ABSTRACT

We investigated the in vitro interaction with and antitumor effect on several murine and human leukemic cell lines of diaziquone (AZQ). L1210 cells accumulated AZQ from Roswell Park Memorial Institute Medium 1640 with or without newborn calf serum by a temperature-dependent and sodium azide-resistant process. AZQ inhibited, in a dose-dependent fashion, [3H]thymidine incorporation into L1210 cells, but this inhibition was slow to develop, requiring approximately 6 hr to become apparent. The minimal inhibitory concentration of AZQ for this process was 0.05 to 0.25 nmol/ml. AZQ was a much less effective inhibitor of L1210 cell [3H]thymidine and [3H]cytosine incorporation in suspension cultures, AZQ inhibited growth of L1210 and HL-60 cells at minimal inhibitory concentrations of 0.5 to 1 nmol/ml. In soft agar cultures, AZQ inhibited HL-60 cell cloning at minimal inhibitory concentrations of 0.1 to 0.3 nmol/ml. AZQ provoked a dose-dependent increase in oxygen consumption when added to intact L1210, HL-60, and K562 cells and was converted to an AZQ anion free radical by these cells. When the aziridine rings of AZQ were opened by acid treatment, the resulting molecule was not accumulated by L1210 cells, did not provoke O2 consumption, did not form free radicals when added to L1210 cells, and was a much less effective inhibitor of [3H]thymidine incorporation by L1210 cells than was AZQ.

INTRODUCTION

AZQ (Chart 1) is a diaziridinyl benzoquinone, one of a group of compounds whose antitumor activity has been known for years (10, 22, 28–30, 33, 35). The exact structure of AZQ was rationally conceived in an effort to create, from a class of compounds with known activity, a drug which also possessed chemical characteristics that would allow it to cross the blood-brain barrier (6, 9, 25). To date, clinical trials have documented AZQ's activity against CNS and other neoplasms (1, 4, 5, 8, 15, 19, 23, 31, 37, 38, 40–42). Clinical and animal pharmacology studies have documented AZQ's plasma, tissue, and CNS pharmacokinetics (2, 36, 39). Still, there is a paucity of data regarding the in vitro activity and mechanism of action of AZQ (32).

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2 To whom requests for reprints should be addressed, at Division of Developmental Therapeutics, University of Maryland Cancer Center, 655 West Baltimore Street, Baltimore, MD 21201.

3 The abbreviations used are: AZQ, diaziquone; CNS, central nervous system; TLC, thin-layer chromatography; ROQ, aziridine ring open quinone product resulting from acid hydrolysis of diaziquone; DMA, dimethyl acetamide; HPLC, high-performance liquid chromatography; ESR, electron spin resonance.

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RESULTS

Inhibition of Cell Growth. Under the conditions used in growth curve experiments, L1210 cells exhibited a short lag period and then grew in exponential fashion for at least 96 hr (Chart 2A). AZQ not only reduced the rate of L1210 cell growth but did so in a dose-dependent fashion (Chart 2A). Although the inhibition of AZQ was apparent as early as 24 hr, evidence of the dose-dependent nature required several doubling times and, therefore, was not observed until 48 hr, after which it became progressively more evident. In control cultures, DMA, used at concentrations obligatorily included in AZQ experiments, had no effect on L1210 cell growth until the small inhibition observed at a concentration of 0.1%, i.e., that DMA concentration associated with AZQ, 10 nmol/ml. The dose-dependent nature of the growth inhibition of AZQ was better assessed when the cell concentrations achieved in AZQ-treated cultures after 72 and 96 hr of incubation were expressed as percentages of those in additive-free control cultures incubated for the same length of time. There was no inhibition of L1210 cell growth at AZQ concentrations less than 0.3 nmol/ml.

Under the culture conditions used, HL-60 cells grew somewhat more slowly than did L1210 cells but still grew exponentially for at least 96 hr (Chart 2B). As with L1210 cells, AZQ produced a dose-dependent decrease in HL-60 cell growth (Chart 2B). In these experiments, HL-60 cells proved about as sensitive to AZQ as were L1210 cells but were more resistant to the effect of DMA than were L1210 cells.

Clonogenic Assays. As with cell growth, AZQ inhibited HL-60 cell cloning in a dose-dependent fashion (Chart 3), and, as with cell growth, 0.3 nmol/ml was the lowest studied AZQ concentration at which cellular growth-inhibitory effects were seen. As with HL-60 cell growth, DMA had no effect on HL-60 cell cloning (Chart 3).

Radio-labeled Macromolecular Precursor Incorporation. AZQ inhibited [3H]thymidine incorporation by L1210 cells more than it did incorporation of [3H]uridine and [3H]valine (Chart 4, A and B). There were several notable aspects of this inhibition. As with the inhibition of cell growth and cloning of AZQ, the drug reduced [3H]thymidine incorporation in a dose-dependent fashion, with the first decrease noted at AZQ concentrations between 0.1 and 0.5 nmol/ml. Although AZQ inhibited [3H]thymidine incorporation, the onset of this inhibition was slow and continued to increase for at least 24 hr after addition of AZQ. AZQ had much less effect on [3H]uridine incorporation by L1210 cells, and, as with [3H]thymidine incorporation, this effect was delayed in onset (Chart 4B). No reduction of [3H]uridine incorporation was observed with AZQ concentrations less than 1 nmol/ml. Concentrations of AZQ as high as 5 nmol/ml had no effect on

Oxygen Consumption and Free Radical Generation. L1210 cells were washed twice in calcium- and magnesium-free Hank's balanced salt solution before being resuspended to 10^7/ml in the same medium. One ml of cell suspension was aerated in the cuvet of a Model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH). Endogenous cellular oxygen consumption at 22° was monitored for 5 min with a Clark-type electrode before drug was added to final concentrations of 10 μM to 1 mM, and drug-induced oxygen consumption was assessed (3).

ESR measurements were made at 22° in an X-band (9.3 GHz) Varian E-line Century Series ESR spectrometer (Varian, Palo Alto, CA) equipped with 100 kHz field modulation. A dual cavity (TE-104) was used with a strong pitch standard (g = 2.0028) in one section and the sample in a flat ESR cell in the other. The strong pitch marker was used to estimate g values.

ASSAY OF DRUG UPTAKE. L1210 cells were washed twice with 0.154 M NaCl and resuspended in fresh Medium A containing 15% newborn calf serum, and 10^7 cells were preincubated for 60 min before addition of drug. The final incubation volume was 1 ml, and all incubations were performed at 37°, in an atmosphere containing 5% CO₂ and 95% humidity. After incubation for specified periods of time with AZQ or DMA, cells were pulsed with 0.1 ml of medium containing either 1 μCi of methyl-[3H]thymidine (2.0 Ci/mmol; New England Nuclear). After incubation for 60 min with radiolabeled material, cells were assayed for incorporation of radioactivity into trichloroacetic acid-precipitable material, as described previously (14).

Assay of Drug Uptake. L1210 cells were washed twice with 0.154 mM NaCl and resuspended in fresh Medium A, and 10^7 cells were preincubated in 16- x 150-mm glass test tubes for 60 min prior to addition of [14C]AZQ. The final incubation volume was 1 ml, and, unless indicated, all incubations were performed at 37° in an atmosphere containing 5% CO₂ and 95% humidity. After incubation, triplicate sets of tubes were centrifuged at 2500 x g for 10 min at 4°, the supernatant was discarded, and radioactivity remaining with the cells was counted.

Efflux Experiments. After incubation with [14C]AZQ (5 nmol/ml) for up to 180 min, L1210 cells were sedimented by centrifugation for 10 min at 2500 x g. The supernatant was discarded, and the cells were resuspended and incubated in 10 ml of fresh, drug-free medium. At specified times after resuspension, cells were again sedimented, the supernatant was discarded, and radioactivity remaining with the cells was counted.

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Chart 2. Growth of L1210 (A) or HL-60 (B) cells incubated with specified concentrations of AZQ as described in "Materials and Methods." Points represent the mean of 5 experiments done in triplicate with L1210 cells and 4 experiments done in triplicate with HL-60 cells; Bars have been omitted for clarity. S.E. was ≤25% in all cases.

Chart 3. Inhibition of cloning of HL-60 cells by AZQ. HL-60 cells were incubated in 0.3% agar with specified concentrations of AZQ or DMA as described in "Materials and Methods." Points, mean of a representative triplicate experiment; bars, S.E.

Radiolabeled valine incorporation into trichloracetic acid-precipitable material (data not shown). DMA, at concentrations obligatorily added with AZQ, had no effect on incorporation of any macromolecular precursor by L1210 cells.

ROQ, the molecule produced by opening of the aziridine rings of AZQ with acid treatment, had greatly reduced ability to inhibit [3H]thymidine incorporation by L1210 cells.

AZQ Accumulation and Efflux from Cells. In initial experiments in which 10⁶ L1210 cells were incubated with 1 (2.3 x 10⁴ dpm) or 10 (2.3 x 10⁵ dpm) nmol of [14C]AZQ, there was no accumulation of radioactivity above the background remaining in the tubes. In all experiments, this background radioactivity was approximately 1% of the total number of dpm added at the start of the experiment. This difficulty was overcome by increasing the number of cells in each tube to 10⁷. This decision was based on the realization that the volume of 10⁶ cells was approximately 1 μl, and the overall incubation volume was 1 ml. Therefore, achievement of a cellular concentration of radioactivity 10-fold greater than that in the medium would result in a number of dpm associated with the cell mass that would only approximate background, and that the way to optimize measurement of intracellular ¹⁴C was to increase the size of the cell mass to be measured.

When 10⁷ L1210 cells were incubated with [¹⁴C]AZQ, they accumulated radioactivity, a process which appeared to slow after approximately 5 hr (Chart 5). The accumulation of radioactivity by cells incubated with [¹⁴C]AZQ was the same in medium with or without newborn calf serum (data not shown). Cellular accumulation of radioactivity was reduced to the same degree by both cooling (4°) and inclusion of 5 mM iodoacetate in the incubation medium (Chart 5). Cellular accumulation of ¹⁴C was also reduced, but to a lesser extent, by inclusion of 10 mM sodium azide in the incubation medium (Chart 5). The accumu-
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Chart 4. Inhibition by AZQ or DMA of [3H]thymidine (A) or [3H]uridine (B) incorporation into trichloroacetic acid-precipitable material by L1210 cells. Cells were incubated with specified concentrations of AZQ or DMA for various times before being assayed for radiolabel incorporation into trichloroacetic acid-precipitable material as described in "Materials and Methods." Points represent the mean ± S.E. of 3 triplicate experiments; bars have been omitted in Chart 4A for clarity. S.E. was <20% of the means in all cases.

The accumulation of 14C by 10^7 L1210 cells increased with increasing extracellular concentrations of [14C]AZQ but plateaued at 7 to 10 nmol/ml (Chart 6). In contrast to the accumulation of 14C by L1210 cells incubated with [14C]AZQ, cells incubated with [14C]-ROQ, accumulated 14C to about the same extent as did cells incubated with [14C]AZQ at 4°. In all experiments, the amount of 14C associated with the cells was very small compared to the total amount of 14C available and never exceeded 2 to 3% of the total radioactivity present in the 1 ml of incubation medium. Even though the total amount of accumulated radioactivity was relatively small, there was always a definite amount of 14C, greater than background, measured in cells assayed immediately after addition of [14C]AZQ (Chart 5).

14C exited rapidly from L1210 cells that had been placed into drug-free medium (Chart 7). This efflux of radioactivity from L1210 cells had a number of differences from cellular accumulation of 14C. 14C was lost from cells much more rapidly than it was accumulated (Chart 7). In addition, resuspension of cells in drug-free medium at 4° or inclusion of iodoacetate or sodium azide in the resuspending medium at 37° had no effect on the rate or amount of radioactivity lost (Chart 7). Finally, when drug efflux was studied after a 3-hr period of accumulation, some radioactivity remained with the cells. In view of the large concentration gradient available to drive radioactivity out of cells and into the medium and the failure to lose any 14C after the initial rapid decline, subsequent experiments were designed to evaluate whether the remaining radioactivity was, in fact, not exchangeable. The radioactivity remaining with the cells after an initial washing and resuspension was not reduced any further after the cells had been sedimented and resuspended in drug-free medium for a second time. The final experiments characterizing radiolabeled AZQ accumulation by and efflux from L1210 cells defined the relative amounts of exchangeable and nonexchangeable 14C associated with the cells after various periods of drug accumulation (Chart 8). As observed previously, total cellular radioactivity increased with progressively longer periods of incubation of cells with [14C]AZQ (5 nmol/ml). This increase, however, reflected the summation of a relatively stable amount of exchangeable 14C and a progressive increase in nonexchangeable 14C, from an almost undetectable amount at the start of the experiment to amounts exceeding the amounts of exchangeable 14C by 6 to 7 hr. Although incubation of cells at 4° or inclusion of 5 mM iodoacetate in the medium did not alter the accumulation of exchangeable 14C, these maneuvers did ablate accumulation of nonexchangeable 14C.

Oxygen Consumption and Free Radical Formation. AZQ has been shown to form a free radical and to stimulate oxygen consumption when incubated with microsomal preparations and NADPH (20). In analogous experiments, the reproducible, endogenous rate of oxygen consumption exhibited by L1210 cells was immediately increased by the addition of AZQ (Chart 9). Stimu-
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**Chart 6.** Accumulation of \(^{14}C\) by L1210 cells incubated with various concentrations of \([^{14}C]AZQ\). L1210 cells \(10^7\) were incubated for 3 hr with various concentrations of \([^{14}C]AZQ\) before cellular accumulation of \(^{14}C\) was assessed as described in "Materials and Methods." Points, mean of 6 values from duplicate experiments, each done in triplicate; bars, S.E.

**Chart 7.** Efflux of \(^{14}C\) from L1210 cells incubated with \([^{14}C]AZQ\) and resuspended in medium without drug. L1210 cells \(10^7\) were incubated for 3 hr at 37\(^\circ\) with \([^{14}C]AZQ\) (5 nmol/ml), and cellular accumulation of \(^{14}C\) was assessed at various times as described in "Materials and Methods." After 3 hr, cells were sedimented and resuspended in 10 ml of medium containing no AZQ. Resuspending medium was either prechilled to 4\(^\circ\) (○) or prewarmed to 37\(^\circ\) (□) or 10 mm sodium azide (△). At various times after resuspension, cells were assayed for retained radioactivity as described in "Materials and Methods." Points, mean of 6 values from duplicate experiments, each performed in triplicate.

**Chart 8.** Accumulation of nonexchangeable \(^{14}C\) by L1210 cells incubated with \([^{14}C]AZQ\). L1210 cells \(10^7\) were incubated at 37\(^\circ\) with \([^{14}C]AZQ\) (5 nmol/ml), and, at various times, total and nonexchangeable cellular \(^{14}C\) was assessed as described in "Materials and Methods." Points, mean of 6 values from duplicate experiments, each done in triplicate. Bars have been omitted for clarity. S.E. was ±20\% of the mean in all cases.

**Chart 9.** Effect of AZQ or doxorubicin on oxygen consumption by L1210 cells. L1210 cells \(10^7\) were resuspended in calcium- and magnesium-free Hanks’ balanced salt solution, and the suspension was aerated. Endogenous oxygen consumption at 22\(^\circ\) was monitored, drug was added (△) to a final concentration of 1 mm, and drug effects on oxygen consumption were assessed with a Clark-type oxygen electrode as described in "Materials and Methods." Similar results were obtained with HL-60 and K-562 cells.
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AZQ is an antineoplastic compound that fulfills a number of the predictions made and expectations held when it was introduced into preclinical and clinical trials (6, 9, 25). Phase I trials have documented the ability to administer AZQ safely to humans (19, 37, 38, 40, 41), and Phase II trials are investigating the antitumor activity of AZQ against CNS (1, 8, 15, 23, 42) and other neoplasms (4, 5, 31, 37, 40, 42). Other studies in humans (2, 8), dogs (11), and monkeys (18) have documented the ability to administer AZQ safely to humans. Our studies are the first to document the in vitro activity of AZQ against tumor cells and indicate that concentrations ≥0.05 nmol/ml are required for growth inhibition. In addition, they indicate that DNA synthesis is more sensitive to AZQ than is RNA synthesis and that protein synthesis is unaffected by AZQ for at least 24 hr. There are 2 other notable aspects of the inhibition of cellular growth of AZQ. (a) Intact aziridine rings appear essential for growth inhibition because ROQ, the molecule produced when the rings of AZQ were opened, proved devoid of growth-inhibitory properties. (b) Inhibition of cell growth and [3H]thymidine incorporation by AZQ is relatively slow to develop as compared to daunorubicin and nor-nitrogen mustard in which inhibition of [3H]thymidine incorporation is easily demonstrable after 1 hr of drug exposure (40).

The cellular accumulation of 14C by cells incubated with [14C]AZQ has a number of interesting aspects. As with AZQ-induced growth inhibition, cellular accumulation of 14C was a relatively slow process and required intact aziridine moieties to occur. In addition, AZQ was not highly concentrated in cells, achieving concentrations approximately 10 times those in the incubation media.

When we examined cell-associated radioactivity more carefully, we found 2 radioactive species: a very rapidly exchangeable portion, which was unaltered by temperature or metabolic inhibitors; and a progressively increasing nonexchangeable portion, the accumulation of which accounted for the progressive rise in cell-associated 14C. This production of nonexchangeable 14C could represent an energy-dependent process, since it was ablated by reduced temperature and the metabolic poison iodoacetate.

Another characteristic of the interaction of AZQ with cells was the rapid stimulation of oxygen consumption and formation of...
AZQ free radicals by all 3 cell lines studied. Both of these processes, which had been observed when microsomal preparations were incubated with AZQ and NADPH (20), occurred much more rapidly than did reduced cell growth, inhibition of \([^{3}H]\)thymidine incorporation, or accumulation of radiolabel by cells. Neither stimulation of oxygen consumption nor free radical formation occurred when the AZQ analogue without intact aziridine rings was used. The occurrence of these 2 events in cellular preparations is notable, because they did not occur when doxorubicin, another drug which enhances oxygen consumption and is converted to a free radical by microsomes (3, 21), was incubated with intact cells. The basis for this difference between AZQ and doxorubicin is unclear and is being studied further in our laboratory.

Our results with respect to the relatively slow occurrence of AZQ-induced growth inhibition and drug accumulation can be contrasted with the pharmacokinetic studies of AZQ in which plasma half-lives of 15 to 30 min have been documented (2, 19, 37, 38). In view of the rapid decline in plasma concentrations of AZQ, it is clear that, despite initial peak plasma concentrations of 1000 to 2000 ng/ml (2, 19, 37, 38), plasma concentrations of AZQ do not remain greater than 100 ng/ml for very long. Rather, our studies would argue for administration of AZQ by continuous infusion. Furthermore, since the total body clearance of AZQ is approximately 500 ml/min (38), the rate of AZQ infusion required to maintain a plasma AZQ concentration of 0.05 to 0.3 nmol/ml (18 to 109 ng/ml) can be calculated easily from the relationship

\[
\text{Steady-state plasma concentration} = \frac{\text{Rate of infusion}}{\text{Total body clearance}}
\]

This rate is 9.1 to 54.5 μg/min, i.e., 13.1 to 78.5 mg/day, or 7.7 to 46 mg/sqm-day for a patient of average size (1.7 sq m). This rate is greater than the maximum tolerated dosage defined by our center in a Phase I trial of 7-day continuous infusion of AZQ in patients with solid tumors (43), but it is compatible with the maximum tolerated dosage defined in other Phase I trials at our center using the same schedule in patients with acute leukemia (26). It must be remembered that our studies have been restricted to leukemic cells and may not be representative of the behavior of AZQ with solid tumor cell cultures. Studies addressing this last issue are near completion in our laboratory (16).

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