BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **231,** 94–98 (1997)
ARTICLE NO. RC965908

Enhanced DNA Alkylation Activities of Hoechst 33258 Analogues Designed for Bioreductive Activation

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Received November 8, 1996

A series of analogues of Hoechst 33258, designed to be subject to bioreductive activation, were synthesized, and interactions between these compounds and pBR322 DNA were investigated. Compounds containing a quinone group reacted with DNA via two possible pathways in the presence of reductants NADH or NADPH: radical cleavage and DNA alkylation. The corresponding dimethoxy compounds, which are not subject to reduction, showed very weak DNA binding ability. The strength of alkylation reaction of the quinone derivatives is related to leaving group ability. Furthermore, the quinone compounds preferentially alkylate DNA at 5′-CG and TG sequences rather than at the AT sites preferred as binding sites of Hoechst 33258.

The design of low molecular weight sequence-specific DNA binding agents has attracted increasing attention in recent years (1, 2). Minor groove binding agents are attractive for the development of such DNA sequence-selective compounds based on naturally occurring netropsin and distamycin. These agents bind DNA at primarily AT rich sequences. Such structures lead themselves, by appropriate structural modification, to the development of sequence-altered analogues, termed lexitropsins. Recently, for example, our group has reported imidazole-containing analogues of netropsin and distamycin have increased selectivity for GC rich sequences (3, 4). Hoechst dye 33258 is also known to bind to double helical DNA and to have ready to access into cells (5, 6). In common with netropsin, Hoechst 33258 binds preferentially at AT-rich DNA sequences with a minimum binding size of four consecutive AT base pairs (7–10). Because most minor groove binding agents are AT specific, it is particular challenging to develop ligands with GC selectivity. High GC contents are commonly found in the genes of mammals, and that a functional role of GC-rich sequences is suggested by their frequent occurrence in genes associated with proliferation (11, 12).

On the other hand, most anticancer drugs are nonselective and kill normal cells at the same time as cancer cells. It is therefore desirable to create agents which recognize and react with disease cells preferentially. Normally, cancer tissues have a reduced blood supply and are hence less well furnished with oxygen (13). This is the basis for the design of bioreductive anticancer drugs. Thus in this study we synthesized two kinds of analogues (Figure 1) of Hoechst 33258: dimethoxy compounds and quinone compounds; the latter of which were anticipated to be subject to bioreductive activation. These results should aid in the design of more selective compounds to recognize AT-GC sites and supply new information to create agents which can target specific sequences in cancer cells.

MATERIALS AND METHODS

Materials

pBR 322 DNA and restriction enzymes, [γ-^{32}P]ATP were purchased from GIBCOBRL company and DUPONT Co. respectively. All microtubes were used after being washed by metal ion-free ultra pure water. All chemical agents were reagent grade. The solutions of compounds **1-7** were prepared fresh immediately before use or used from frozen stock solution.

Methods

DNA cleavage reactions. Plasmid pBR322 DNA was used in these experiments. All 20 μl samples include 10 μM Or 100 μM compound 2-7 were incubated with DNA and 1 mM NADH or NADPH at 37°C for different reaction times. 5 μM desferrioxamine were added to each sample to remove traves of iron (14). After ethanol precipitation 15 μl BPB-glycerol dye were added and loaded on to a 0.5×TBE buffer in a 1% agarose gel.

Ethidium displacement assay (15). To 2 ml of ethidium bromide buffer (10 mM Tris-HCl and 10 mM NaCl, pH 7.4), was added 20 μl of DNA solution (A_{260}=2), and the maximum fluorescence was measured on a Turner 430 spectrofluorometer. Excitation and emission wavelengths were set at 550 and 600 nm at room temperature. Aliquots of a 10 mM stock drug solution were then added to the DNA-ethidium solution, and the fluorescence was measured after each addition until 50% reduction of fluorescence had occurred. If

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0006-291X/97 $25.00

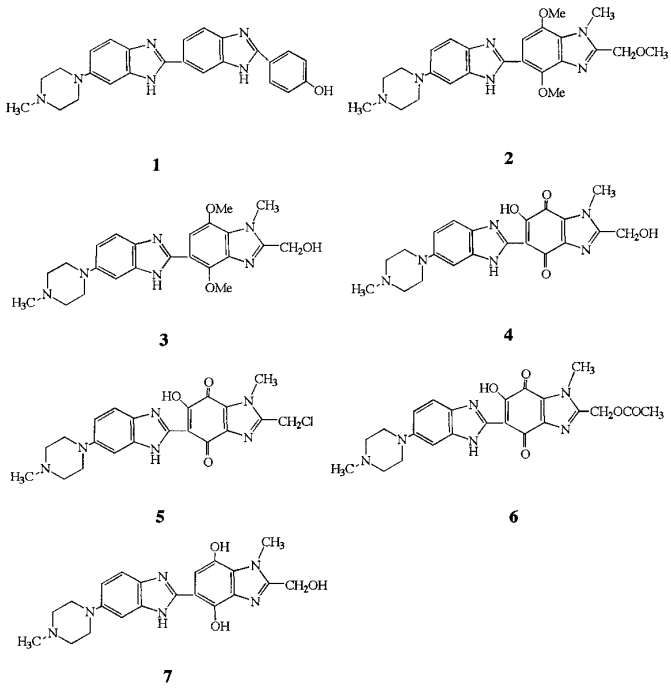


FIG. 1. Chemical structures of Hoechst 33258 (**1**) and Hoechst analogues (**2–7**).

the 10 mM stock solution lowered the percent fluorescence too quickly, the solution was further diluted to 1 mM or 0.1 mM prior to titration. The apparent binding constant was calculated from $K_{Et\text{-}Br}[EtBr]=K_{app}[drug]$, where [drug]=the concentration of drug at a 50% reduction of fluorescence and K_{EtBr} and [EtBr] are known. The concentration of EtBr is 1.3 mM and K_{EtBr} for Poly(dA-dT)·Poly (dA-dT) is 9.5×10^6; for Poly(dG-dC)·Poly(dG-dC) is 9.9×10^6, and for T4 coliphage DNA is 10.0×10^6.

Sequencing of DNA alkylation reactions. The linearized DNA was 5′-end labeled at the *Hin*dIII site using [γ-^{32}P]ATP and T4 polynucleotide kinase under standard conditions, then the labeled DNA fragments were treated with restriction enzyme *Eco*RV. The necessary fragments were separated using a preparative electrophoresis apparatus system. DNA cleavage reactions were carried out by combining labeled DNA, calf thymus DNA and the drug in buffer (10 mM Tris-HCl, pH 7.0). 5 μM desferrioxamine were added to each samples to remove traces of iron (14). After incubation for 20 hr at 20 °C, the samples were heated at 95 °C for 30 min and a piperidine treatment performed to half of the samples. The solutions were then lyophilized and resuspended in formamide loading dye. Electrophoresis of the samples was performed on a slab 10% polyacrylamide sequencing gel at 2200 volts and 25 °C.

RESULTS AND DISCUSSION

The ligand-DNA interactions were investigated using three kinds of reductants: dithiothreitol (DTT), NADH and NADPH. None of the compounds cause any DNA cleavage in the presence of DTT but marked cleavage reactions were observed in the presence of NADH or NADPH. Figure 2 shows the results for Hoechst 33258, compounds 2, and 4-7 incubated with NADH (lanes 3, 6, 9, 11, 13) and NADPH (lanes 4, 7, 10, 12, 14). The percentage of open-circled (OC) form and linear form related to reaction time are shown in figure 3. Compounds 4-6 behaved differently from compounds 2-3. The OC form DNA increased a little after incubating with 2, even after adding reductant NADH (Fig. 3). However, compounds 4-6 behaved differently with or without reductant. Linear form DNA could also be observed in lanes 3 and 6 after reaction with NADH. While the percentage of linear form does not increase with reaction time however the OC form increases during 64 hr incubation (Fig. 3A). It is noteworthy that NADH and NADPH affect the DNA cleavage reaction differently: stronger cleavage was observed in the presence of NADH. The reduction potentials for NADH and NADPH are −0.32V and is −0.34 respectively. It is therefore plausible that the redox potential of quinone compounds may be a factor that could affect the above reactions. The DNA cleavage activity order is 5>6>4>2>3 as determined by measuring by ultra scan XL. Thus the quinone compounds have higher activity than the dimethoxy compounds; a stronger leaving group (Cl-) shows high reactivity than a medium group (OCOCH3) and also a weak one (OH-). Because of the generation of the OC and linear forms after long time incubation, we suggest compounds 4-6 cleave DNA by two pathways. One is radical cleavage and the other is the result of alkylation activity. As shown in Fig. 3, the fact that the supercoiled form DNA does not disappear entirely means there is another kind of cleavage reaction occurring i.e. DNA alkylation. The possible mechanism of DNA alkylation is shown in Scheme 1. Neither catalase nor superoxide dismutase, expected to trap hydrogen peroxide and superoxide anion respectively showed any effect on the cleavage

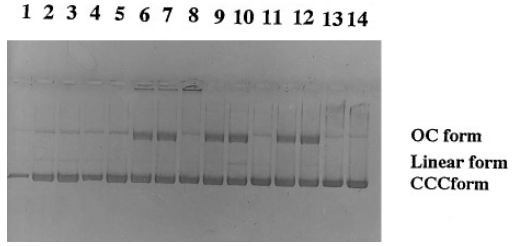


FIG. 2. 1% agarose electrophoresis results of the compounds **2, 4–7** incubating with 0.5 μg pBR322 DNA plus 1 mM NADH and NADPH at 20°C for 2 hr. Lane 1, Intact DNA; lanes 2–4, **4** incubating without reductant, with 1 mM NADH and 1 mM NADPH, respectively; lanes 5–7, **5** incubating without reductant, with 1 mM NADH and 1 mM NADPH, respectively; lanes 8–10, **6** incubating without reductant, with 1 mM NADH and 1 mM NADPH, respectively; lanes 11–12, **2** incubating without reductant, with 1 mM NADH; lanes 13, 14, **7** incubating without reductant, with 1 mM NADH; lane 15, intact DNA plus 10 mM NADH.

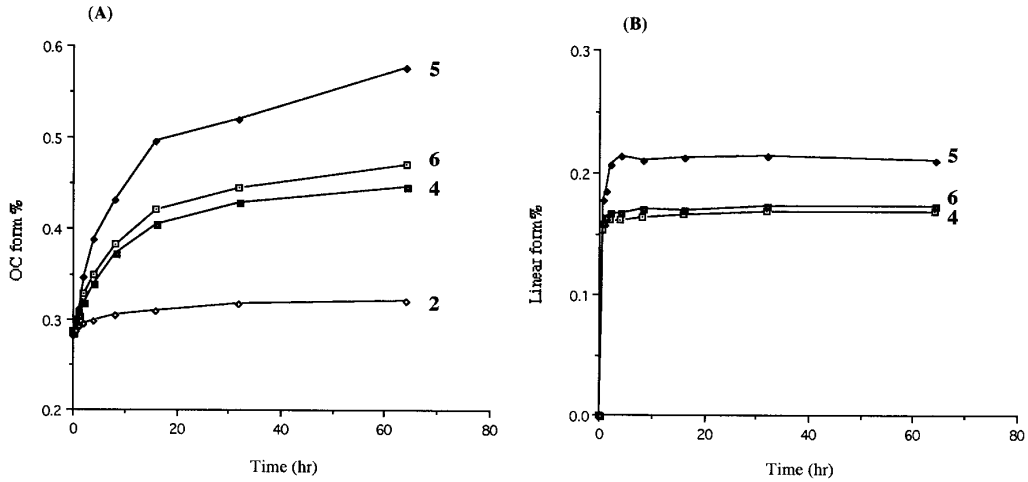
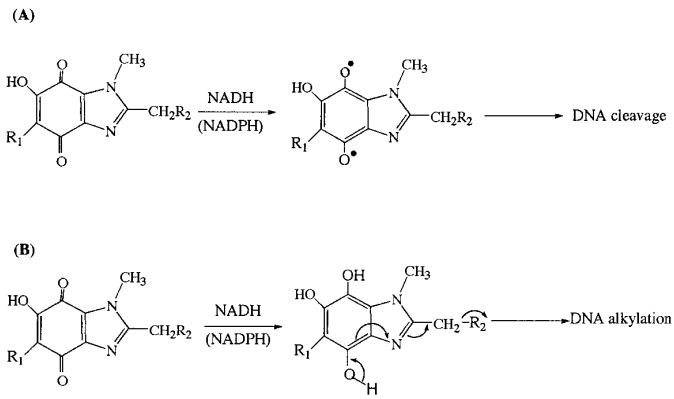


FIG. 3. The increase of OC form DNA (A) and linear form (B) of pBR322 DNA incubation with compounds related to reaction time.

by these compounds. This strongly suggests the alkylation mechanism rather than the radical mechanism is the predominant mode of action of compounds 4-6.

The C_{50} values of Hoechst 33258 and compounds 2-7 with binding to calf thymus DNA, T4 coliphage DNA, poly(dA-dT)·Poly(dA-dT) and poly(dG-dC)·poly(dG-dC) were determined using the ethidium displacement assay (15) and calculated from C50 (C50 value is the concentration of the required total ligand concentration to achieve a 50% reduction in fluorescence intensity). The values of Kapp for 2-6 are lower than that of Hoechst 33258 for poly(dA-dT)·poly(dA-dT). This lower affinity could be due to the loss of one benzimidazole group. The Kapp values for quinone analogues 4-6 to poly(dG-dC)·poly(dG-dC) are higher than that of poly(dA-dT)·poly(dA-dT), suggesting that these analogues can accept GC base pairs. The Kapp values of poly(dG-dC) for 5 is 1.5 times that of 6, and 3.0 times that of 4. This is accord with the proposal that the



SCHEME 1. Possible free radical mechanism (A) and alkylation (B) mechanism for bioreductive Hoechst 33258 analogues.

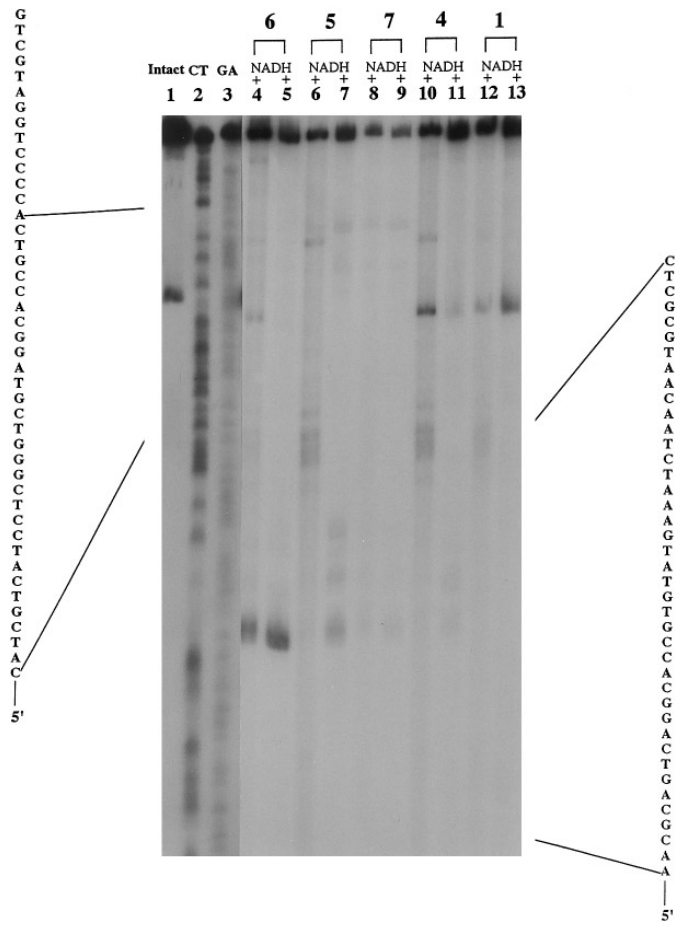


FIG. 4. Autoradiogram for a 10% gel electrophoresis of a 5′-end labeled *Hin*dIII-*Eco*R V fragment (156 bp) of pBR322 DNA reacted with compounds **1, 4–7** plus 1 mM NADH. Lanes 4–13, 10 μM **6, 5, 7, 4, 1** incubated with the fragment in presence of NADH at 20°C for 20 hr; further 1 M piperidine treatment were performed in lanes 4, 6, 8, 10, 12. Lane 1 indicates intact DNA. Lanes 2 and 3 show the Maxam-Gilbert reaction for C+T and G+A, respectively.

TABLE 1

C_{50} and K_{app} Values of Compounds **1–6** Binding with Poly(dA-dT)·Poly(dA-dT), Poly(dG-dC)·Poly(dG-dC), and T_4 Coliphage DNA

	Poly(dA-dT)·Poly(dA-dT)		Poly(dG-dC)·Poly(dG-dC)		T_4 coliphage DNA	
Compounds	C_{50}	K_{app} ($\times 10^6$)	C_{50}	K_{app} ($\times 10^6$)	C_{50}	K_{app} ($\times 10^6$)
1	0.775	15.94	[a]	[a]	2.846	4.568
2	25.282	0.49	13.912	0.93	109.048	0.119
3	[b]	[b]	[b]	[b]	205.61	0.0632
4	26.49	0.466	4.209	3.06	132.80	0.0979
5	38.58	0.320	1.393	9.24	456.27	0.0285
6	83.64	0.148	1.938	6.64	113.87	0.114

[a] The fluorenscence spectrum increases when drugs are added.
[b] The fluorenscence spectrum decreases at first but increases after a certain drug concentratior.

nature of the leaving group (Cl) is a key point to affect the strength of DNA alkylation. The large apparent binding constant for T4 coliphage DNA for Hoechst 33258 gave evidence of its minor groove selectivity, because the major groove of T4 coliphage DNA is blocked by α-glycosylation of the 5-(hydroxymethyl)cytidine residue. However values for T4 coliphage DNA for 5 is nearly 200 times (161.5 times) lower than that of Hoechst 33258. This also suggests that 5 and 6 and 4 do not bind in minor groove directly and probably bind via the major groove of DNA. This has yet to be confirmed by footprinting study.

In order to further investigate the mechanism of this DNA cleavage reaction, we compared the cleavage activity of compounds 2-7 with Hoechst 33258 using a 156 base pair restriction fragment (Figure 4). Lane 4 and 5 show the result after incubating with Hoechst 33258 and proceed further heat and piperidine treatment. Because Hoechst binds B-form DNA at AAATT site, it does not react with this fragment. However our compounds specifically bind to G residues of this fragment. Compound 5, strongly binds to 5′-T**G**AAA, 5′-CCGT**G,** 5′-GC**G**CT, C**A**TC**G,** CACC**G;** compounds 6 and 4, bind to 5′-T**G**AAA, 5′-CCGT**G,** 5′-GC**G**CT. It is evident that these compounds preferred to attack the 5′-CG sequences. Recently several studies on the structure of Hoechst 33258-DNA complexes including X-ray crystallography (16–18) and NMR (19, 20) have been reported. They provide evidence for the minor groove binding Hoechst-DNA complex via an ensemble of intermolecular van der Waals contacts and hydrogen bonding interactions. Our group recently reported that a Hoechst 33258 analogue N1-alkoxyalkyl-bis-benzimidazole has increased G binding ability but this compound still predominantly binds to AATT region (21). Replacing one benzimidazole group by a benzoquinone, as in 4, 5, and 6 decreases the ability to bind to AATT regions. Due to the low Kapp constant to T4 coliphage DNA and G sequence specificity, we suggest that the quinone compounds react via the major groove but near to the minor groove because of binding to AACGC region. This structural aspect will be examined by NMR study and X-ray crystallography.

In summary analogues of Hoechst 33258 bearing a quinone group and designed to be subject to bioreductive activation have been synthesized. After incubating with reductants (NADH or NADPH) the quinone compounds interact with DNA by a two alternative mechanisms: radical cleavage and DNA alkylation. The results strongly suggested that the alkylation mechanism is the main mode of DNA binding of compounds 4-6. Conversely, the dimethoxy compounds hardly intereact with DNA compared to Hoechst 33258. As may be seen by a comparison of K_{app} of T_4 coliphage DNA of 4-6 (Table 1). This suggests that the quinone compounds 4-6 bind via the major groove. Furthermore the quinone compounds were observed to preferentially bind to G residues of 5′-CG and TG sequences rather than AT sequences from polyacrylamide electrophoresis. Therefore the binding to DNA is dominated by the alkylation event at the primary nucleophilic site of DNA G-NT rather than by the inherent AT recognizing properties of the bis-benzimidazole moiety. The quinone compounds preferentially alkylate **G** bases of 5′-CG sequences in contrast to Hoechst 33258 which recognizes AATT sequences. It is of interest that compounds in this study show strong affinity to G sites contrasted with the natural products distamycin and netropsin. These results supply information which may prove useful to design of agents able to recognize both AT and GC sequences.

ACKNOWLEDGMENT

This study was supported by a grant (to J.W.L.) from the National Cancer Institute of Canada.

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