Mutagenicity of Quindoxin, Its Metabolites, and Two Substituted Quinoxaline-Di-N-Oxides

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The quinoxaline-di-N-oxides carbadox, olaquindox, and quindoxin, which are potent antibacterial agents, were tested for mutagenicity in the Salmonella microsomal system. They all induced base pair substitutions and frameshift mutations in Salmonella, and occurred independently of the presence of a rat liver microsomal fraction in the test system. Mutagenicity was dependent on the presence of their N-oxide groups, since quinoxacline, a completely reduced derivative of quindoxin, was not mutagenic, whereas the partially reduced quinoxaline-N-oxide exhibited a lower mutagenic activity than quindoxin. recA and uvrB Salmonella were found to be more susceptible to mutagenic quinoxacline derivatives than wild-type strains. The mutagenicity of quinoxaline-di-N-oxides was enhanced under anaerobic incubation as was the antibacterial activity. These results suggest that both the antibacterial and mutagenic activity of quinoxaline-di-N-oxides depend upon the same bacterial activation mechanism.

Quinoxaline-di-N-oxides (QdNO's) are known as potent antibacterial agents which act on several gram-positive and -negative species (6, 7, 12). For this reason they have been used as growth promoters in agricultural stock farming (11, 13, 15). Two QdNO derivatives, carbadox and olaquinoxin, are currently added to pig and cattle feeds in several countries. A third substance, quindoxin, was withdrawn from commercial use several years ago.

The antibacterial effect of QdNO's has been the subject of various publications. The results indicate that QdNO's act as inhibitors of deoxyribonucleic acid (DNA) synthesis which subsequently results in DNA breakdown in bacteria (10, 24). Suter et al. (24) have investigated the mode of action of some QdNO's in more detail. They demonstrated that a bacterial enzyme, QdNO reductase, is needed for the activation of these compounds. Escherichia coli mutants deficient in the function of this enzyme exhibit a 30-fold-higher resistance to QdNO's than the isogenic wild-type strain. Furthermore, it is known that the bactericidal activity of QdNO action is drastically increased under anaerobic growth conditions since the presence of oxygen suppresses activation of these compounds (24).

These results indicate that reduction of QdNO's is needed for their activation as DNA attacking agents.

Two metabolites of bacterial quinoxin reduction, quinoxaline-N-oxide (QNO) and quinoxaline, have been identified that do not show any antibacterial activity (24). According to Suter et al. (24), an unstable radical is the active intermediate in the reduction of N-oxide groups. Electron spin resonance experiments demonstrated the formation of free radicals during QdNO reduction.

Recently, carbadox (21, 26), olaquindox, and quindoxin (29) were shown to be mutagenic in bacterial and yeast screening systems. The mutagenicity of these compounds was detected in the absence of an exogeneous mammalian enzyme activation system which is usually needed to activate a large number of indirect mutagens (2).

It seemed possible that both the antibacterial and the mutagenic activity of the QdNO's result from the same reduction process. To investigate this phenomenon, we compared the antibacterial and mutagenic properties of carbadox, olaquindox; and quindoxin as well as the two metabolites QNO and quinoxaline. The structure of the QdNO's and the metabolites are shown in Fig. 1.)

MATERIALS AND METHODS

Bacteria. All bacteria used in this study were derivatives of Salmonella typhimurium. The set of strains for mutagenicity testing (TA98, TA100,
TA1535, TA1537, and TA1538) and the strain TA1978 were obtained from B. N. Ames (University of Berkeley, Berkeley, Calif.) and maintained with routine testing as described (4). TA1537 and TA1538 were used for the detection of frameshift mutants, and TA1535 served for the identification of base pair substitutions. The derivatives of TA1538 and TA1535, i.e., TA98 and TA100, carry the "repair plasmid" pKM101, an R46 derivative which increases the susceptibility of its host to a number of mutagens (18). All TA tester strains used in this work carry a uvrB deletion and are deep rough (rfa). TA1978 is a uvr+ derivative of TA1538. The strains NK875 (recA) and NK995 (rec+) which were used in the repair test were kindly provided by N. Kleckner (Harvard University, Cambridge, Mass.).

Chemicals. Carbox (Fortigro, Mecadox; Pfizer) and olaquindox (Bayo-N-ox; Bayer) were obtained from the manufacturers. Quinoxalin (Grifas; Imperial Chemical Industries) was generously provided by W. Suter (Sandoz Ltd., Basel, Switzerland). QNO was from Ferak (Berlin, Federal Republic of Germany), and quinoxaline was from Aldrich. All quinoxaline derivatives were stored in the dark and protected from daylight to avoid photoactivation. The known mutagens which were used as routine controls in the mutagenicity tests were: 9-aminocacridine hydrochloride (Merck-Schuchardt), 2-aminofluorene, methyl methan sulfonate (Aldrich), and 4-nitroquinoline-N-oxide (Fluka). The substances were dissolved in dimethyl sulfoxide (Merck), except for 9-aminocacridine hydrochloride and low concentrations of olaquindox (0.1%), which were dissolved in double-distilled water.

Mutagenicity test. The Salmonella microsomal assay which was developed and extensively described by Ames et al. (4) was used with the following modifications: bacterial stock cultures were prepared by dilution of an overnight culture grown in L broth (16) with an equal amount of 87% sterile glycerol. These cultures were kept at −80°C and were stable for at least 18 months. Freshly grown late-exponential cells (1 × 10^6 to 2 × 10^8 bacteria per ml) were taken for each experiment. Difco media were used for the cultivation of bacteria.

The minimal plates for the mutagenicity test were poured as a 1:1 dilution of medium 56 (19), supplemented with glucose (0.2%) and thiamine (0.2 μg/ml), with 4% agar. The top agar (0.8% agar) was prepared as described (4) and supplemented with the same amounts of glucose and thiamine as the minimal plates.

For experiments with mammalian metabolic activation of the test compound, Aroclor 1254-induced rat liver homogenates (S-9) were obtained as deep frozen stocks from Litton Bionetics and kept at −80°C. The S-9 was supplemented for use with mineral salts, nicotine adenine dinucleotide phosphate, and glucose-6-phosphor (Merck) as originally described (4) as S-9 mix. For each plate, 0.5 ml of S-9 mix was added to 2.0 ml of molten top agar. If metabolic activation was not required, the S-9 mix was replaced with an equal amount of an adequate mix containing no S-9 fraction. The test compound solutions and the mix were always freshly prepared immediately before the experiment. The activity of the S-9 mix was routinely checked with strain TA98 and 2-aminofluorene since this substance needs activation to become mutagenic.

The spontaneous his+ revertant rates and the positive response with known mutagens (4-nitroquinoline-N-oxide for TA98, TA100, and TA1538; methyl methan sulfonate for TA1535; and 9-aminocacridine hydrochloride for TA1537) were routinely controlled. For the determination of mutagenicity, the numbers of revertants per nanomole of substance were compared as described (17).

Repair test. The repair test was performed as
described (3), except that rich media (L broth with 2% agar and L top agar [1%]) were used instead of minimal media. A 10-µl volume of test substance dissolved in dimethyl sulfoxide was spotted on a sterile filter disk (6-mm diameter) placed in the center of the agar plate. The zones of killing were measured after overnight incubation of the plates at 37°C.

**Determination of minimal inhibitory concentration.** The minimal inhibitory concentrations for the tested compounds were determined on L-plates containing serial twofold dilutions of the substance by using the method of Suter et al. (24). About 10^5 bacteria per plate were taken as inoculum and distributed with a loop.

**Anaerobic growth conditions.** For anaerobic growth, the plates were incubated in a GasPak 150 system (Becton Dickinson and Co., Cockeysville, Md.). After 16 h of anaerobic incubation at 37°C, the minimal plates were further incubated until colonies were visible (36 to 48 h later). The minimal plates for comparing anaerobic and anaerobic mutagenesis were additionally supplemented with biotin (2.5 µg/ml). This modification was necessary to avoid the diffusion of biotin from the top agar overlayer into the plates during the prolonged incubation period.

**RESULTS**

**Mutagenicity of the QdNO's carbadox, olaquindox, and quindoxin.** The *Salmonella* microsome assay described by Ames and co-workers (1, 4) was used to test for mutagenicity. The results obtained with the two strains carrying the repair plasmid pKM101 are presented in Fig. 2. All three QdNO's were identified as potent inducers of base pair substitutions (TA100) and frameshift mutations (TA98). A dose-response relationship for the increase of mutation rates was found with all substances tested. At higher doses than indicated in Fig. 2, bacterial growth on the plates was inhibited.

The induction of mutations occurred in the absence of a rat liver microsomal fraction (S-9 mix) in the test system. Olaquindox and carboxox were equally mutagenic whether S-9 mix was added to the plates or not. The mutagenic activity of quindoxin was slightly enhanced by the addition of S-9 mix.

The results in Fig. 2 indicate that carbadox was the most efficient mutagen of the three tested. However, olaquindox was only 3-fold less mutagenic per nanomole, and quindoxin was 18-fold less active than carbadox. The mutagenic threshold for each substance and the strain TA100 was calculated as the concentration which resulted in doubling the spontaneous revertant rate. This was 5 nmol per plate for carbadox, 12 nmol per plate for olaquindox, and 50 nmol per plate for quindoxin.

In addition to TA98 and TA100, their parental strains TA1538 and TA1535 and the strain TA1537 were used in the mutagenicity screening. TA1537 was found to be as susceptible as TA98 for the detection of frameshift mutations induced by the tested QdNO's, but TA1538 and TA1535 exhibited only a low susceptibility to these compounds (data not shown). The addition of S-9 mix to the plates did not enhance the response with these tester strains.

**Mutagenicity of the reduced quindoxin metabolites quinoxaline and QNO.** The results obtained when quinoxaline was tested with TA98 and TA100 are summarized in Table 1. No mutagenic activity of quinoxaline could be detected up to 30.7 µmol per plate. This result was also obtained with strains TA1535, TA1537, and TA1538. Higher concentrations than indicated in Table 1 were too toxic for the bacteria to be tested. Similar results were obtained when the system was supplemented with rat liver S-9 mix. Therefore, quinoxaline did not exhibit any mutagenic activity in the *Salmonella* system.

Figure 3 presents the results of QNO tested with TA98 and TA100. QNO did induce base pair substitutions and frameshift mutations but with lower efficiency than the three QdNO's. The data shown in Fig. 3, QNO was found to be 18-fold less mutagenic than quindoxin. The lowest detectable mutagenic dosage for TA100 with QNO was at 1.4 µmol per plate. Addition of S-9 mix to the test system led to an increase of induced mutations at higher QNO concentrations. This effect was not further investigated.

The mutagenicity of QNO could also be detected with the strains TA1537, TA1535, and TA1538. TA1537 was as susceptible as TA98 to the induction of frameshift mutations by QNO. The strains TA1535 and TA1538 exhibited a significant response with QNO, but were much less susceptible than their pKM101-carrying derivatives (data not shown).

**Susceptibility of *Salmonella* repair-deficient strains to QdNO's and quindoxin metabolites.** It is known that DNA repair-deficient bacteria show an increased susceptibility to DNA-damaging agents. Consequently, susceptibility tests with recA, polA, and uvrB mutants have been introduced as a tool in mutagen screening (3, 14, 23).

We used a simple repair test to demonstrate the response of repair-deficient *Salmonella* to the QdNO's and quindoxin metabolites investigated in this study. The results of these tests are presented in Table 2. All compounds which were mutagenic had an enhanced effect on recA and uvrB mutants. QNO affected only the repair-deficient strains but not the wild types, whereas none of these strains was susceptible to quinoxaline (100 µg). When higher amounts of quinox-
Fig. 2. Mutagenicity of carbadox (A), olaquindox (B), and quindoxin (C) with S. typhimurium TA98 (●) and TA100 (○) with (−−−) and without (−) metabolic activation. The numbers of spontaneous his<sup>+</sup> revertants have been subtracted from the numbers of his<sup>+</sup> colonies plotted against the amount of test compound per plate.

aline (1 mg) were tested the mutant and wild-type strains were found to be equally susceptible. This finding corresponds with the negative result of the mutagenicity test with quinoxaline.

Comparison of mutagenicity with the antibacterial activity of carbadox, olaquindox, and quindoxin. The antibacterial activity of carbadox, olaquindox, and quindoxin was
The tests studied an summarized in (uvrB) TA1538 (uvrB, pKM101"). Quantitative results were obtained by the determination of minimal inhibitory concentration. The tests were performed under aerobic and anaerobic growth of bacteria; the results are summarized in Table 3. The uvrB mutants exhibited an increased susceptibility to the QdNO’s compared to the wild-type strain TA1978. The presence of the plasmid pKM101 in TA98 and TA100 which codes for an “error-prone” repair system (20) diminished the susceptibility of its uvrB host but did not restore the wild-type resistance level.

As previously observed with E. coli, the antibacterial activity of the tested QdNO’s was enhanced under anaerobic conditions. When the aerobic minimal inhibitory, concentrations of the QdNO’s for TA98 (TA100) were compared, carbadox was found to be 4-fold stronger than olaquindox and 13-fold stronger than quindoxin.

**Table 1. Mutagenicity test with quinoxaline and the strains TA98 and TA100 without metabolic activation (S-9 mix)**

<table>
<thead>
<tr>
<th>Quinoxaline (μmol per plate)</th>
<th>his⁺ revertants per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA98</td>
</tr>
<tr>
<td>0</td>
<td>15.8 ± 2.9*a</td>
</tr>
<tr>
<td>0.07</td>
<td>15.1 ± 3.5</td>
</tr>
<tr>
<td>0.7</td>
<td>14.0 ± 4.1</td>
</tr>
<tr>
<td>3.8</td>
<td>15.7 ± 6.4</td>
</tr>
<tr>
<td>7.6</td>
<td>12.6 ± 4.5</td>
</tr>
<tr>
<td>15.4</td>
<td>11.5 ± 1.8</td>
</tr>
<tr>
<td>30.7</td>
<td>9.3 ± 3.2</td>
</tr>
</tbody>
</table>

*a Average number of colonies per plate ± standard deviation.

In its antibacterial activity. These findings paralleled the results obtained in the mutagenicity tests where carbadox was identified as the most efficient mutagen followed by olaquindox and quindoxin. However, under anaerobic incubation, TA98 (TA100) became 30-fold more susceptible to carbadox and quindoxin but only 8-fold more susceptible to olaquindox than under aerobic growth.

To study the influence of oxygen limitation on the mutagenic activity of these QdNO’s, anaerobic and aerobic mutagenesis was compared in strain TA100. The results of these tests are summarized in Fig. 4. Also under anaerobic test conditions, carbadox represented the strongest mutagen among the three QdNO’s. Its mutagenic activity was about sevenfold higher compared to the aerobic mutagenicity test. The mutagenic effect of olaquindox, however, was not drastically stimulated (1.3-fold), whereas quindoxin was about 13-fold enhanced in its mutagenic activity than under aerobic conditions. In accordance with the highly increased antibacterial activity of carbadox and quindoxin under oxygen limitation, the toxicity of these compounds in the anaerobic mutagenicity test was markedly increased. Therefore, higher amounts than 3.8 nmol per plate for carbadox and 30.8 nmol per plate for quindoxin could not be tested.

When anaerobic and aerobic conditions were compared, a strong relationship between antibacterial and mutagenic activity of the QdNO’s was found. Both activities could not be separated.

**DISCUSSION**

It has been demonstrated in this study that
the QdNO's carbadox, olaquindox, and quinoxalin act as efficient mutagens in the Salmonella test system. Previous results (24) have shown that bacterial DNA is attacked directly by activated QdNO. This process leads to the destruction of the DNA template and inhibition of DNA synthesis. According to the mode of action of QdNO's, repair-deficient Bacillus subtilis (21) and E. coli (24, 26) were found to be more susceptible to these compounds than wild-type strains. In the present study, similar results were obtained with uvrB and recA Salmonella mutants.

With quinoxalin and its metabolites QNO and quinoxalin, a striking dependence on the presence of N-oxide groups for mutagenicity was found. This result corresponded with the antibacterial activity of QNO. The lack of this activity in the study of Suter et al. (24) may be explained by the low concentration of QNO (62 × 10⁻⁴ M) tested on a wild-type strain. In the repair test presented here, even high amounts of QNO (100 μg per plate) did not inhibit the growth of wild type but did with repair-deficient bacteria. The reason for the low mutagenic activity of QNO compared to quinoxalin is not known. The finding of Suter (W. Suter, Ph.D. thesis, University of Zurich, Zurich, Switzerland, 1977) that reduction of QNO to quinoxalin is not as efficient as the reduction of QdNO to QNO in bacteria could explain this result. It is also possible that the activated form of QNO is less mutagenic than the activated form of quinoxalin.

The three QdNO's tested in this study exhibited significant differences in their mutagenic potency. Suter et al. (24; Suter, Ph.D. thesis) reported that carbadox is more rapidly reduced by bacteria than olaquindox or quinoxalin. This corresponds to the finding presented here that the activity of carbadox in the mutagenicity and minimal inhibitory concentration assay was higher than that of the other two QdNO's. Therefore the increased efficiency of carbadox compared to olaquindox and quinoxalin can be related to a better activation of this compound by bacterial reductases. It is known that oxygen suppresses the activity of QdNO reductase (Suter, Ph.D. thesis). This may explain the intensified activity of the QdNO's under anaerobic conditions. The antibacterial and mutagenic activity of the QdNO's was increased under anaerobic incubation with bacteria. The results presented here suggest that the same bacterial ac-

<table>
<thead>
<tr>
<th>Test compound</th>
<th>MIC (nmol/ml) for strain</th>
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<tbody>
<tr>
<td></td>
<td>TA1978 (uvr⁺)</td>
</tr>
<tr>
<td>Carbadox</td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>14.3</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0.9</td>
</tr>
<tr>
<td>Olaquindox</td>
<td>57</td>
</tr>
<tr>
<td>Aerobic</td>
<td>14.2</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>11.6</td>
</tr>
<tr>
<td>Quinoxalin</td>
<td>185</td>
</tr>
<tr>
<td>Aerobic</td>
<td>11.6</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>11.6</td>
</tr>
</tbody>
</table>

a Strain TA100 shows adequate results as TA98.

The QdNO's bacterial activity was tested with N-oxide groups for mutagenicity. Previous results (24) have shown that bacterial DNA is attacked directly by activated QdNO. This process leads to the destruction of the DNA template and inhibition of DNA synthesis. According to the mode of action of QdNO's, repair-deficient Bacillus subtilis (21) and E. coli (24, 26) were found to be more susceptible to these compounds than wild-type strains. In the present study, similar results were obtained with uvrB and recA Salmonella mutants.

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**TABLE 2. Repair tests with uvrB and recA S. typhimurium strains**

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Conc (μg per diak)</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide</td>
<td><em>a</em></td>
<td>TA1978 (uvr⁺) TA1538 (uvrB) NK995 (rec⁺) NK875 (recA)</td>
</tr>
<tr>
<td>Crystal violet</td>
<td>10</td>
<td>&lt;6 &lt;6 &lt;6 &lt;6</td>
</tr>
<tr>
<td>4-NNO ⁴</td>
<td>10</td>
<td>21 20 12 13</td>
</tr>
<tr>
<td>Carbadox</td>
<td>100</td>
<td>22 28 16 32</td>
</tr>
<tr>
<td>Olaquindox</td>
<td>100</td>
<td>28 32 26 34</td>
</tr>
<tr>
<td>Quinoxalin</td>
<td>100</td>
<td>25 31 17 36</td>
</tr>
<tr>
<td>QNO</td>
<td>100</td>
<td>&lt;6 15 &lt;6 15</td>
</tr>
<tr>
<td>Quinoxaline</td>
<td>100</td>
<td>&lt;6 &lt;6 &lt;6 &lt;6</td>
</tr>
<tr>
<td>Quinoxaline</td>
<td>1,000</td>
<td>11 11 7 7</td>
</tr>
</tbody>
</table>

a —, 10 μl of dimethyl sulfoxide per plate.

b Zones of inhibition smaller than the diameter of the filter disk (6 mm) could not be detected and are designated as <6.

c Strains TA1978 and TA1538 show an increased susceptibility to crystal violet and other substances because of their lipopolysaccharide defect (3).

d-4-NNO, 4-Nitroquinoline-N-oxide.

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**TABLE 3. Minimal inhibitory concentration (MIC) for carbadox, olaquindox, and quinoxalin**

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<td>Quinoxalin</td>
<td>185</td>
</tr>
</tbody>
</table>

a Strain TA100 shows adequate results as TA98.
tivation mechanism is responsible for both activities.

For a number of mutagens, deactivation mediated by mammalian liver enzymes has been described (5, 9). We have shown in this work that simultaneous incubation of bacteria with S-9 mix did not result in deactivation of the tested QdNO's. However, a QdNO reductase-like activity has been detected in rat liver cell extracts (Suter, Ph.D. thesis). Ohta et al. (21) found deactivation of carbadox when it was preincubated with rat liver S-9 mix and tested thereafter on TA100. Therefore it seems possible that the QdNO's are metabolized by rat liver cells via activation of these compounds analogous to the bacterial metabolism. This would explain the lack of effect on QdNO in a system containing both S-9 mix and bacteria.

If there is an analogous QdNO reductase system in mammalian cells, the question arises whether these substances represent potential hazards for human and animal health. Until now no results were available on the mutagenicity of QdNO's in mammalian in vivo and in vitro systems. In a eucaryote system using mitotic gene conversion in yeast, a positive response with carbadox, olaquindox, and quindoxin was obtained (26).

Mutagenic compounds are suspected as potential carcinogens (17, 22). Only quindoxin has been further investigated and detected as a carcinogen in rats (25). It has also been reported that this substance causes contact eczema in humans (8).

Considering the identical mode of action of quindoxin and the other two QdNO's investigated in this study, further mutagenicity and carcinogenicity tests with carbadox and olaquindox should be carried out. This may be of special importance in view of the large quantities of these compounds produced for use in animal nutrition.

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LITERATURE CITED


