Mode of Action of the Bioreductive Alkylating Agent, 2,3-Bis(chloromethyl)-1,4-naphthoquinone

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SUMMARY

The bioreductive alkylating agent, 2,3-bis(chloromethyl)-1,4-naphthoquinone (CMNQ), has been shown to inhibit the growth of Sarcoma 180 ascites cells in vivo. Evidence for the reductive activation of this agent via the mitochondrial respiratory chain was provided by CMNQ-induced oxidation of reduced nicotinamide adenine dinucleotide; the interaction was shown to be on the substrate side of the site of rotenone inhibition. Consistent with the concept that reductive activation of CMNQ to a hydroquinone results in the generation of an alkylating species (i.e., a quinone methide), the finding that radioactivity from [14C]CMNQ present in Sarcoma 180 ascites cells was associated with DNA, RNA, and protein for a period of up to 72 hr after exposure of tumor-bearing animals to this agent. Inhibition of the incorporation of [3H]thymidine, [3H]uridine, and [14C]leucine into DNA, RNA, and protein, respectively, of Sarcoma 180 ascites cells was produced by this agent, with DNA biosynthesis being the most susceptible. The inhibitory effect of CMNQ on the formation of DNA was, at least in part, the result of a prevention of the conversion of thymidine to its nucleotide forms. This action was due to (a) a drug-induced decrease in intracellular levels of adenosine 5'-triphosphate, presumably resulting from uncoupling of oxidative phosphorylation by CMNQ; and (b) a partial loss of thymidine kinase activity in Sarcoma 180 cells, which did not appear to be due to direct inhibition of the enzyme by the drug. Although the primary event produced by CMNQ at the mitochondrial level appeared to be release of respiratory control, other effects on mitochondrial metabolism occurred. These included inhibition of reduced nicotinamide adenine dinucleotide and succinoxidase activities, as previously demonstrated, and mitochondrial swelling, which suggested interaction of CMNQ with the inner mitochondrial membrane. These findings indicate a variety of biochemical lesions are associated with the antineoplastic activity of CMNQ and demonstrate a relationship between the effects of this drug on mitochondrial respiratory control and DNA biosynthesis.

INTRODUCTION

The antineoplastic agent, mitomycin C, a potent inhibitor of the synthesis of nucleic acids (10), acts as a bifunctional alkylating agent which adds across both strands of the DNA double helix to cause cross-linking (6, 7). Reduction of the benzoquinone ring of the mitomycin molecule to a dihydroquinone is essential for both alkylation and inhibition of nucleic acid synthesis and is accomplished enzymatically by a quinone reductase that requires NADPH as the reducing agent (21). Kinoshita et al. (11, 12) have postulated that the carbamyl group and the aziridine ring of the mitomycins are not strictly essential for alkylating activity; on the basis of this suggestion, we proposed that relatively simple benzo- and naphthoquinones possessing similar properties might function in an analogous way (14). It was visualized that following enzymatic reduction of these quinones to dihydroquinones, spontaneous degradation would occur to a reactive intermediate (i.e., a quinone methide) with the potential to bind covalently to critical cellular macromolecules. To test this concept, a number of benzo- and naphthoquinones with 1- or 2-side chains capable of alkylation following reduction have been synthesized and shown to possess potent antitumor activity against the murine neoplasms Adenocarcinoma 755 and Sarcoma 180 (13-15, 17). Chemical evidence was obtained to substantiate the existence of a quinone methide as an intermediate following sodium borohydride reduction of the model compound 2,3-dimethyl-5,6-bis(acetoxymethyl)-1,4-benzoquinone (16).

We have speculated that the latent alkylating potential of these compounds, the expression of which is dependent upon reduction, may be particularly useful against hypoxic cells of solid tumors, in that it is reasonable to assume that these neoplastic cells, distal to blood vessels, would have intracellular conditions conducive to reduction.

This report presents studies on the metabolic lesions created by a representative bioreductive alkylating agent of this series, CMNQ, a derivative of this class that has shown antineoplastic activity against both Sarcoma 180 and Adenocarcinoma 755 (17). The findings have shown that CMNQ causes inhibition of the synthesis of nucleic acids and proteins, interferes with the conversion of thymidine to its nucleotide forms, interacts with cellular nucleic acids and proteins, causes inhibition of respiration and release of respiratory control, induces mitochondrial swelling, and presents evidence for reductive activation of CMNQ by mitochondria.

MATERIALS AND METHODS

Experiments were performed on 9- to 11-week-old female CD-1 mice (Charles River Breeding Laboratories, Wilmington, Massachusetts; Charles River Breeding Laboratories, Wilmington, Massachusetts) that were 9- to 11-week-old at the time of induction. The animals were fasted for 24 hr before injection and then given ad libitum access to food and water throughout the experiment. Mice were killed by cervical dislocation, with the time of death noted to the nearest 15 min. The animals were sacrificed by cervical dislocation, with the time of death noted to the nearest 15 min.

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1 This research was supported in part by USPHS Research Grants CA-02817 and CA-16359 from the National Cancer Institute.

2 The abbreviations used are: CMNQ, 2,3-bis(chloromethyl)-1,4-naphthoquinone; HBHM, heavy bovine heart mitochondria; DNP, 2,4-dinitrophenol.
tion, Mass.). Transplantation of Sarcoma 180 ascites cells was carried out by i.p. injection into each mouse of about 6 × 10^6 cells obtained from a donor mouse bearing a 7-day tumor growth. CMNQ was prepared for administration in fine suspension by homogenization in a glass tissue grinder, containing a small amount of water and a few drops of 20% Tween 80, and by dilution to the proper concentration with distilled water. To determine the effects of this agent on Sarcoma 180 under conditions used in the in vivo biochemical studies, a single dose of CMNQ was injected i.p. into mice bearing 6-day tumor implants. The neoplastic population was collected from the peritoneal cavities of animals by extensive washing at selected intervals after exposure to a given drug dose and the number of cells was determined with a Coulter Model A particle counter.

To determine the effects of CMNQ on the synthesis of nucleic acids and protein, 6-day tumor-bearing animals were given i.p. injections of a single dose of either 10, 15, or 30 mg CMNQ per kg. Control tumor-bearing mice received no drug, but were given an equal volume of 0.9% NaCl solution. At selected intervals of time thereafter, either [3H]thymidine (200 μg/mouse, 10 × 10^6 cpm/μg), [3H]uridine (200 μg/mouse, 17.6 × 10^6 cpm/μg), or [3H]-[14C]leucine (125 μg/mouse, 4.26 × 10^6 cpm/μg) was given i.p. and 1 hr was allowed for incorporation. Cells were processed and radioactivity was determined as previously described (2). The specific activity of the nucleic acids was expressed as cpm/μg mole of deoxynucleotide or ribose after determination of the content of these carbohydrates in the deoxyribose using diphenylamine or orcinol (20), respectively, and the specific activity of protein as cpm/mg of protein was determined by the method of Lowry et al. (18), with bovine serum albumin (Fraction V) as the standard.

The effects of CMNQ on the conversion of [3H]thymidine to deoxynucleotide forms was measured by treating mice bearing 6-day implants of Sarcoma 180 i.p. with a single 30-mg/kg dose of this agent. One hr later, cells were collected, washed with Fischer’s medium to remove erythrocytes, and resuspended in Fischer’s medium containing 10% horse serum at a concentration of approximately 10^6 cells/ml. [3H]Thymidine (2 × 10^6 cpm; 0.37 μCi/μmole) was added and the suspension was incubated with shaking at 37° for 10 min. The cells were layered onto 0.25 M sucrose and centrifuged at 1600 × g for 2 min. The cell pellet was extracted 3 times with 0.4 M perchloric acid, and the extracts were collected and neutralized to pH 7 with potassium hydroxide and frozen. Separation of thymidine from thymine deoxynucleotides was accomplished on columns of epichlorohydrin triethanolamine cellulose and the radioactivity therein was determined. CMNQ was dissolved in dimethyl sulfoxide for enzymatic assays; the concentration of dimethyl sulfoxide in these assays did not exceed 0.57%.

Rat liver mitochondria were isolated by differential centrifugation according to the method of Johnson and Lardy (9) using as the isolation buffer 0.25 M sucrose, which was 1 mM with respect to ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid and 3.4 mM with respect to Tris-HCl (pH 7.4). The rat liver mitochondria used in the swelling experiments were isolated as described above, washed with a buffer containing 0.175 M KCl and 0.025 M Tris-HCl (pH 7.4) as described by Bhuvaneswari and Dakshinamurti (1), and suspended in medium containing 0.15 M KCl and 0.02 M Tris-HCl (pH 7.4).

Rat liver mitochondrial respiration was measured polarographically on a Gilson Oxygraph as described by Estabrook (8).

The effects of the test compounds on ADP-stimulated, DNP-stimulated, and State 4 respiration were assayed by adding the various test compounds in 10 μl of ethanol.

To assess the effect of the various test compounds on mitochondrial swelling, the suspension of mitochondria was adjusted so that 20 μl (approximately 0.5 mg protein) diluted to 3 ml with suspending medium gave an initial absorbance of 0.9 to 1.0 at 520 nm. Swelling was initiated with 2 mM potassium phosphate and 12 μM thyraxine and was followed by continuous recording of the change in absorbance at 520 nm in 1-cm cuvets at 30° on a Cary 14 spectrophotometer (1).

HBHM were isolated by differential centrifugation (22). Aged mitochondria were prepared by repeated freezing and thawing of HBHM at a concentration of protein of 30 mg/ml.
in 0.25 M sucrose-0.05 M Tris-HCl (pH 7.4).

The oxidation of NADH was followed spectrophotometrically at 340 nm following the addition of naphthoquinones. All assays were carried out in the presence of antimycin A and KCN. The assay system was described by Ruzicka and Cittadini et al. (3). Oxygen consumption was measured polarographically in the same buffer.

Bioreduction of CMNQ was initiated by the addition of 0.5 mg of aged mitochondrial protein to a total reaction medium of 3 ml containing 80 mM NaCl, 5 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 4.5 mM sodium phosphate (pH 7.4), 0.167 μM NADH, and 20 μM CMNQ; the reaction was monitored by measuring absorption at 242 nm with a Cary 14 spectrophotometer. Chemical reduction of CMNQ was accomplished by the addition of 0.5 μM sodium dithionite to the same buffer without NADH and with the CMNQ concentration adjusted to 50 μM. The reduced spectrum of CMNQ was determined by measuring absorption between 200 and 350 nm.

ATP levels were measured by repeatedly washing Sarcoma 180 ascites cells with respiration buffer until the erythrocytes were removed. Cells were suspended at a concentration of 115 x 10⁶ cells/ml of respiration buffer and 20 mM succinate, to give a final concentration of 11.5 x 10⁶ cells/ml as described by Cittadini et al. (3). Oxygen consumption was measured polarographically in the same buffer.

Tumor cell respiration was determined by suspending Sarcoma 180 cells in respiration buffer [154 mM NaCl, 6.2 mM KCl, and 10 mM morpholinopropane sulfonate, (pH 7.4)] at a concentration of 7 to 9 x 10⁷ cells/ml as described by Cittadini et al. (3). Oxygen consumption was measured polarographically in the same buffer.

Bioreduction of CMNQ was initiated by the addition of 0.5 μM of aged mitochondrial protein to a total reaction medium of 3 ml containing 80 mM NaCl, 5 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 4.5 mM sodium phosphate (pH 7.4), 0.167 μM NADH, and 20 μM CMNQ; the reaction was initiated by the addition of the naphthoquinone to be tested.

RESULTS

The growth inhibitory effects of a single dose of CMNQ on Sarcoma 180 ascites cells were measured under conditions used in the biochemical studies conducted in vivo to allow comparisons to be made between these studies. This was accomplished by determining the number of ascites cells present in the peritoneal cavities of mice bearing 6-day accumulations of this neoplasm at selected intervals of time after the i.p. injection of a single dose of CMNQ (Chart 1). Administration of 10 mg CMNQ per kg to tumor-bearing mice caused inhibition of the proliferation of Sarcoma 180; treatment with a higher dosage level of this agent (15 mg/kg) resulted in cell death, as demonstrated by a loss in the number of ascites cells present in the peritoneal cavities.

Since we have hypothesized (14, 15) that the action mechanism of compounds such as CMNQ involves bioreduction in vivo of the parent quinone to a dihydroquinone, which then spontaneously decomposes to generate a reactive quinone methide capable of alkylation, it was of importance to determine whether CMNQ interacts with cellular macromolecules. To accomplish this, 11 mg [14C]CMNQ per kg were injected i.p. into animals bearing 5- to 7-day implants of Sarcoma 180. Cells were collected at subsequent intervals and radioactivity associated with purified DNA, RNA, and protein was determined (Table 1). Three hr after exposure of cells to [14C]CMNQ, 8% of the total radioactivity found in the combined nucleic acid and protein fractions was associated with DNA and 22% with RNA. The radioactivity from this agent associated with DNA remained relatively constant for up to 72 hr, while considerable loss of radioactivity occurred from the RNA and protein fractions during this period.

To determine the potential involvement of inhibition of the syntheses of nucleic acids and protein in the tumor-inhibitory properties of CMNQ, the effects of this compound on these processes were measured by the incorporation of [3H]thymidine, [3H]uridine, and [14C]leucine into cold acid-insoluble material. The association of radioactivity from [14C]CMNQ with DNA, RNA, and protein of Sarcoma 180 ascites cells exposed to this agent was measured (Table 1). The growth inhibitory effects of a single dose of CMNQ on Sarcoma 180 ascites cells were measured under conditions used in the biochemical studies conducted in vivo to allow comparisons to be made between these studies. This was accomplished by determining the number of ascites cells present in the peritoneal cavities of mice bearing 6-day accumulations of this neoplasm at selected intervals of time after the i.p. injection of a single dose of CMNQ (Chart 1).

Table 1

<table>
<thead>
<tr>
<th>Time after CMNQ (hr)</th>
<th>No. of samples</th>
<th>DNA fraction</th>
<th>RNA fraction</th>
<th>Protein fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2</td>
<td>9.8</td>
<td>26.8</td>
<td>85.2</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>10.2</td>
<td>11.4</td>
<td>47.2</td>
</tr>
<tr>
<td>24</td>
<td>7</td>
<td>12.4</td>
<td>15.2</td>
<td>42.8</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
<td>8.6</td>
<td>8.2</td>
<td>28.6</td>
</tr>
<tr>
<td>48</td>
<td>2</td>
<td>7.8</td>
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<td>30.4</td>
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<td>60</td>
<td>2</td>
<td>10.4</td>
<td>11.8</td>
<td>27.6</td>
</tr>
<tr>
<td>72</td>
<td>2</td>
<td>9.6</td>
<td>8.8</td>
<td>18.6</td>
</tr>
</tbody>
</table>
insoluble material of Sarcoma 180 ascites cells in vivo. The results are shown in Table 2. Inhibition of the incorporation of thymidine into acid-insoluble material occurred when tumor-bearing mice were treated with the drug 1 hr before the injection of radioactive thymidine. The degree of inhibition was dose dependent, increasing with elevation of the quantity of CMNQ to which the neoplastic cells were exposed. Blockade of DNA biosynthesis persisted for at least 12 hr. Uridine incorporation into RNA was considerably less susceptible to the action of CMNQ and significant inhibition (41%) of RNA synthesis was obtained only at a level of 30 mg/kg. This degree of interference with RNA biosynthesis persisted for up to 3 hr after the drug was administered, and the rate of incorporation of uridine into acid-insoluble material was normal at 12 hr after the administration of CMNQ. A similar low level of inhibition of the synthesis of cellular protein was produced by this agent.

The relationship between the inhibition of [3H]thymidine incorporation into DNA by CMNQ and the effects of this agent on the formation of thymine deoxyribonucleoside triphosphate from this radioactive tracer was measured by determining the distribution of radioactivity from [3H]thymidine in cold acid-soluble nucleotides of Sarcoma 180 cells. This was accomplished by exposing neoplastic cells to CMNQ by injecting 30 mg/kg i.p. into tumor-bearing animals. One hr later, cells were collected and incubated in vitro in Fischer's medium in the presence of [3H]thymidine for 10 min. The distribution of radioactivity in acid-soluble and acid-insoluble fractions was measured and the findings are shown in Chart 2. CMNQ caused pronounced inhibition of thymidine incorporation into DNA, which appeared to be due to interference by this agent with the conversion of thymidine to its nucleotide forms. In contrast, menadione (2-methyl-1,4-naphthoquinone), included as a control, had little or no effect on these processes.

That the inhibitory effect by CMNQ on the synthesis of thymine nucleotides was directed, at least in part, at the level of thymidine kinase was shown by determining the total activity of this enzyme in extracts of cells treated in vivo with either 15 or 30 mg CMNQ per kg (Table 3). Significant depression (62%) of enzymatic activity was produced by the higher dosage level of CMNQ, while menadione was not inhibitory. This decrease in the cellular level of thymidine kinase activity did not appear to be due to direct inhibition of enzymatic activity, since addition to cell-free extracts of up to 2 x 10^{-4} M CMNQ, both in the absence and presence of the reducing agent sodium borohydride, did not significantly decrease kinase activity.

Depression of thymidine kinase activity in situ appeared to be due in part to a decrease in the availability of ATP. Thus, exposure of cells to CMNQ in vitro for 1 hr resulted in a pronounced lowering of cellular levels of ATP, a phenomenon directly related to the concentration of this agent (Table 4). Incubation of Sarcoma 180 cells for 3 hr, under these conditions, in the absence of drug resulted in an 88% decrease in cellular ATP levels; this finding indicated that the concentration of ATP cannot be maintained in these cells for extended time periods under the in vitro conditions used.

Since these initial experiments indicated that ATP levels could be decreased by exposure of neoplastic cells to CMNQ in vitro and a previous report from this laboratory (15) demonstrated inhibition of heavy bovine heart mitochondrial respiration by this agent, it was of importance to investigate in detail the effects of CMNQ on mitochondrial function. Chart 3 compares the effects of mitomycin C with CMNQ and menadione on the respiration of Sarcoma 180 ascites cells. Menadione represents a compound capable of being bioreduced to a hydroquinone without the ultimate generation of a reactive quinone methide species analo-

### Table 2

**Effects of CMNQ on the incorporation of [3H]thymidine, [3H]uridine, and [14C]leucine into DNA, RNA, and protein, respectively, of Sarcoma 180 ascites cells**

<table>
<thead>
<tr>
<th>Time after drug (hr)</th>
<th>Drug dose (mg/kg)</th>
<th>[3H]Thymidine into DNA</th>
<th>[3H]Uridine into RNA</th>
<th>[14C]Leucine into protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>67.5</td>
<td>86.5</td>
<td>94.2</td>
</tr>
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<td>61.8</td>
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<td>30</td>
<td>27.5</td>
<td>59.4</td>
<td>61.1</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>85.0</td>
<td>87.3</td>
<td>102.2</td>
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<td>93.6</td>
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<td></td>
<td>30</td>
<td>46.6</td>
<td>113.2</td>
<td>85.9</td>
</tr>
</tbody>
</table>
The effect of pretreatment of Sarcoma 180 ascites cells with CMNQ and menadione on the cellular content of thymidine kinase activity

Mice bearing 6-day growths of Sarcoma 180 were given a single i.p. injection of either CMNQ or menadione at the indicated dose levels. One hr later, the cells were collected, cell-free extracts were prepared, and thymidine kinase activity was measured as indicated in "Materials and Methods." Each value is the mean of analyses from at least 3 separate preparations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity (nmoles/30 min/mg)</th>
<th>% control activity</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>8.58</td>
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</tr>
<tr>
<td>CMNQ 15mg/kg</td>
<td>6.86</td>
<td>80</td>
</tr>
<tr>
<td>CMNQ 30mg/kg</td>
<td>3.22</td>
<td>38</td>
</tr>
<tr>
<td>Menadione 30mg/kg</td>
<td>9.12</td>
<td>106</td>
</tr>
</tbody>
</table>

Table 3

Chart 3. Polarographic tracing of Sarcoma 180 tumor cell respiration. The reaction was initiated by the addition of Sarcoma 180 cells to respiration buffer in the absence or presence of either mitomycin C, menadione, or CMNQ at the indicated concentrations. —, control respiration; ---, alterations in the cellular respiration rate produced by the various inhibitors.

Effect of CMNQ on ATP levels of Sarcoma 180 ascites cells

Sarcoma 180 cells were incubated in the absence and presence of various concentrations of CMNQ for 1 hr at 37°. At that time the reaction was terminated and the ATP concentration was measured as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (µM)</th>
<th>pmoles ATP/ml</th>
<th>% control activity</th>
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<tbody>
<tr>
<td>None</td>
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<td>5900</td>
<td>100</td>
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<tr>
<td>CMNQ</td>
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<td></td>
<td>250</td>
<td>520</td>
<td>9</td>
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</table>

Table 4

Action of Bioreductive Alkylating Agents

Chart 4. Polarographic tracing of rotenone-inhibited Sarcoma 180 tumor cell respiration. The reaction was initiated by the addition of Sarcoma 180 cells to respiration buffer. The point of addition and concentrations of rotenone, KCN, and CMNQ are as indicated.

Chart 5. Comparative effects of CMNQ and menadione on ADP-stimulated respiration in rat liver mitochondria. The polarographic tracings represent different experiments in which CMNQ and menadione were used at the various concentrations indicated. Mitochondria were added (2.5 mg mitochondrial protein) prior to the addition of the substrate, which was a mixture of 5.0 mM glutamate, 1.0 mM malate, and 1.0 mM malonate (GMM). Respiration was stimulated by the addition of 600 nmoles ADP and DNP was used as an uncoupling agent at a concentration of 0.12 mM. The ADP/O ratio observed with this system was 2.5 to 2.6.

In order to characterize in more detail the effects of CMNQ on mitochondrial processes, its effects were studied in isolated rat liver mitochondria. The data presented in Chart 5 represent a comparison of the effects of CMNQ and menadione on NADH-linked ADP-stimulated respiration (State 3) in rat liver mitochondria. These results indicated that at a concentration of $1.25 \times 10^{-5}$ M CMNQ, an apparent release of respiratory control was produced as the State 3 to State 4 transition became less pronounced (i.e., the State 4 respiration rate was increased). At a level of $2.5 \times 10^{-5}$ M CMNQ, this effect was even more pronounced and the subsequent addition of DNP did not stimulate respiration.
When the concentration of CMNQ was increased further to either $6.25 \times 10^{-8}$ or $1.25 \times 10^{-4} \text{ M}$, an initial burst of oxygen uptake was observed followed by concomitant inhibition of respiration. Similar results were not obtained when menadione was tested at a similar range of concentrations. Thus, at all levels tested, respiratory control was depressed by menadione (i.e., the State 4 respiratory rate was enhanced), but the subsequent addition of DNP always stimulated respiration. This is consistent with the report (4) that demonstrates that menadione is an artificial electron acceptor capable of bypassing coupling Site 2 in rat liver mitochondria. Menadione reductase (EC 1.6.99.2) has been shown to be required for this phenomenon, since dicoumarol, a potent inhibitor of this enzyme, completely depresses menadione-mediated oxygen consumption in rat liver mitochondria treated with rotenone (4). The effect of dicoumarol on CMNQ-mediated respiration in the presence of rotenone was found to be much less pronounced (data not shown), suggesting that this enzyme may not be involved in CMNQ activation. With CMNQ, DNP stimulation of respiration over the State 4 rate was prevented at a concentration of CMNQ of $2.5 \times 10^{-8} \text{ M}$ or greater (Chart 5). These findings suggest that the primary mitochondrial event, at relatively low levels of CMNQ (i.e., $1.25$ to $2.5 \times 10^{-8} \text{ M}$) was uncoupling of oxidative phosphorylation, as evidenced by the increased rate of State 4 respiration. Higher concentrations of CMNQ (i.e., $6.25 \times 10^{-8} \text{ M}$ and greater) appeared to activate electron transport with subsequent respiratory chain inhibition. Similar effects were observed when CMNQ was added to mitochondria respiring without added ADP (Chart 6) or in mitochondria uncoupled with DNP (Chart 7); these data indicate that the effect of a relatively high concentration of CMNQ (i.e., $6.25 \times 10^{-8} \text{ M}$) was independent of the coupling mechanism. Similar results were observed with the same concentration of CMNQ when succinate was used as the substrate.

The effects of CMNQ on phosphate plus thyroxine-induced swelling of mitochondria are depicted in Chart 8. Since succinate was used as the substrate, Chart 8 represents mitochondrial swelling mediated by coupling Sites 2 and 3. The swelling of untreated control mitochondria had essentially reached the maximum when $60 \mu\text{M}$ ATP and $\text{Mg}^{2+}$ were added (Curve 0). At a concentration of $1.25 \times 10^{-5} \text{ M}$ CMNQ, swelling was partially inhibited, but the subsequent addition of ATP and $\text{Mg}^{2+}$ permitted swelling to approach maximum levels. At a level of $2.1 \times 10^{-5} \text{ M}$ CMNQ or greater, however, rapid swelling was induced by the addition of CMNQ prior to the addition of the mixture of thyroxine and phosphate. At $2.1 \times 10^{-5} \text{ M}$ CMNQ, the addition of ATP promoted some additional mitochondrial swelling, but the degree of enlargement attained did not reach the maximum size occurring in control mitochondria. ATP had no effect when the CMNQ was added at the highest level used (i.e., $1.25 \times 10^{-4} \text{ M}$). In contrast, at all concentrations of menadione tested, mitochondrial swelling did not occur without the addition of thyroxine and phosphate, although the rate of swelling was faster in the presence of menadione than in that occurring in control preparations. Maximum enlargement was always reached when ATP was added. Similar results were observed when glutamate, malate, and malonate were used as substrates and all 3 coupling sites were participating in the swelling process. At a concentration of $2.1 \times 10^{-5} \text{ M}$ CMNQ, swelling was initiated by CMNQ (Chart 8) in the absence of thyroxine and phosphate, and at a level of $6.25 \times 10^{-8} \text{ M}$ of this agent, an increased burst of oxygen consumption and subsequent electron transport chain inhibition was observed (Chart 5). In both cases, the maximum effect was produced by $1.25 \times 10^{-4} \text{ M}$ CMNQ (Charts 5 and 8).

To further evaluate the significance of both the increased

![Chart 7](chart7.png)

Chart 7. Comparative effects of CMNQ and menadione on DNP-stimulated respiration in rat liver mitochondria. Conditions were as described in Chart 5.

![Chart 6](chart6.png)

Chart 6. The stimulatory effects of CMNQ and menadione on rat liver mitochondria in the absence of ADP. Conditions were as described in Chart 5.
respiratory rate and the subsequent inhibition produced by 6.25 x 10^{-6} M CMNQ, it was important to determine whether this agent participated directly in electron transport. The data presented in Chart 9 represent the oxidation of exogenous NADH by aged mitochondria in the presence of antimycin and cyanide with CMNQ acting as the terminal electron acceptor. Similar experiments were conducted with menadione, and these data are also presented in Chart 9. The results indicate that both CMNQ and menadione increased the rate of oxidation of NADH by HBHM; however, the stimulation of NADH oxidation by CMNQ was considerably greater than that produced by menadione, which barely enhanced the oxidation of NADH above the nonenzymatic rate. In addition, following the initial rapid rate of NADH oxidation produced by CMNQ, a decrease in NADH oxidation to the nonenzymatic rate occurred. The observation that additional NADH and CMNQ had little effect on NADH oxidation at this time, suggests that the decrease in the rate of oxidation of CMNQ was not due to limited NADH or CMNQ. Furthermore, rotenone did not inhibit the oxidation of NADH induced by CMNQ (Chart 9).

The UV spectra of the reduced and oxidized forms of CMNQ are presented in Chart 10. Dithionite reduction of CMNQ produced a peak absorbance at 242 nm. The change in absorbance allowed a measurement of the degree of bioreduction of CMNQ by NADH in the presence of HBHM by determination of the rate of increase in absorbance at 242 nm (Chart 11). A slight increase in absorption was observed when NADH was added to HBHM in the absence of CMNQ (Chart 11, Curve A); this change is presumed to be due to secondary absorption of NADH. However, when CMNQ was added to HBHM in the presence of NADH, a marked increase in absorption at 242 nm occurred and was followed by a decrease to the rate occurring in the absence of the naphthoquinone (Chart 11, Curve B).

**DISCUSSION**

The concept of bioreductive alkylation has as its primary basis a directed attack on hypoxic cells of solid tumors (i.e., those cells traditionally resistant to antineoplastic agents and irradiation). The hypothetical utility of agents of this type against hypoxic neoplastic cells is based upon the expectation of high reducing potential for these cells. The potential bioreductive quinone alkylating agent CMNQ, used in this investigation, is envisioned upon reduction to spontaneously generate a quinone methide alkylating species. Previous studies by this laboratory (16), using the related compound 2,3-dimethyl-5,6-bis(acetoxymethyl)-1,4-benzoquinone, have provided evidence for the generation of a quinone methide upon reduction of this agent in vitro with sodium borohydride. The data presented in Chart 9 of this investigation demonstrate that CMNQ is capable of being reduced by NADH via the respiratory chain and that its site of interaction is on the substrate side of rotenone inhibition. NADH-juglone reductase and NADH-ferricyanide reductase activities have similarly been shown to be insensitive to rotenone and piericidin A (19). An important difference between juglone and CMNQ is that the latter compound inhibits the respiratory chain following reduction, whereas juglone does not. These findings are consistent with the generation of a reactive inhibitory quinone methide.
from thymidine into its nucleotide forms was markedly decreased in cells treated with CMNQ (Chart 2). This inhibitory activity, as shown in Table 3, was partly due to a decrease in cellular thymidine kinase activity. That the depression in thymidine kinase activity of cells treated with CMNQ was not due to direct inhibition of the enzyme by this agent was suggested by the insensitivity of thymidine kinase in cell-free extracts to relatively high concentrations of CMNQ in the absence and presence of the reducing agent sodium borohydride. Thus, the decrease in cellular levels of thymidine kinase activity would appear to be due to either a decrease in synthesis or increase in catabolism of this enzyme. However, it is also conceivable that the conformation of thymidine kinase in situ differs from that in cell-free extracts in a manner that would render the catalyst susceptible to direct inhibition by CMNQ in its native intracellular state.

The depression of the conversion of thymidine to its nucleotide forms in Sarcoma 180 cells treated with CMNQ might also be in part the result of a drug-induced deficiency of ATP, since incubation of these cells in vitro with CMNQ produced a marked lowering of cellular levels of this nucleotide. The drug-induced decrease in the intracellular concentration of ATP is consistent with the findings presented in Chart 5, that the primary mitochondrial event produced by this agent is at the level of the coupling mechanism. The data, however, are not consistent with the view that CMNQ acts solely on mitochondria by release of respiratory control, because DNP, an established uncoupler of oxidative phosphorylation, inhibits thyroxine and phosphate-induced swelling of mitochondria when energy is derived from the respiratory chain but not when it comes from ATP, whereas CMNQ induces mitochondrial swelling under these conditions. The latter finding suggests that CMNQ may interact with the inner mitochondrial membrane.

The production of a new peak in the UV absorption spectrum of CMNQ at 242 nm, which is caused by its reduction by NADH in the presence of HBHM particles, was followed by inhibition of reduction (Chart 11, Curve B); this phenomenon is paralleled by similar stimulatory followed by inhibitory effects of CMNQ on respiration (Chart 5), mitochondrial swelling (Chart 8), and NADH oxidation (Chart 9), suggesting that these events are associated with the generation of a quinone methide that ultimately produces inhibition.

The finding that the related bioreductive alkylating agent mitomycin C did not affect intact Sarcoma 180 ascites cells, whereas CMNQ caused inhibition, indicates significant differences in the biochemical actions of these agents and encourages the further development of naphthoquinone bioreductive alkylating agents.

REFERENCES


The relatively wide spectrum of inhibitory effects produced by CMNQ in Sarcoma 180 ascites cells is also consistent with the concept that the compound is capable of generating an alkylating species. Thus, inhibition of the biosynthesis of DNA, RNA, and protein was produced by this agent, with DNA biosynthesis being the most susceptible of these processes to the action of this agent. Also consistent with the idea that this material was capable of alklylation is the finding that significant amounts of radioactivity from [14C]CMNQ was associated with these macromolecules for up to 72 hr after exposure of Sarcoma 180 cells to this agent.

The site of action of CMNQ on the DNA biosynthetic pathways appeared to be directed, at least in part, at the level of thymidine kinase. Thus, the passage of radioactivity

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Chart 10. UV absorption spectra of oxidized and reduced forms of CMNQ. UV spectra were determined on a Cary 14 dual-beam spectrophotometer. Spectrum A, obtained by adding CMNQ at a concentration of 50 μM to a solution containing 80 mM NaCl, 5 mM Tris-HCl (pH 7.4), 10 mM MgCl2, and 4.5 mM sodium phosphate (pH 7.4). The blank cuvet contained the same buffer. Spectrum B (reduced form of CMNQ), obtained under the same conditions, except that 0.5 μM sodium dithionite was added to both the sample and blank cuvets.

Chart 11. Bioreduction of CMNQ by a beef heart mitochondrial NADH-linked reaction. CMNQ reduction was followed by measuring the absorbance at 242 nm in a Cary 14 dual-beam spectrophotometer. Conditions were as described in "Materials and Methods." Curve A, NADH (0.167 mM); Curve B, NADH (0.167 mM) + CMNQ (20 μM).


Mode of Action of the Bioreductive Alkylating Agent, 2,3-Bis(chloromethyl)-1,4-naphthoquinone


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