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A CHELATING AGENT POSSESSING CYTOTOXICITY AND ANTIMICROBIAL ACTIVITY: 7-MORPHOLINOMETHYL-8-HYDROXYQUINOLINE

Ai Yu Shen[a]*, Chi Pien Chen[b] and Steve Roffler[c].

[a]Department of Pharmaceutical Science, Foo Yin Institute of Technology, Kaohsiung County 831. [b]Department of Pharmacy, Ta Jen Pharmaceutical College, Pingtung, [c]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China.

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Summary

7-Morpholinomethyl-8-hydroxyquinoline (MO-8HQ), which like 8-hydroxyquinoline (8HQ) readily forms a chelate, was synthesized and found to possess cytotoxicity and antimicrobial activity. Both 8HQ and MO-8HQ were cytotoxic to human carcinoma cell lines at micromolar concentrations. MO-8HQ also inhibited DNA synthesis of tumor cells at micromolar concentrations, suggesting that MO-8HQ might chelate metals necessary for the enzymatic catalysis of DNA biosynthesis. MO-8HQ was more active against Gram positive bacteria than Gram negative bacteria and its potency correlated with iron chelation. An "unsaturated" chelate with a MO-8HQ to Fe ratio of 2:1 exhibited greater antibacterial activity than MO-8HQ alone. Among the organisms tested, *Micrococcus flavus* was most susceptible with a MIC of 3.9 μg/ml. MO-8HQ also exhibited anti-fungal activity at 7-15 μg/ml. MO-8HQ:Fe chelate markedly increased the susceptibility of *Escherichia coli* to deoxycholate. Addition of Ca^{2+} or Mg^{2+} reversed the sensitivity of bacteria to deoxycholate as well as to rifampicin. It is suggested that MO-8HQ exerts its biological activity as a membrane-active agent through metal ion chelation.

Key Words: cytotoxicity, antimicrobial activity, chelate, MO-8HQ

Since the discovery that specific metals are active constituents of many enzymes, biological and pharmacological chelating agents have been of increasing interest. 8-Hydroxyquinoline (8HQ) can chelate bivalent cations because of the location of the hydroxyl group relative to the ring nitrogen (Fig. 1). The hydroxyl group acts as an acid, dissociating to–O^-. Bivalent cations are bound by the dissociated acid group and the lone pair of electrons borne by the nitrogen atom. We have synthesized an analog of 8HQ (Fig. 1) that contains a morpholinomethyl group. The similar molecular structures of 8HQ and MO-8HQ suggested a common chelating activity. Previous studies have shown that 8HQ and its derivatives suppress the growth of melanoma cells, *Ehrlich* ascites tumor cells and P_{388} leukemia cells (1, 2). Cytotoxic activity may involve the

Correspondence to: Ai Yu Shen, Associate Professor, Department of Pharmaceutical Science, Foo Yin Institute of Technology, Kaohsiung County 831, Taiwan, Republic of China. Tel: 886-7-7811151. FAX : 886-7-7826146

chelation of metals necessary for the biological activity of critical enzymes. The cytotoxicity of MO-8HQ as well as its effect on cellular synthesis of DNA and RNA were therefore examined.

8HQ and its derivatives also exhibit antimicrobial activity. These compounds are used as fungitoxicants for dermatophytoses and as antiamebic agents. Their biological activity has been attributed to their ability to chelate metals essential for metabolism (3 - 5). Gram negative bacteria have an outer membrane composed of an asymmetric lipid bilayer with large lipopolysaccharide (LPS) molecules restricted to the outer leaflet (6, 7). The presence of divalent cations within the membrane appears to be critical for stabilization of the strong negative charge of the core oligosaccharide chain of the LPS molecules (8). We hypothesized that MO-8HQ could affect the outer membrane of Gram negative bacteria. Most studies of the outer membrane have been carried out in *E.coli*. The antimicrobial activity of MO-8HQ and its interaction with Ca^{2+} and Mg^{2+}, were therefore studied in this bacteria.

Methods

Preparation of MO-8HQ

The melting point of MO-8HQ was determined with a Yanagimoto MP-3 micromelting apparatus and was uncorrected. Infrared spectra were obtained on a Shimadzu IR-408 spectorphotometer. Nuclear magnetic resonance spectra were recorded on a Varian Gemini T-300 spectrometer at the National Sun Yat-sen University, Kaohsiung, and are expressed in parts per million (δ) with tetramethylsilane used as an internal standard. Mass spectra obtained for the purposes of structure confirmation were obtained on a Jeol JMS-HX 110 mass spectrometer at the National Sun Yat-sen University, Kaohsiung. Elemental analysis was performed on a CHN-O-Rapid Heraeus Elemental Analyzer at the National Cheng-Kung University, Tainan. Thin layer chromatography was carried out on precoated silica gel F_{254} chromatographic plates (20 × 20cm ; 0.2mm).

7-Morpholinomethyl-8-hydroxyquinoline (MO-8HQ). MO-8HQ was prepared according to the Mannich reaction (9). Briefly, an aqueous solution of 37% formaldehyde was stirred into a solution of morpholine alcoholic solution. After 30 min of continuous stirring, a solution of 8HQ in ethanol was added and heated to reflux for 2 h. The product was purified by chromatography using ethylacetate and methanol (9:1) as eluent. The hydrochloride salt was recrystallized from absolute ethanol with ether. Mp 213-215℃. IR (KBr) : 3400, 3000, 2800, 2400 cm^{-1}. ^{1}H-NMR (DMSO-d_6); δ : 3.2 (broad, 4H, CH_2NCH_2), 3.9 (broad, 4H, CH_2OCH_2), 4.6 (s, 2H, Ar-CH_2-N), 7.6 (d, $1H_5$, Ar), 7.8 (d, $1H_3$, Ar), 7.9 (d, $1H_6$, Ar), 8.5 (d, $1H_4$, Ar), 9.0 (d, $1H_2$, Ar). MS; 158 (88: M^+), 159 (100). Anal. Calcd for $C_{14}H_{16}N_2O_2 \cdot 2HCl \cdot H_2O$ (334): C 50.30 H 5.98 N 8.38 O 14.37 Found: C 50.22 H 6.01 N 8.44 O 14.58. The NMR spectrum of the product corresponded to the structure of MO-8HQ. Morpholine predominantly reacts at the more highly substituted 7-position according to the mechanism of the Mannich reaction (9). The NMR spectra of 8HQ and MO-8HQ exhibited different absorption peaks in the 7.2-7.3 ppm region. The disappearance of the proton in position 7 of MO-8HQ indicated substitution of the morpholinomethyl group at position 7 of 8HQ.

Cytotoxicity test

Reagents and cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company, St. Louis, Mo. [^{3}H]-thymidine and [^{3}H]-uridine (50

Ci/mmol) were purchased from ICN Biomedicals Inc., Costa Mesa, CA. Hep G2 human hepatoma and Colo 205 human colon carcinoma cells were obtained from the American Type Culture Collection, Rockville, MD. KB nasopharynx carcinoma cells were obtained from the

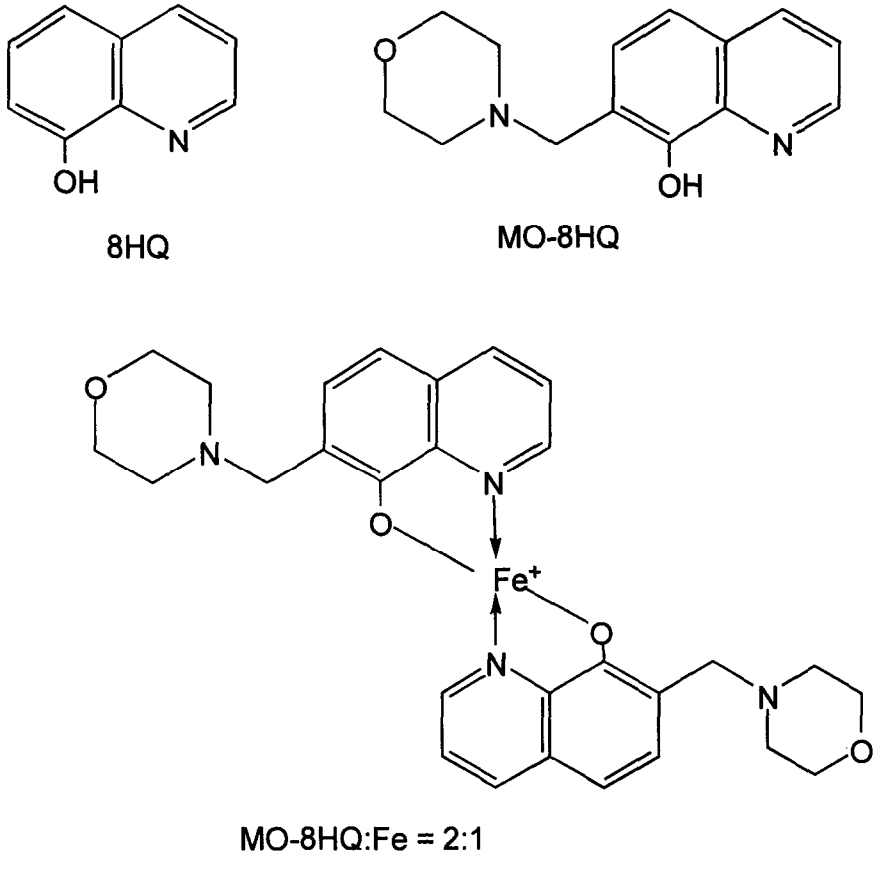

FIG 1

Chemical structure of 8-hydroxyquinoline (8HQ), 7-morpholinomethyl-8-hydroxyquinoline (MO-8HQ) and iron binding MO-8HQ (2:1).

National Cancer Institute of the United States. TSGH 8302 human cervical carcinoma cells were obtained from the Tri-Service General Hospital, Taipei, Taiwan. Cells were maintained in RPMI 1640 or DMEM (Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Methods. Cytotoxicity was tested against KB, TSGH 8302, Colo 205 and Hep G2 cells by the MTT assay as described (10, 11). Tumor cells (1.6×10^4 /ml) were treated with various concentrations of MO-8HQ or 8HQ in 96-well plates for 4 days. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added to each well for another 4 to 6 h. The optical density of each well was measured at 545 and 690 nm using a Titertek Multisk plate reader. The cytotoxicity of the tested compounds were calculated as follows:

$$\text{Cytotoxicity} = [1 - \frac{A_{545}\,(\text{tested}) - A_{690}(\text{tested})}{A_{545}\,(\text{control}) - A_{690}(\text{control})}] \times 100$$

IC_{50} values were determined by plotting the cytotoxicity index versus the concentration of the tested compound.

Inhibition of DNA and RNA synthesis. DNA and RNA synthesis of cell cultures were determined as described (12). Briefly, Hep G2 cells were plated overnight in 96 well microtiter plates at 2 x 10^4 cells per well. Serial dilutions of MO-8HQ in medium containing 5% bovine serum were added to cells for 24-48 h at 37℃. Cells were subsequently washed once with sterile phosphate buffer solution (PBS) and incubated for 2 h with 1 μCi/well of [^{3}H]-thymidine or [^{3}H]-uridine for DNA and RNA synthesis determinations, respectively. Radioactivity of trichloroacetic acid precipitated and washed RNA or DNA was measured in a Beckman LS 6000 series liquid scintillation counter.

Antimicrobial activity

Test organisms. The microbial strains used in this investigation were cultures obtained from the American Type Culture Collection (ATCC), U.S.A. *Escherichia coli* KH 683 was a clinical isolate obtained from Chung-Ho Memorial Hospital, Kaohsiung Medical College, Taiwan. All strains were maintained in semi-solid agar before use.

Methods. Antibacterial activity was assayed by the agar dilution method (13) using Mueller-Hinton medium (Difco Laboratories). Prior to testing, the bacteria were inoculated into 2 ml of tryptic soy broth (TSB, Difco Laboratories) and incubated for 16-18 h at 37℃. The cultures were then adjusted with sterile normal saline to 2-5 x 10^7 colony forming units (CFU) per ml. Drug containing media was prepared by adding 1 volume of diluted MO-8HQ solution (in 50% dimethylsulfoxide) into 9 volumes of autoclaved Mueller-Hinton agar that had been cooled to 50 ℃. Agar plates containing drug and control plates without drug were spot inoculated with 10^4 CFU with a Steer Replicator (14) within 30 min after preparation of the inocular. The agar plates were allowed to stand until the inoculum spots were completely absorbed and were then incubated at 37℃ for 20-22 h. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of drug at which no visible growth of organisms was observed.

Antifungal activity was measured as described above except that yeast morphology agar (Difco Laboratories) was used at 27℃.

Effect of MO-8HQ on bacterial susceptibility to deoxycholate. The *E. coli* KH 683 strain was grown in tryptic soy broth, harvested in the stationary phase of growth and washed twice with normal saline. 10^5 CFU was suspended in saline and 1.5 x 10^{-4} M MO-8HQ with or without 7.5 x 10^{-5} M ferric chloride solution was added for 60 min at 37℃. Samples were removed after 0, 15, 30, and 60 min and bacterial colonies were determined after plating overnight at 37℃ on MacConkey agar (Difco Laboratories) containing 0.085% sodium deoxycholate.

Effect of MO-8HQ on bacterial susceptibility to rifampicin. E. coli was grown to a density of 10^8 CFU per ml in tryptic soy broth. An inoculum of 3 x 10^5 CFU was added to 1 ml of the same medium with or without EDTA (5x10^{-5} M) , MO-8HQ (40 μg/ml), rifampicin (4.5 μg/ml) or

supplemental calcium (1.5 mM) at 37℃. Samples were removed after 0, 2 and 4 h and bacterial colonies were measured by plating overnight at 37℃ on tryptic soy agar.

Reversal of MO-8HQ-Fe mediated deoxycholate susceptibility. The test was modified from the method described by Ellison (15). Briefly, *E. coli* was cultured and treated with deoxycholatye as described above. A 10^5 CFU inoculum of *E. coli* was first incubated with 1.5 x 10^{-4} M MO-8HQ + 7.5 x 10^{-5} M $FeCl_3$ in 1.0 ml normal saline solution for 10 min at 37℃. Solutions were then added separately to each tube to the final concentrations of 1.5 x 10^{-4} M $CaCl_2$, 1.5 x 10^{-4} M $MgSO_4$, 10^{-4} M K_2HPO_4, 1.5 x 10^{-4} M $CaCl_2$ + 10^{-4} M K_2HPO_4, and 1.5 x 10^{-4} M $MgSO_4$ + 10^{-4} M K_2HPO_4. Samples were removed 15 or 30 min later and bacterial colonies were determined by plating overnight at 37℃ on MacConkey agar.

U.V. absorption spectra. The U.V. absorption spectra of MO-8HQ was determined in a Jasco model 7800 spectrophotometer. $MgCl_2 \cdot 6H_2O$ (Merck), $MnCl_2 \cdot 4H_2O$ (Merck), $CaCl_2 \cdot 2H_2O$ (Merck) or $FeCl_3 \cdot 6H_2O$ (Merck) were added to MO-8HQ in phosphate buffer solution for U.V. spectrophotometer determinations. The ratio of MO-8HQ-to-Fe was determined by the method of continuous variation (20). In brief, the absorbance of complexes at various mole fractions were substracted from the corresponding values expected without complex formation. The difference was then plotted against mole fraction to estimate the molar ratio of the complex.

Results

Cytotoxicity

The cytotoxic activities of 8HQ and MO-8HQ against Hep G2, Colo 205, TSGH 8302 and KB human carcinoma cells are shown in Table 1. Both 8HQ and MO-8HQ inhibited cell growth in a concentration dependent manner with half maximal activities in the micromolar concentration range. TSGH 8302 cells were most sensitive to 8HQ whereas all 4 cell lines exhibited similar sensitivities to MO-8HQ. DNA synthesis of Hep G2 cells was inhibited by MO-8HQ within 24 h. However, higher concentrations of drugs and longer incubation times were required to inhibit RNA synthesis in Hep G2 cells (Table 2).

Antimicrobial activity

Because of the broad spectrum of activity exhibited by 8HQ (1-5), great effort has been expanded to improve the activity of 8 HQ by modifying its structure. The morpholino derivative of naphthol has been shown to possess potent antimicrobial activity compared to other naphthol derivatives (17) as well as moderate activity against HIV (human immunodeficiency virus) (18). Since both 8HQ and naphthol are phenolic compounds, we synthesized MO-8HQ to determine if its potency could be increased.

Both MO-8HQ and 8HQ were more active against Gram positive than Gram negative bacteria. Modification of 8HQ increased its antimicrobial activity. MO-8HQ was more active than 8-HQ against Gram positive strains of *Bacillus subtilis* 6633 and *Bacillus cereus* 11778 (Table 3). All Gram positive bacteria were more sensitive to MO-8HQ:Fe (2:1) chelate. For example *Staphylococcus* were insensitive to MO-8HQ but were inhibited by 5.8 μg/ml MO-8HQ:Fe (2:1). *Bacillus* and *Micrococcus* were sensitive to both MO-8HQ and MO-8HQ:Fe (2:1) with MIC values ranging from 4–8 μg/ml. In contrast, all Gram negative bacteria with the exception of *Bordetella bronchiseptica* were insensitive to both MO-8HQ and MO-8HQ:Fe (2:1).

Table 4 shows that MO-8HQ was toxic to fungi at low concentrations. The MIC to yeast such as *Candida albicans* and *Saccharomyces cerevisiae* was 15.6 μg/ml, whereas the filamentous *Penicillium raistrickii* displayed a MIC of 7.8 μg/ml. 8HQ displayed less antifungal activity than MO-8HQ.

TABLE 1

Cytotoxicity of MO-8HQ and 8HQ as Determined by the MTT Assay

Cell line	IC_{50} (μg/ml)	
	8HQ	MO-8HQ
Hep G2 (heptoma)	9.6	6.8
Colo 205 (colon cancer)	9.7	5.0
TSGH 8302 (cervical cancer)	1.2	4.4
KB (nasopharynx cancer)	4.5	4.3

Each experiments were carried out in quadruplicate and repeated once.

TABLE 2

The Effect of MO-8HQ on DNA and RNA Synthesis in Hep G2 Cells

	Incubation time (h)	IC_{50} (μM)
DNA	24	11.8
	48	5.9
RNA	24	>100
	48	11.9

Each experiment was carried out in triplicate and repeated once.

Effect of MO-8HQ on E. coli susceptibility to deoxycholate. EDTA and polyionic agents can sensitize Gram negative bacteria to detergents. Previous studies have demonstrated that other factors can sensitize Gram negative bacteria to deoxycholate (19). Experiments were thus performed to investigate whether MO-8HQ or MO-8HQ:Fe (2:1) could sensitize bacteria to deoxycholate. As shown in Fig. 2, incubation of *E .coli* with 50 μg/ml MO-8HQ had minimal effect on bacteria survival after plating on MacConkey agar containing 0.085% deoxycholate. In contrast, a 2:1 chelate of MO-8HQ and iron significantly increased the sensitivity of *E. coli* to deoxycholate. After 15 min incubation, the bacterial survival was reduced to $16.1 \pm 3.8\%$ and almost all bacteria were killed after 60 min exposure to MO-8HQ:Fe (2:1) followed by

TABLE 3

Minimal Inhibitory Concentrations of MO-8HQ:Fe and MO-8HQ Against Bacteria

Strain	Bacteria (Gram + or -)	MIC (μg/ml)		
		MO-8HQ:Fe (2:1)	MO-8HQ	8HQ
ATCC 6633	*Bacillus subtilis* (+)	5.8	7.8	31.3
ATCC 11778	*Bacillus cereus* (+)	5.8	7.8	31.3
ATCC 12228	*Staphylococcus epidermidis* (+)	5.8	62.5	>62.5
ATCC 25923	*Staphlococcus aureus* (+)	7.8	62.5	>62.5
ATCC 10240	*Micrococcus flavus* (+)	3.9	7.8	7.8
ATCC 9341	*Sarcina lutea* (+)	31.3	62.5	>62.5
ATCC 4617	*Bordetella bronchiseptica* (-)	7.8	15.6	62.5
ATCC 25922	*Escherichia coli* (-)	>62.5	62.5	>62.5
ATCC 27853	*Pseudomonas aeruginosa* (-)	>62.5	>62.5	>62.5
ATCC 6539	*Salmonella typhi* (-)	>62.5	>62.5	>62.5
ATCC 10031	*Klebsiella pneumoniae* (-)	>62.5	>62.5	>62.5
ATCC 8100	*Serratia marcescens* (-)	62.5	62.5	>62.5
ATCC 12022	*Shigella flexneri* (-)	62.5	>62.5	>62.5
ATCC 13048	*Enterobacter aerogenes* (-)	>62.5	62.5	>62.5
ATCC 13315	*Proteus vulgaris* (-)	31.3	62.5	>62.5

MO-8HQ:Fe was at the molar concentration ratio of 2:1. (+) indicates Gram positive bacteria; (-) indicates Gram negative bacteria.

plating on agar containing deoxycholate. Clinical *E. coli* strains as well as *Pseudomonas aeruginosa* have been reported to be similarly sensitized to deoxycholate by transferrin (15). These results indicate that MO-8HQ:Fe (2:1) might alter the Gram negative outer membrane and therefore increases bacterial susceptibility to deoxycholate.

Effect of MO-8HQ on E. coli susceptibility to rifampicin. The outer membrane of Gram negative bacteria acts as an effective permeability barrier to rifampicin to establish high levels of resistance (20). LPS, a characteristic component of the outer membrane strongly binds divalent cations (21). The effect of Ca^{2+} on the cytotoxicity of MO-8HQ and rifampicin to *E. coli* was thus investigated. Table 5 shows that addition of 1.5 mM Ca^{2+} did not affect the growth of *E. coli*. Ca^{2+} did not alter the sensitivity of bacteria to MO-8HQ, but it significantly decreased the ability of both MO-8HQ and EDTA to sensitize *E. coli* to rifampicin. The LPS interactions in the outer membrane are weakened by the removal of divalent cations by EDTA, causing the LPS monolayer to become more permeable to hydrophobic molecules (such as rifampicin) that are normally excluded by the hydrophilic LPS barrier (6, 7). The similar increase in rifampicin sensitivity by EDTA and MO-8HQ suggests that MO-8HQ damages the outer membrane of Gram negative bacteria in a manner similar to EDTA.

Reversal of MO-8HQ:Fe exposed E. coli to deoxycholate susceptibility. The outer membrane damage induced by EDTA can be reversed by addition of a large excess of Ca^{2+} or Mg^{2+} (6).

TABLE 4

Minimal Inhibitory Concentrations of MO-8HQ Against Fungi

Strain	Fungi	MIC (μg/ml)	
		MO-8HQ	8HQ
ATCC 10231	*Candida albicans*	15.6	31.3
ATCC 36232	*Candida albicans*	15.6	31.3
ATCC 2366	*Saccharomyces cerevisiae*	15.6	15.6
ATCC 9763	*Saccharomyces cerevisiae*	15.6	31.3
ATCC 10490	*Penicillium raistrikii*	7.8	7.8

Similar studies were performed to evaluate whether susceptibility to deoxycholate induced by MO-8HQ:Fe (2:1) could be reversed. This was tested by treating cells with MO-8HQ:Fe (2:1) and then holding the exposed cells in solutions containing Ca^{2+}, Mg^{2+} or phosphate before plating the cells on agar that contained deoxycholate. Reversal of deoxycholate susceptibility occured to varying degrees in different solutions, but the greatest recovery was observed when the cells were held in solutions containing both Ca^{2+} or Mg^{2+} and phosphate. Table 6 shows that *E. coli* exposed to MO-8HQ:Fe (2:1) only gradually recovered in Mg^{2+} or Ca^{2+} medium with the percentage of viable bacteria increasing to $42.9 \pm 7.8\%$ and $47.7 \pm 4.9\%$ respectively after 30 min. Recovery significantly increased in the presence of both Ca^{2+} or Mg^{2+} and phosphate. For example, bacteria viability increased to $89.3 \pm 7.8\%$ after 30 min in Ca^{2+} + phosphate medium. The ability of *E. coli* to recover from MO-8HQ:Fe (2:1) mediated susceptibility to deoxycholate indicates that healing of the membrane damage occurred. It is suggested that healing may be related to the incorporation and stabilization of new molecules in the outer membrane. Phosphate improved the recovery of exposed cells, suggesting that phosphate plays a role in the incorporation of new LPS molecules or in the enhancement of bacterial metabolism.

Characterization of the chelating properties of MO-8HQ. MO-8HQ is able to chelate polyvalent cations because of the location of the hydroxyl group relative to the ring nitrogen. Chelation of polyvalent cations by MO-8HQ was measured by the change in U.V. absorption in the presence of Mg^{2+}, Mn^{2+}, $Ca^{2+}$and Fe^{3+}. Addition of Mn^{2+} (Fig. 3a), Mg^{2+} (Fig. 3b), Ca^{2+} and Fe^{3+} caused changes in the absorption spectrum with increased extinction at 240-260 nm. Univalent cations (Na^{+}, K^{+}), in contrast, did not affect the absorption spectrum (data not shown). We also examined whether the MO-8HQ:Fe (2:1) chelate was the predominant species in solution. Fig. 4 shows that the mole fraction of MO-8HQ was about 0.67, is indicating a 2:1 complex of MO-8HQ to iron. A maximum increase in the extinction at 252 nm was also observed with 2 moles of MO-8HQ and 1 mole of $FeCl_3$. These data suggest that two moles of MO-8HQ are bound per $FeCl_3$ in solution.

Discussion

The mechanism of cytotoxicity induced by MO-8HQ was explored by examining its ability to inhibit DNA and RNA synthesis in Hep G2 cells. MO-8HQ inhibited DNA synthesis in the micromolar concentration range. DNA determinations closely reflect the number of cells undergoing growth inhibition either due to inhibition of cell division or cell death (22). This

TABLE 5

Effect of Ca^{2+} on the Ability of EDTA and MO-8HQ to Enhance the Susceptility of *E. coli* to Rifampicin

Treatment	Log (difference in no. of bacteria after 4 h)	
	-Ca^{2+}*	+1.5 mM Ca^{2+}
TSB	3.0±0.1	3.1±0.1
EDTA ($5x10^{-5}$ M)	3.1±0.1	3.2±0.1
MO-8HQ (40 μg/ml)	2.1±0.1[a]	2.1±0.1
Rifampicin (4.5 μg/ml)	2.1±0.2	2.8±0.1
Rifampicin (4.5 μg/ml)+EDTA ($5x10^{-5}$ M)	0.8±0.1[b]	2.8±0.1[c]
Rifampicn (4.5 μg/ml)+MO-8HQ (40 μg/ml)	0.7±0.2[b]	1.5±0.2[c]

Values are mean values ± S.E.M. of seven experiments. All statistics are based on the paired *t*-test. The significance of results is indicated as follows : (a) $p<0.05$ versus TSB, (b) $p<0.05$ versus rifampicin, (c) $p<0.001$ versus experiments without added Ca^{2+}. * : Ca^{2+} free

result suggests that MO-8HQ cytotoxicity was mediated by the inhibition of nucleoside synthesis in Hep G2 cells. 8HQ has been reported to inhibit RNA synthesis in yeast by chelation of bivalent cations required for RNA polymerase activity (23). The 8HQ derivatives, clioquinol, and its metal complex have been shown to directly bind to Hela cell DNA (24). 8HQ was reported to inhibit DNA synthesis in fission yeast (25). Because of the similarities of the molecular structures of 8HQ and MO-8HQ, a common mode of action of these compounds is expected. MO-8HQ may inhibit cellular processes requiring bivalent cations. Results showing inhibition of DNA synthesis by MO-8HQ suggest that it might form a chelate with metals necessary for enzymes which catalyzes DNA biosynthesis. Ribonucleotide reductase may be a candidate because this enzyme contains two iron ions which are required for activity (26). Other investigators (27, 28) have reported that iron bound to 8HQ has strong toxic properties and that it may cause substantial DNA-strand breakage and lipid peroxidation in living cells. Reactive oxygen metabolites might be involved in the toxic action. Thus, cytotoxicity may be accompanied by membrane lipid peroxidation. More detailed studies will be necessary to clarify the precise mechanism of MO-8HQ cytotoxicity .

MO-8HQ was active against Gram positive bacteria and its activity involved the chelation of metal ions (ferric ions). Experiments conducted in Mueller-Hinton agar revealed that iron ions alone were not harmful to the organisms, whereas MO-8HQ:Fe at the molar ratio of 2:1 was toxic. The present study is consistent with a report that 8HQ-Fe (1:1) or 8HQ-Fe (2:1) chelates were toxic whereas the 8HQ-Fe (3:1) chelate was nontoxic (29). Thomas et al (30) also indicated that iron is an active participant in oxidative damage to endothelial cells. Balla et al (31) demonstrated that 8HQ forms lipophilic chelates with iron and rapidly transfers the metal across the intact plasma membrane of endothelial cells, increasing their sensitivity to oxidants. The above observations suggest that MO-8HQ:Fe (2:1) mediated antimicrobial activity might be related to the action of a metal-bound oxyl radical formed from MO-8HQ:Fe (2:1) complexes in Gram positive bacteria. However, Gram negative bacteria were much less susceptible to MO-8HQ and

TABLE 6

Reversal of MO-8HQ:Fe (2:1) Induced Susceptibility to Deoxycholate by Incubation of *E. Coli* with Different Salt Solutions

	Percentage of bacteria remaining			
	N	0 min	15 min	30 min
NaCl (control)	6	91.8±7.2	85.6±4.2	83.3±5.8
$CaCl_2$ (1.5×10^{-4}M)	7	17.4±4.4	40.7±7.5*	47.0±4.9**
$MgSO_4$ (1.5×10^{-4}M)	7	14.5±4.3	33.1±7.7*	42.0±7.8**
K_2HPO_4 (10^{-4}M)	9	17.1±5.2	25.1±6.2**	30.2±5.9**
$CaCl_2$ (1.5×10^{-4}M) + K_2HPO_4 (10^{-4}M)	5	75.5±6.6	80.0±7.2**	89.3±7.8*
$MgSO_4$ (1.5×10^{-4}M) + K_2HPO_4 (10^{-4}M)	5	48.9±6.7	69.4±5.9*	78.3±3.9**

Values represent the percentage of the bacteria inoculum remaining after incubation with MO-8HQ and subsequent plating on MacConkey agar that contained 0.085% deoxycholate. The 0 min results indicate the percentage of bacteria that was present immediately after the 10 min incubation with 40 μg/ml of MO-8HQ:Fe (2:1) for 10 min. The values are means ± S.E.M. N, number of tests. *$p<0.001$, **$p<0.05$ by paired *t* test comparing the percentage of bacteria remaining at 0 min with the percentage at 15 and 30 min.

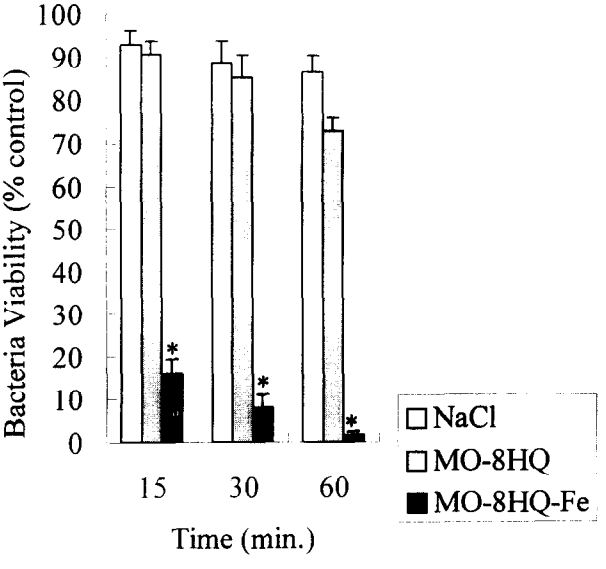

FIG. 2

Effect of MO-8HQ and MO-8HQ:Fe (2:1) on *E. Coli* susceptibility to deoxycholate. *E.coli* were treated for 15, 30, and 60 min with NaCl, MO-8HQ or MO-8HQ:Fe (2:1) before plating on MacConkey agar containing 0.085% deoxycholate. Colonies were counted 24 h later. Each value represents mean ± S.E.M. for six experiments. The paired *t* test was used for the statistical analysis. *$p<0.05$ comparing MO-8HQ with MO-8HQ:Fe (2:1).

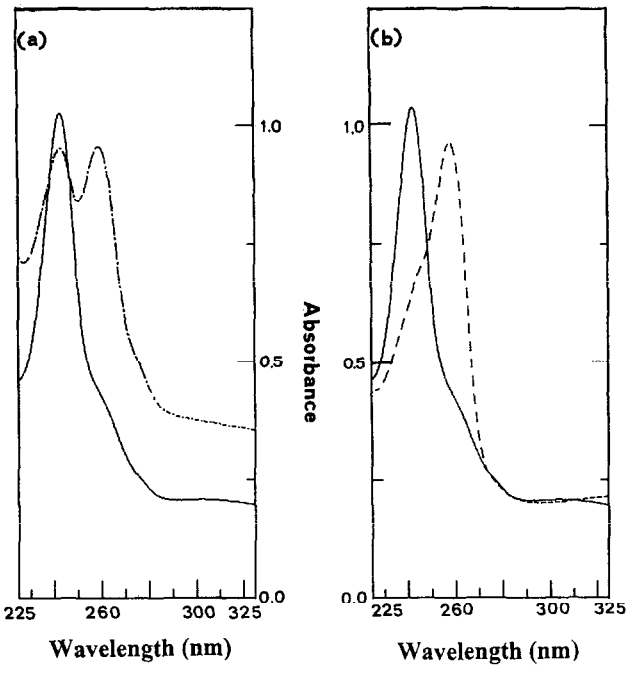

FIG. 3

0.02 mM MO-8HQ in phosphate buffer solution, pH 7.3, alone (—) and plus 50 mM $MnCl_2$ (-·-·) (a) or 50 mM $MgCl_2$ (---) (b).

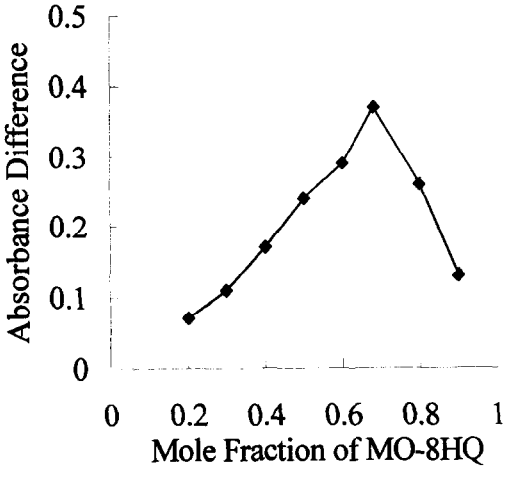

Fig. 4

Plot of absorbance difference against mole fraction of MO-8HQ. Spectrophotometric absorbance was determined at 240 nm. The absorbance of MO-8HQ:Fe obtained at various mole fractions after 30 min incubation were substracted from the absorbance values of MO-8HQ. The curve indicating a 2:1 complex of MO-8HQ:Fe predominates in solution.

MO-8HQ:Fe (2:1). The outer membrane of Gram negative bacteria may serve as a selective barrier that hinders the passage of MO-8HQ:Fe (2:1) into the cell.

It is clear that the outer membrane of Gram negative bacteria is important for resistance to host defence factors (32, 33). In enteric Gram negative bacteria, which live in the intestinal tract of animals, the outer membrane has developed into a very effective barrier, protecting cells from the detergent action of bile salts and degradation by digestive enzymes (18). The outer membrane of enteric and some Gram negative bacteria also acts as a strong permeability barrier to many antibiotics (e. g., rifamycins, lincomycin) that are effective against other bacteria (7). Many studies have shown the importance of cations in the organization of the outer membrane of bacteria. Among the most convincing are those which show that the outer membrane can be disorganized by removing divalent cations with chelators (30). The LPS component of the outer membrane is known to contain inorganic (Na^+, K^+, Mg^{2+}, Ca^{2+}) and organic cations (8). By binding membrane-stablizing cations, EDTA releases LPS from the outer membrane, concurrently increasing membrane permeability to hydrophobic molecules and sensitizing bacteria to membrane-active agents (7). The presence of Ca^{2+} remarkedly decreased the ability of both MO-8HQ and EDTA to sensitize *E. coli* to rifampicin. This result suggests that the ability of MO-8HQ to chelate these ions may be important for the observed effects. These findings are related to the studies of Ellison and his coworkers who found that LPS release by lactoferrin could be blocked by concurrent addition of Ca^{2+} and Mg^{2+}, and that Ca^{2+} also blocked the ability of lactoferrin to increase the susceptibility of *E. coli* to rifampicin (15). The demonstration of on additive interaction between MO-8HQ and rifampin may involve alteration of the outer membrane permeability by MO-8HQ, although other explanations are not excluded. It is reasonable to speculate that MO-8HQ might act as a membrane active agent whose effects are modulated by Ca^{2+} and Mg^{2+}. MO-8HQ may thus play a valuable role as an antibiotic adjuvant. In addition, MO-8HQ:Fe (2:1) significantly increased the susceptibility of *E. coli* to deoxycholate. Sensitivity was reversed in the presence of Ca^{2+} or Mg^{2+}. These results are consistent with the concept that Ca^{2+} and Mg^{2+} are important to the stability of the Gram negative outer membrane. Chelation of bivalent cations by MO-8HQ was demonstrated by a change in its U.V. absorption spectrum in the presence of bivalent cations (Fig. 3).

In conclusion, the present investigation shows that MO-8HQ is a chelating agent that possesses cytotoxicity and antimicrobial properties. It can sensitize bacteria to both a hydrophobic antibiotic and a surface-active detergent. These activities are modulated by Ca^{2+} and Mg^{2+}. In addition, the mechanism of cytotoxicity may involve inhibition of DNA synthesis.

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