Comparison of the Structural and Cytotoxic Activity of Novel 2,5-Bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone Analogues

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ABSTRACT

Eight analogues of 2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone have been synthesized and tested for cytotoxicity against four different leukemic and lymphomic cell lines. For K562 and BSM cells, the toxicity could be correlated with the ease of reduction of the compounds as determined by the one-electron reduction potentials and the electron spin resonance detection of the reduced compounds produced by the cells. The cell toxicity could also be correlated with the efficiency of the compounds to form cross-links in DNA. However, no such correlations could be observed for the L1210 and Raji cells, although the activity of the NADPH dependent reducing enzymes in these cells was similar to that in the others. It is believed that for the L1210 and Raji cells, the influx/efflux of the different compounds may be more important to the cytotoxicity than their reduction or alkylation.

INTRODUCTION

Bioreductive activation is a process in which compounds are reduced by cellular enzymes to produce more active forms. Several antitumor quinones including Adriamycin, mitomycin C, and aziridinyl benzoquinones have the potential to be activated by this process (e.g., Refs. 1 and 2). However, despite hundreds of publications, it has been difficult to prove the role of the antitumor activity of these quinones or in the production of toxic side effects.

AZQ2 (D2 in Fig. 1), has undergone Phase I, II, and III trials and shows activity in progressive glioma of the central nervous system as well as variable activity in other central nervous system tumors (3, 4). It was originally developed in an effort to find a drug which can cross the blood-brain barrier and which also has the ability to undergo alkylation reactions via activation of the aziridine moieties. This drug has been studied extensively from the point of view of bioreductive activation inasmuch as the quinone moiety is readily reduced to relatively stable semi-quinone/hydroquinone species (5, 6). Recent results from our laboratory have shown that although AZQ is taken up slowly into certain cells, it readily forms semiquinone free radicals which appear in the medium, presumably by a controlled influx/efflux mechanism (7).

It is surprising that even though several diaziridinyl benzoquinones have been tested in vitro (e.g., Ref. 8), AZQ is the only carbamoyl ester analogue that has undergone cell toxicity testing. We have recently synthesized several AZQ analogues using novel techniques (9). This work has now been extended to increase this range and to report on the cytotoxicities of all of these compounds and their uses in studying bioreductive activation.
The plates were then removed and 50 μl of a 3-mg/ml solution were then put into 3 wells of a 96-well microtiter plate and then incubated at 37°C in an atmosphere of 95% air/5% CO2 for 5 to 7 days. The plates were then removed and 50 μl of a 3-mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide in phosphate buffered saline was added to each well and then incubated for 3 h. The medium from each well was then aspirated and 200 μl of DMSO were added. The plates were agitated and then read on the multiscan using a wavelength of 540 nm. Growth inhibition curves were constructed using the mean and standard deviation of the triplicate values. From these curves, the concentration of drug necessary to inhibit cell growth to 50% of the control was determined for each drug.

One-Electron Reduction Potentials

The one-electron reduction potentials of the drugs at pH 7 were measured using the pulse radiolysis apparatus at the Paterson Institute. The system consisted of a tungsten analyzing lamp and a Kratos UV/VIS multichromator. Microcells with an absorbance path length of 2.5 cm were used throughout. The method of measuring one-electron reduction potentials is similar to that previously reported (5). Essentially the drug radicals, which are rapidly generated by the pulse technique, are allowed to come into equilibrium with compounds of known reduction potentials. The equilibrium constant is measured before the decay of the radicals and from these values, the reduction potential can be determined.

DNA Cross-Linking Assay

The fluorescence assay used to determine DNA cross-linking was similar to that reported previously (12, 13) with some slight modifications. This assay relies on the fact that heat denatured DNA shows a loss in fluorescence due to the release of ethidium bromide from the double strands. However, if a drug covalently cross-links the DNA strands, then heat denaturation is prevented. The investigation of the cross-linking was determined at pH 4 because it has been shown that the reaction of reduced AZQ with DNA is pH dependent (7). The interactions at more neutral pH values are extremely weak and are barely detectable by this method. The incubation mixture consisted of 20 mM TRIS buffer, 200 mM potassium phosphate buffer (pH 7.4), 2 mM ascorbic acid, and 100 μg DNA ( calf thymus; Sigma) in a total volume of 3.0 ml. This mixture was incubated for 40 min in a sealed tube at 37 ± 1°C. This incubation time was chosen as a result of previous experiments and represents the time when the interaction between reduced AZQ and DNA is at a maximum while there is minimal contribution from acid assisted ring opening (5). On completion of the incubation time, the pH was adjusted to 7.0 with phosphate buffer (200 mM, pH 9). DNA (5 μg) was removed and added to a solution containing ethidium bromide (0.5 μg/ml), tripotassium phosphate (20 mM, pH 11.8), and EDTA (0.5 mM). The difference in the fluorescence readings before and after heating were then determined as described previously (7) using an excitation wavelength of 525 nm and an emission wavelength of 580 nm.

NADPH-requiring Enzymes

The relative activities of the reducing enzyme systems which require NADPH in the different cell lines were determined using a modification to the previous method (14). The cells were washed/resuspended in phosphate buffer and then sonicated using an MSE ultrasonicator for three periods of 10 s. Cellular protein was measured using the Bio-Rad assay. The extracts (40 μg protein/ml) were then incubated at 37°C with 1 mM NADPH and 35 μM acetylated ferricytochrome c in a total volume of 3.0 ml (50 mM potassium phosphate buffer, pH 7.5). The rate of reduction of the acetylated cytochrome c was determined by the increase in absorption at 550 nm. The results from the different cell lines are summarized in Table 2.

ESR

Spectra were obtained at an incident microwave power of 10 mW. The magnetic field was modulated at 100 kHz with an amplitude of 0.8 G. Identical instrument settings were used throughout the study. All spectra were obtained in an aqueous flat cell. Aliquots of drug (0.5–5 mM) were incubated with a sample of cell suspension (10⁴–10⁵ cells/ml) in supplemented RPMI, as with the cell toxicity studies for 1 h at 37°C prior to measurement (14).

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RESULTS

The measured cell toxicities of the individual compounds are shown in Fig. 2 in the form of the inverse of the concentration of drug necessary to inhibit cell growth to 50% of control. The K562 and the BSM cell lines are more responsive to the compounds and show a definite trend in the activities. The D1 compound is the most toxic while there is a distinct decrease in toxicity down to D7. The derivatives D8 and D9 then show relative increases in toxicities.

The one-electron reduction potentials at pH 7 of the different compounds and the standards used are shown in Table 1. The errors in the determinations are mainly due to the relatively slow rates of equilibria between some of the compounds and the standards.

The intensity of the ESR signals from D1, D2, and D3 incubated with the cells showed a trend in the order BSM > K562 > L1210 > Raji. Whereas large, distinct ESR signals could be detected from the BSM and K562 cell lines, only small signals could be detected in the Raji cells even after extensive signal averaging. However, when the whole cells were disrupted by sonication, the signals obtained were substantially increased in intensity. These results are summarized in Fig. 3 for the parent drug, AZQ.

A comparison of the signals from all of the compounds was made with K562 and BSM intact cell suspensions. The intensity of the signals followed the order D1 > D2 > D3 > D4 with no detectable signals for the D5, D6, and D7 compounds. The asymmetrical compounds, D8 and D9, gave well defined ESR signals of intensity similar to those of the D1 and D2 compounds. The results are illustrated in Fig. 4.

The results from the cross-linking of DNA by the reduced compounds D1 to D9 are summarized in Fig. 5. In Fig. 5, D1 was regarded as 100% cross-linked and all of the other compounds are normalized to this value.

DISCUSSION

The efficiency of a quinone to undergo bioreductive activation and function as an alkylating agent should depend on the ease at which the quinone can be reduced by the intracellular enzymes and the stability of the reduced forms of the quinone in the presence of oxygen. We have previously demonstrated that the rate at which an enzyme system reduces a quinone strongly depends on the one-electron reduction potential of the Q/Q+ couple (5). The one-electron reduction potentials in Table 1 would predict that whereas most of the compounds should be easily reduced, the D6 and D7 compounds are relatively more difficult. Also, because the reduction potential of the O2/O2- couple is ~155 mV, the reduced forms of the D8 and D9 compounds should be less stable in the presence of oxygen than the other compounds. Thus, if alkylation of important cellular targets and hence prevention of replication occurs only when the compounds are in a reduced state, then the toxicities should be dependent on the reduction potentials. This is indeed the approximate order of toxicities observed for the K562 and BSM cell lines (Fig. 2).

The ESR experiments also served to qualitatively confirm the above trend. Signals could be detected for the D1 to D4 and the D8 and D9 compounds in the K562 and BSM cells, showing...
Table 1  One-electron reduction potential values for the series D, to D9

<table>
<thead>
<tr>
<th>D</th>
<th>E_r/mV</th>
<th>Standards used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-44.2 ± 5°</td>
<td>Oxygen</td>
</tr>
<tr>
<td>2</td>
<td>-61.0 ± 15</td>
<td>Oxygen, naphthazarin</td>
</tr>
<tr>
<td>3</td>
<td>-67.0 ± 3</td>
<td>Oxygen</td>
</tr>
<tr>
<td>4</td>
<td>-78.7 ± 9</td>
<td>Oxygen</td>
</tr>
<tr>
<td>5</td>
<td>-65.9 ± 15</td>
<td>Oxygen, naphthazarin</td>
</tr>
<tr>
<td>6</td>
<td>-167.2 ± 20</td>
<td>Oxygen, menadione</td>
</tr>
<tr>
<td>7</td>
<td>-125.2 ± 30</td>
<td>Oxygen, menadione</td>
</tr>
<tr>
<td>8</td>
<td>-61.9 ± 4</td>
<td>Oxygen</td>
</tr>
<tr>
<td>9</td>
<td>-80.6 ± 2</td>
<td>Oxygen</td>
</tr>
</tbody>
</table>

* Mean ± SD.

Fig. 3. Comparison of the intensity of the ESR signals from AZQ (D2) incubated with the four cell lines, a, BSM; b, K562; c, L1210; d, RAJI; and e, RAJ1 sonicated. The cell numbers used for all cell types were 5 x 10^6 and the experiments were carried out aerobically in flat cells, taking due care to ensure that the contents did not settle. Signals were recorded over identical time periods and averaged 10 times.

that they are reduced to semiquinone radicals. However, no discernible signals could be detected for the other compounds indicating that they are not efficiently reduced in the cells and/or they are unstable with respect to reoxidation by oxygen. Several preliminary experiments using K562 and BSM cells under hypoxic conditions showed that there was a small increase in the size of the ESR signals from these drugs which might imply in the first instance that reoxidation does occur in the presence of oxygen. However, it is also possible that the redox status of the cell can also change under conditions of hypoxia.

It has been demonstrated using alkaline elution techniques that the DNA interstrand cross-linking ability of AZQ can be correlated with the measured toxicities in several human cell lines (15). The DNA cross-linking results from the present study show that some of the compounds in the reduced state are very efficient at cross-linking DNA. The extent of reduction within the cells is expected to be an important factor.

Fig. 4. ESR signals obtained from incubating some of the compounds with K562 cells. Conditions were as described in Fig. 3.
which of the two factors, steric hinderence or reduction poten
crast decrease. However, there is
to determine which of the two factors, steric hinderence or reduction poten-
tial, has the most important influence on cell toxicity because they are both interrelated. The reduction potentials of the
analogues will be dependent on the structure of the carbamoyl
esters side chains and it is to be expected that as the side chains
become more bulky then the reduction potentials should de-
crease.

The L1210 and the Raji cells were not as sensitive towards the
compounds as the K562 or BSM cell lines. The one-line
signals observed in Fig. 3, c and d, are typical ESR signals
obtained from signal saturation, in this case, due to power and
possibly modulation broadening. The ESR signals were also much weaker in the intact L1210 and Raji cells which might at
first suggest that the ability of these cell lines to reduce the
compounds to reactive forms is decreased. However, there is
some evidence from this study which shows that this is not
necessarily the case. The activities of the NADPH requiring
reducing enzymes have been found to be similar in all of the
cell lines tested (Table 2) and it is generally believed that the
NADPH requiring systems and in particular, NADPH-cyto-

However, the relative efficiency of cross-linking DNA should
also depend on the structure of the compounds. It is expected
that the more bulky carbamoyl esters in the series and in
particular the D₆ and D₇ compounds will experience more steric
restraints than the others. However, it is difficult to determine
which of the two factors, steric hinderence or reduction potential,
has the most important influence on cell toxicity because they are both interrelated. The reduction potentials of the
analogue will be dependent on the structure of the carbamoyl
ester side chains and it is to be expected that as the side chains
become more bulky then the reduction potentials should de-
crease.

Table 2 NADPH dependent reducing ability of the four cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Rate of reduction* (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSM</td>
<td>8.8 ± 0.5*</td>
</tr>
<tr>
<td>K562</td>
<td>7.3 ± 0.05</td>
</tr>
<tr>
<td>L1210</td>
<td>6.7 ± 0.35</td>
</tr>
<tr>
<td>Raji</td>
<td>8.3 ± 1.1</td>
</tr>
</tbody>
</table>

* Mean of two determinations.
* Mean ± SD.

REFERENCES


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