-sequence selectivity increased as the spacer-chain length decreased below four methylene units. Most strikingly, long spacer compounds reacted strongly at 5'-GT-3' sequences, whereas this reaction was almost completely suppressed when the spacer length was reduced to two or three methylenes. Mono- and difunctional compounds of a given spacer length showed no consistent difference in DNA-sequence preference.

Introduction

Rational anticancer drug development faces the fundamental difficulty that most drugs have multiple sites of action and it is not known which of these may contribute to therapy and which may contribute solely to toxicity. A strategy to cope with this dilemma would select compounds that have the most restricted range of actions while not suffering reduced antitumor activity in preclinical test systems. Just as medicinal chemists seek to purify a single active chemical species from an active mixture, one may seek to isolate as nearly as possible a single effective action mechanism. In addition to possible reduction of toxicity, this would reduce the chance of unpredictable interactions in drug combination therapies.

The nitrogen mustards are notorious for their multiplicity of reaction sites which may include all biomolecules bearing accessible nucleophilic groups. These nonspecific drugs nevertheless remain among the most useful chemotherapeutic agents. Surely most of the reactions are useless as far as therapy is concerned, and some could be sources of side effects. Among the multiplicity of reactions of nitrogen mustards, there may be a single class (perhaps a quantitatively minor one) that is responsible for the therapeutic effects. If the effective reactions could be enhanced and the unnecessary reactions suppressed, a substantial improvement in therapy might be anticipated.

Alkylation of DNA is generally accepted as the most likely reaction class responsible for the antitumor activity of most nitrogen mustards. A first step, therefore, would be to suppress non-DNA reaction sites. This can be accomplished by adding a DNA-binding moiety, for example, an intercalating ring system, to the drug molecule. Creech et al.[1] prepared a variety of mustards linked through aliphatic chains to heterocyclic ring systems that could bind to DNA by intercalation. A number of interesting observations were made in tests of the ability of the compounds to prolong the life of mice inoculated with Ehrlich ascites tumor cells (intraperitoneal drug administration on days 1-3 following tumor inoculation). The most effective compounds increased survival time (relative to a control of 16 days) by a factor of at least 2.6 at the optimal dose. A striking observation was that, contrary to the common nitrogen mustards which require difunctionality for good antitumor activity, some of the heterocyclic nitrogen mustards were almost equally effective as mono- or difunctional variants. This was true, however, only if the heterocyclic ring was fully aromatic; if one of the rings of an acridine-like structure was saturated and therefore nonplanar and unlikely to be able to intercalate, only the difunctional mustard was active. Moreover, with difunctional compounds, the optimal dose was lower when intercalation was possible. Thus, the ability to intercalate seemed to increase potency and to eliminate the requirement for difunctionality.

After targeting of DNA, a further level of selectivity could be for reaction in particular base-sequence environments. The most preferred base for nitrogen mustards is guanine, particularly the N^7 position which is the most electronegative position in DNA.[2] Since much of the mammalian genome appears to be chimeric for G/C-rich and A/T-rich regions in different types of genes and regulatory sequences,[3] selectivity for one or the other type of region could be biologically important. Study of the sequence selectivity of several types of nitrogen mustards revealed enhanced reactivity at the G-N^7 position located in sequence environments that would be expected to enhance the electronegative potential at the reaction site.[4]

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The most electronegative base triplet is GGG (with the reaction site at the central G), and these tended to be the most reactive sequences for most nitrogen mustards. Selectivity for GGG sequences would greatly favor reaction in G-rich regions, including G-string regulatory sequences.[5] It is therefore of interest to identify nitrogen mustards with maximum selectivity for GGG sequences.

Among the nitrogen mustards studied in our previous investigations of DNA-sequence selectivity, quinacrine mustard showed unusual behavior.[4,6] Quinacrine mustard, a DNA intercalator, consists of a substituted acridine ring linked via a hydrocarbon chain to a bis(2-chloroethyl)-amine moiety. Quinacrine mustard had greater sequence discriminatory ability than any of the other mustards studied. The sequences most preferred were 5′-GGR-3′ and 5′-GTR-3′ (R = purine), where the reaction is at the first G. G or T in the +1 position was equally effective, and the further enhancement by purine, as opposed to pyrimidine, in the +2 position was of a lesser degree. The requirement for G or T at +1 was attributed to an incompatibility of the amino group of C or A in this position with the hydrocarbon spacer chain stretching across the intervening base pair from the intercalated acridine ring system to the alkylation site.[4] A drug design objective would be to maximize the selectivity of a DNA-intercalating nitrogen mustard for sequences such as 5′-GGG-3′ and to minimize the reactivity with 5′-GT-3′-type sequences.

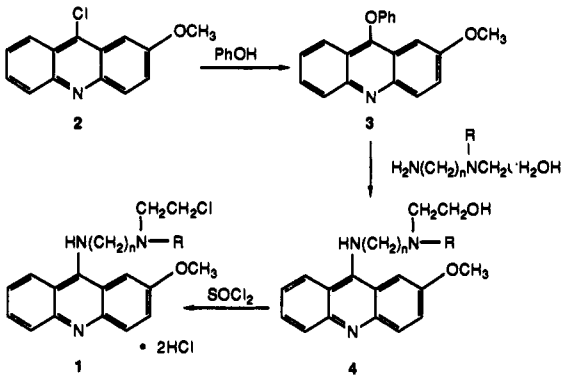
We therefore prepared a series of *N*-alkyl-*N*-(2-chloroethyl) acridine mustards **1a–o** with hydrocarbon chain lengths varying from two to six methylene units. The corresponding bis(2-chloroethyl) mustards were also prepared for comparison. Only achiral spacer groups were chosen to avoid ambiguity due to asymmetry. The 2-methoxyacridine nucleus chosen for the investigation was based on the quinacrine mustard results and also on the data of Creech et al.[1] which suggested that the 2-methoxy substituent was useful for high antitumor activity while a 6-chloro substituent (as in quinacrine mustard) may reduce potency. We find that the length of the spacer chain strikingly affects the reactivity at certain DNA sites, whereas the nature of the alkylating group has relatively little effect.

Syntheses

The mustard derivatives were prepared starting from the known 9-chloro-3-methoxyacridine. One of us recently reported an improved synthesis of this compound.[7] Preparations of the required diamine-based side chains, in general, proved to be quite difficult. These compounds could not be readily purified due to their high boiling points, high polarity, and significant water solubility. All of the required C2 and C3 *N*-(2-hydroxyethyl) derivatives were known compounds.[8-11] The C4 to C6 *N*-methyl compounds were best prepared by alkylating *N*-methylethanolamine with the required Ω-bromo nitrile[10] followed by lithium aluminum hydride reduction. The corresponding *N*-ethyl derivatives were similarly prepared. The bis[*N*-(2-hydroxyethyl)] compounds were also known compounds.[8,9,12]

Coupling of 9-chloro-3-methoxyacridine (**2**) with the respective diamine side chains was carried out in the presence of phenol. This proved to be the most successful method for the preparation of the required hydroxyethyl intermediates. This procedure involved formation of an

Scheme 1



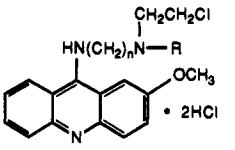
intermediate, 3-methoxy-9-phenoxyacridine (**3**),[13] which, without isolation, was heated with a slight excess of the diamine. This procedure allowed the couplings to be carried out without the need for a large excess of the diamines, avoiding difficulties resulting from protonation of the side-chain nitrogens observed upon direct coupling of the diamines with 9-chloro-3-methoxyacridine. The resulting hydroxyethyl derivatives **4** were purified by preparative thin-layer chromatography. These were then directly converted to the corresponding mustard dihydrochlorides upon extended treatment with excess thionyl chloride at room temperature[9] (Scheme 1). All of these mustard derivatives were crystalline and exhibited the expected physical and spectroscopic properties (Table 1).

Results

The abilities of the compounds to react with guanine-N^7 positions in duplex DNA at sequences 5′-GG-3′ and 5′-GT-3′ were assessed in a GC-rich region in the 5′ flank of the c-myc gene. Comparison with the parent nitrogen mustard, bis(2-chloroethyl)methylamine (HN2), showed that all of the acridine nitrogen mustards had greater sequence selectivity in this region. This is illustrated in Figure 1 for difunctional compounds having the shortest and longest connecting chains (C2-bis and C6-bis). Similar results were obtained with the monofunctional compounds. In the DNA segment shown in Figure 1, there are several instances of consecutive G's and a single instance of 5′-GT-3′. The two G's in the sequence-5′-CGGT-3′ (arrows) were representative of other instances observed qualitatively in other parts of the sequencing gel. These two consecutive G's reacted with nearly equal intensities with HN2 (open circles). C2-bis selectively suppressed the reaction at 5′-GT-3′ (Figure 1A, arrows), while C6-bis reacted more strongly at 5′-GT-3′ than at the preceding G (Figure 1B, arrows).

All of the acridine nitrogen mustards showed greater selectivity than HN2 in that there were fewer sites of intense reaction and greater disparity between strong and weak sites. Moreover, this disparity was greater with C2-bis than with C6-bis (Figure 1). This was most notably true when G was followed on the 3′ side by C, which generally results in a weak site for most nitrogen mustards.[4,6] The degree of suppression at 5′-GT-3′ sites was generally greater with the short spacer-chain than with the long spacer-chain compounds. (Of the four instances of 5′-GC-3′ included in Figure 1A, one instance—the rightmost band in the figure—was exceptionally strong, and no other instance of this type was seen elsewhere in the sequencing gels.)

Table 1. Analytical Data on Mustard Derivatives[a]



compd, no.	R	n	yield (%) hydroxyethyl intermediate	yield (%) mustard salt	mp, (°C) mustard salt
C2-Me, 1a	CH_3	2	69	50	200 dec
C3-Me, 1b	CH_3	3	69	44	215 dec
C4-Me, 1c	CH_3	4	55	56	225 dec
C5-Me, 1d	CH_3	5	65	42	213 dec
C6-Me, 1e	CH_3	6	63	49	110–115
C2-Et, 1f	CH_3CH_2	2	74	58	225 dec (lit.[b] mp 246–248)
C3-Et, 1g	CH_3CH_2	3	89	44	222 dec (lit.[b] mp 229.5–230)
C4-Et, 1h	CH_3CH_2	4	81	28	176 dec
C5-Et, 1i	CH_3CH_2	5	52	50	175 dec
C6-Et, 1j	CH_3CH_2	6	68	32	154 dec
C2-bis, 1k	$ClCH_2CH_2$	2	44	38	225 dec
C3-bis, 1l	$ClCH_2CH_2$	3	95	23	215–216 (lit.[b] mp 219.5–221)
C4-bis, 1m	$ClCH_2CH_2$	4	53	33	210 dec
C5-bis, 1n	$ClCH_2CH_2$	5	34	28	185 dec
C6-bis, 1o	$ClCH_2CH_2$	6	38	53	136 dec

[a] Satisfactory elemental analyses (C,H,N) obtained were within ±0.4% of theoretical values. [b] Previously reported by Creech et al., ref 1.

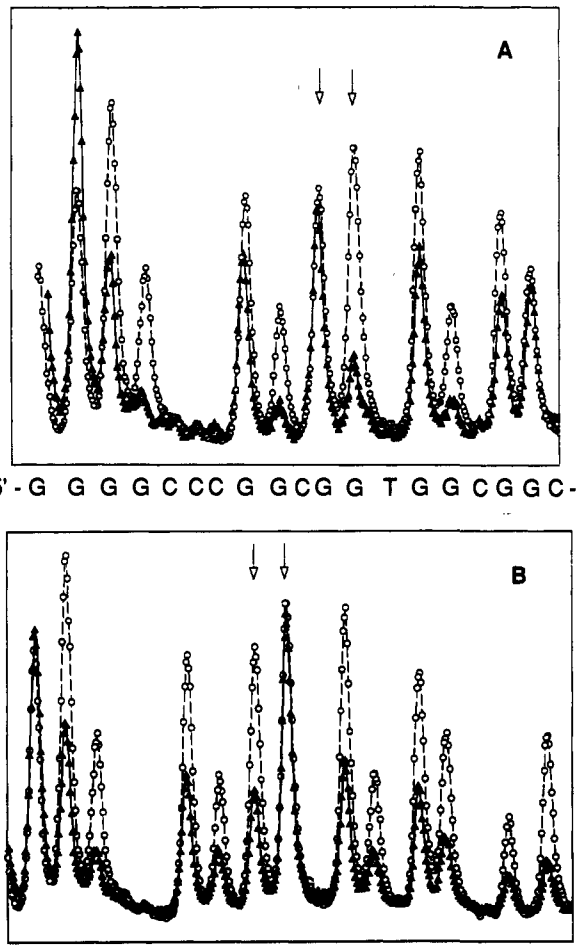


Figure 1. Reactivities of acridine nitrogen mustards having a short (A) or a long (B) spacer chain compared with the reactivity of HN2: open circles, 4 μM HN2, and filled triangles, 0.05 μM C2-bis (A) or 0.05 μM C6-bis (B). Reactions at individual bases are shown in a GC-rich region upstream of the c-myc gene P1 transcription start. Arrows indicate sites that illustrate the strong dependence on spacer-chain length.

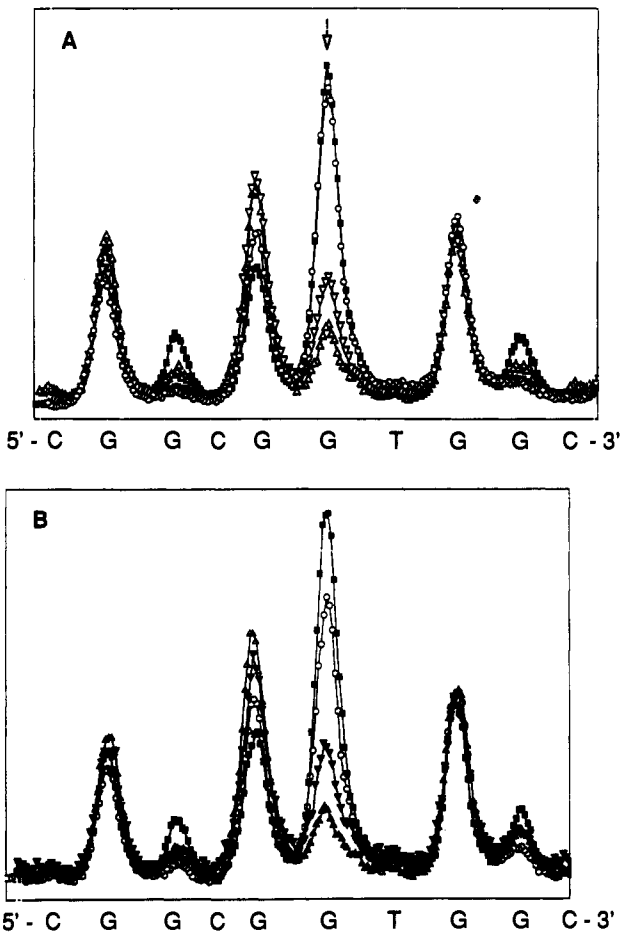


Figure 2. Dependence of reactivity at a 5′-GT-3′ site (arrow) on the length of the spacer chain in a series of difunctional (A) and monofunctional (B) acridine nitrogen mustards. (A): triangles, C2-bis; inverted triangles, C3-bis; circles, C4-bis; and filled squares, C6-bis. (B) triangles, C2-Me; filled inverted triangles, C3-Me; circles, C4-Me; and filled squares, C5-Me. Reaction conditions are as in Figure 1. Drug concentrations were 0.05 μM for the C*n*-bis compounds and 0.1 μM for the C*n*-Me compounds.

The dependence on length of the spacer chain is shown for the difunctional series in Figure 2A and for the monofunctional Me series in Figure 2B. In both series, the major part of the transition from intense to weak

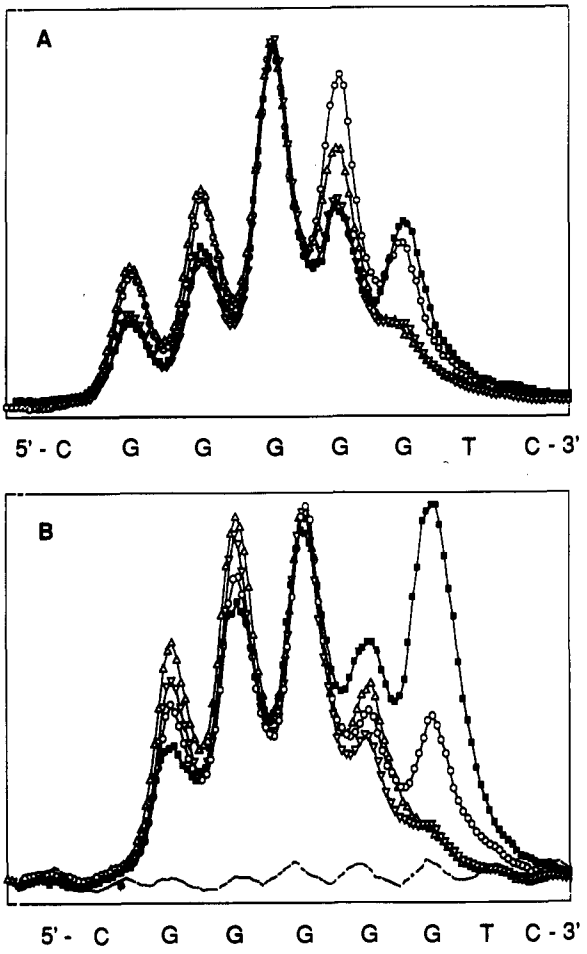


Figure 3. Dependence of reactivity in a 5′-GGGGGT-3′ sequence on the length of the spacer chain in a series of difunctional (A) and monofunctional (B) acridine nitrogen mustards. (A) and (B) are from separate experiments. (B) also shows a base-line control in the absence of drug. (A) triangles, C2-bis; inverted triangles, C3-bis; circles, C4-bis; and filled squares, C6-bis. (B) triangles, C2-Et; inverted triangles, C3-Et; circles, C4-Et; and filled squares, C5-Et. Drug concentrations were 0.05 μM for the C*n*-bis compounds and 0.1 μM for the C*n*-Et compounds.

reaction at 5′-GT-3′ (arrow) occurred when the spacer-chain length was reduced from four to three carbons.

Another example of this behavior is illustrated in Figure 3 in an instance of 5′-GT-3′ occurring at the end of a run of five G's. The suppression of the reactions at 5′-GT-3′ became nearly complete when the connecting-chain lengths were reduced to less than four. Although relative reaction intensities were usually similar in the methyl, ethyl, or 2-chloroethyl series, the high intensity of the reaction of C5-Et with the 5′-GT-3′ in Figure 2B and the more moderate reaction of C6-bis with this site in Figure 2A suggest that there may be special cases where subtle conformational factors can confer unique reactivity.

As a preliminary investigation of their biological properties, the chain-length series C2-Et through C6-Et and C2-bis through C6-bis were assayed for cytotoxic potency against human colon carcinoma HT-29 cells (Table 2). Both series exhibited an optimum in cytotoxic potency at a chain length of four. The difunctional compounds exceeded the monofunctionals by factors of approximately 10–20 in cytotoxic potency against this cell line.

Discussion

Extensive studies of acridine derivatives have demonstrated binding to DNA by intercalation between base pairs. All of the acridine nitrogen mustards synthesized in the current work would be expected to be capable of intercalation. Once the molecule is bound to DNA, the tethered nitrogen mustard moiety could react with the DNA at neighboring nucleophilic sites, depending on the steric accessibility of these sites which would depend upon the local DNA sequence. A two-step reaction sequence of noncovalent binding followed by alkylation is consistent with 10–100-fold lower concentrations of the acridine nitrogen mustards, compared with HN2, that yielded comparable guanine-N^7 reaction intensities. It is therefore plausible to expect that the acridine nitrogen mustards would react in cells more selectively with DNA, as opposed to other nucleophilic molecules.

Table 2. Cytotoxicity to HT-29 Cells[a]

chain length[b]	monofunctional (μM)	difunctional (μM)
2	1.10	0.11
3	0.37	0.02
4	0.25	0.01
5	0.70	0.03
6	0.75	0.06
quinacrine mustard	0.05	
quinacrine	55.0	

[a] Human colon carcinoma HT-29 cells were exposed to the drug for 30 min at 37 °C. Values tabulated are the drug concentrations (μM) required to inhibit colony formation by 50%. [b] The chain-length series of monofunctional compounds were C2-Et through C6-Et; the series of difunctionals were C2-bis through C6-bis.

There was no consistent dependence of reactivity with the generally strong 5′-GG-3′ sites on the length of the spacer chain between the acridine and mustard moieties. Hence, simultaneous intercalation and alkylation of at least these sites does not seem to be limited by shortness of the spacer chain. Acridines may also bind externally to DNA, but this occurs in the minor groove from which guanine-N^7 in the major groove would be inaccessible. In the previously proposed reaction model for quinacrine mustard, alkylation would occur at the second base toward the 5′ end from the intercalation site.[4] With spacer chains as short as two carbons, however, this model is not feasible without major structural distortion of the DNA. Alternatively, the short-chain compounds may react with an immediately adjacent guanine on either side. Since the short-chain compounds alkylated the two G's in 5′-GGT-3′ sequences to different extents, however, the reactions may depend also on flanking bases that could affect the reaction site conformation.

Although the mechanism remains unknown, the current results show that, in this series of compounds, base-sequence preference is markedly influenced by spacer-chain length, particularly at 5′-GT-3′ sequences. By reducing the chain to two or three carbons, reactions at most 5′-GT-3′ sites were suppressed while reactions at 5′-GG-3′ sites remained strong. A quantitative evaluation of a greater number of reaction sites will be presented elsewhere. Studies in intact cells remain to be carried out to test the predicted increased selectivity of the short-chain compounds for reaction in G-rich regions of the genome.

A series of aniline mustards linked to 9-aminoacridine were recently investigated by Prakash et al.[14] These compounds differ from those of the current work in that they are aromatic *N*-mustards and may therefore differ in alkylation mechanism.[15] Moreover, the reduced flexibility of the connecting chain due to the aniline ring would

alter the steric accessibility for alkylation from an intercalated complex. In a series of aniline mustards linked from the *para* position by a straight hydrocarbon chain to 9-aminoacridine, Prakash *et al.* found little discrimination between different G's in DNA. The only discrimination noted was a slightly increased reactivity in runs of G. Increased length of the hydrocarbon chain resulted in general diminution of reactivity. The strong sequence preferences exhibited by our compounds were not seen with the corresponding aniline mustards. When the spacer chain was modified by replacing C with O at the link to aniline, Prakash et al. obtained enhanced reactivity at 5′-GT-3′ sites, an interesting reversal of pattern relative to our findings. The addition of a tertiary butyl group to the acridine ring conferred selectivity of their aniline mustards for 5′-GT-3′ and 5′-GG-3′ sequences. The bulky tertiary butyl group would interfere with ordinary intercalation which might occur only in a distorted DNA helix. We do not have a unified explanation to account for all of the bindings by Prakash et al. and those reported in the current work.

The enhanced selectivity for reaction at successive G's and the suppression of reaction at 5′-GT-3′ that we have observed with our short spacer-chain compounds have not previously been reported, to our knowledge, for any other chemotherapeutic alkylating agents. Our findings show that enhanced reactivity and sequence selectivity can be conferred to nitrogen mustards by connecting the alkylating group to an intercalating group and that the nature of the connecting chain can have a major effect.

Preliminary investigation of biological properties revealed an optimum in cytotoxic potency at a connecting-chain length of four (Table 2). The same optimum length was seen in the monofunctional C2-Et to C6-Et series as in the difunctional C2-bis to C6-bis series, although the latter exhibited (10–20)-fold greater potency. We do not know whether these relationships would apply generally to different cell types or whether the greater DNA-sequence selectivity of the short-chain compounds would enhance differential cytotoxicity against some cell types. Therapeutic potential, of course, will depend more on differential cytotoxicity than on potency.

Experimental Section

Melting points were obtained on a Fisher Mel-Temp apparatus and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 1600 series FTIR. 200-MHz ^{1}H NMR spectra were recorded with a Varian XL 200 spectrometer. GC was carried out with a Hewlett-Packard Model 5880A. Microanalyses were performed by Desert Analytics, Tucson, AZ. Tetrahydrofuran was distilled from benzophenone–sodium. Other commercial reagents were used without further purification.

General Synthetic Procedures. The preparation of 9-[*N*-[4-[*N*-(chloroethyl)-*N*-methylamino]butyl]amino]-2-methoxyacridine, dihydrochloride (1c) illustrates the procedures used for the preparation of the mustards (1a–o).

***N*-Methyl-*N*-(2-hydroxyethyl)-4-aminobutyronitrile.** To *N*-methylethanolamine (2.3 g, 30 mmol) at 45–50 °C was added dropwise with vigorous stirring over 30 min 4-bromobutyronitrile (1.48 g, 10 mmol). The mixture was heated to 110 °C for 30 min. After the mixture had cooled, water (20 mL) was added and the mixture was extracted with chloroform. The combined organic layers were dried over sodium sulfate. Removal of solvent under reduced pressure and bulb-to-bulb distillation afforded the hydroxyethyl butyronitrile as a colorless liquid, 1.61 g, 57% yield. ^{1}H NMR ($CDCl_3$): 3.62 (t, 2H), 2.79 (bs, 1H), 2.57 (m, 4H), 2.43 (t, 2H), 2.26 (s, 3H), 1.84 (m, 2H). IR (neat): 3418, 2947, 2846, 2801, 1463, 1424, 1036 cm^{-1}.

***N*-(2-Hydroxyethyl)-*N*-methyl-1,4-butanediamine.** To an ice-cooled suspension of lithium aluminum hydride (598 mg, 15 mmol) in dry tetrahydrofuran (60 mL) was slowly added the nitrile (0.71 g, 5 mmol) in dry tetrahydrofuran (5 mL). The mixture was heated to reflux overnight. After the mixture had cooled, the following were added in succession: water (0.57 mL), a 15% NaOH solution (0.57 mL), and water (1.71 mL). Chloroform (20 mL) was added and the solution decanted from inorganic salts. The residue was washed with chloroform, the organic layers were combined, and the solvent was removed affording the diamine as an oil, 0.605 g, 83% yield. ^{1}H NMR ($CDCl_3$): δ 1.48 (complex, 7H), 2.24 (s, 3H), 2.42 (t, 2H), 2.52 (t, 2H), 2.71 (t, 2H), 3.58 (t, 2H). IR (neat): 3286, 2937, 2852, 2795, 1601, 1463 cm^{-1}.

9-[*N*-[4-[*N*-(Hydroxyethyl)-*N*-methylamino]butyl]amino]-2-methoxyacridine (4c). A mixture of 9-chloro-3-methoxyacridine[7] (225 mg, 0.924 mmol) and phenol (450 mg, 4.79 mmol) was heated in a hot water bath for 30 min. The mixture was cooled to room temperature and *N*-(2-hydroxyethyl)-*N*-methyl-1,4-butanediamine (200 mg, 1.37 mmol) was added. The resulting mixture was heated in a hot water bath for 1.5 h. After cooling, the mixture was diluted with $CHCl_3$ (15 mL) and washed with 1 N NaOH (10 mL). The resulting organic layer was washed twice with water and dried over Na_2SO_4. Removal of solvent and purification via preparative thin-layer chromatography (silica–ethyl acetate) afforded the hydroxyethyl derivative as a viscous orange liquid, 146 mg, 45% yield. ^{1}H NMR ($CDCl_3$): δ 1.62 (m, 2H), 1.78 (m, 2H), 2.22 (m, 3H), 2.40 (t, 2H), 2.50 (t, 2H), 3.32 (b s, 1H), 3.58 (t, 2H), 3.72 (t, 2H), 3.98 (s, 3H), 4.78 (b s, 1H), 7.44 (m, 3H), 7.66 (t, 1H), 8.08 (m, 3H). IR($CHCl_3$): 2942, 1631, 1560 cm^{-1}.

9-[*N*-[4-[*N*-(Chloroethyl)-*N*-methylamino]butyl]amino]-2-methoxyacridine, dihydrochloride (1c). Thionyl chloride (5 mL, 68.5 mmol) was carefully added to the hydroxyethyl derivative 4c (240 mg, 0.677 mmol) in an ice-cooled flask. The mixture was allowed to stir at room temperature overnight and was poured into anhydrous ether, and the resulting orange precipitate was filtered via suction. The precipitate was dried under reduced pressure to remove any residual thionyl chloride. The resulting crude dihydrochloride salt was dissolved in hot absolute ethanol and precipitated with ether. Drying under reduced pressure afforded the salt 1c as an orange solid, 169 mg, 56% yield, mp 225 °C dec, homogeneous on TLC (silica–methanol). ^{1}H NMR ($CDCl_3$/DMSO): δ 1.92–2.17 (complex, 4H), 2.88 (d, 4H), 3.20–3.58 (complex, 4H), 4.04 (s, 3H), 4.24 (m, 2H), 7.46–7.66 (m, 2H), 7.92 (m, 3H), 8.15 (s, 1H), 8.52 (d, 1H). IR (KBr): 3412, 3236, 2954, 1625 cm^{-1}. Elemental analysis calcd for $C_{21}H_{28}N_3OCl_3$ (444.82): C, 56.70; H, 6.12; N, 9.45. Found: C, 56.43; H, 6.42; N, 9.06.

Reaction of DNA with Nitrogen Mustards. Human c-myc DNA was labeled with ^{32}P at the 5′ end of the XhoI site at position 464, and a fragment uniquely end-labeled in the coding strand was isolated by electrophoresis and electroelution. Reactions with nitrogen mustards and analyses of guanine-N^7 alkylation sites were carried out as previously described[6] with minor modifications. Reactions were carried out for 1 h at 25 °C in 25 mM triethanolamine (pH 7.2)–1 mM Na_3EDTA and were stopped with 0.75 M sodium acetate (pH 7.0)–0.5 M mercaptoethanol–50 μg/mL t-RNA (final concentrations). The DNA was precipitated with 2.7 volumes of 95% ethanol, resuspended in 0.3 M sodium acetate–1 mM EDTA, reprecipitated again, washed with cold ethanol, and lyophilized. Sites of guanine-N^7 alkylation were converted to strand breaks by treatment with 1 M piperidine at 90 °C for 15 min.[16] After lyophilization, water was added and the sample was lyophilized again; this was repeated so as to complete the removal of piperidine. The DNA was then dissolved and denatured in 80% formamide–10 mM NaOH–1 mM EDTA. The samples were then loaded onto 6.8% polyacrylamide gels and electrophoresed in Tris–borate–EDTA (pH 7.4) at 50 °C for between 3.5 and 8 h. Dried electrophoretic gels were counted in a Betascope 603 analyzer (Betagen Corp.). Counts were recorded at 0.4-mm intervals along a lane (nominal resolution 1.5 mm). Digitized outputs were plotted by means of a custom computer program after applying small corrections for proximal strand cuts.

Nitrogen mustards have been reported also to alkylate adenines in DNA.[14,17] The method used in the current work, however, was optimized for guanine alkylations and did not disclose adenine alkylations. Using the method applied by Prakash et al.[14] to disclose adenine alkylations, we found that the acridine mustards do alkylate some of these sites (data not shown), but their sequence dependence has not been determined.

Cytotoxicity Assays. Human colon carcinoma HT-29 cells were grown in Eagle's modified essential medium supplemented with 15% heat-inactivated fetal bovine serum (Advanced Biotechnologies, Inc., Columbia, MD). Exponential phase cells (2×10^5/mL) were plated and 16–20 h later were treated with various drug concentrations for 30 min at 37 °C in medium containing 1% serum. At the end of the drug treatment period, the cells were washed twice with complete medium, trypsinized, and plated in triplicate at 10^2, 10^3, and 10^4 cells/25-cm^2 flask. At least two independent experiments were performed with each compound. The colony-forming efficiency of untreated cells was 40–65%.

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