Azomycin Riboside, a Sugar Homologue of Misonidazole with Favorable Radiosensitizing Properties

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ABSTRACT

Azomycin riboside, a congener of the radiosensitizing agent, misonidazole, sensitized hypoxic V79 hamster lung fibroblast cells to radiation and was cytotoxic to these cells. Radiosensitization effectiveness depended on the conditions of hypoxic cell exposure to azomycin riboside (concentration of the latter, time, and temperature). Cytotoxicity and radiosensitizing effects resulting from exposure to azomycin riboside under anaerobic conditions were not manifested under aerobic conditions. The results presented demonstrate that azomycin riboside is an hypoxic cell radiosensitizer with effectiveness similar to or slightly better than misonidazole under in vitro conditions. The aglycone, azomycin, was weakly cytotoxic and a poor radiosensitizer in the V79 cell system. We have tested the idea that the differences in the efficacies of azomycin and its riboside as hypoxic cell radiosensitizers may be qualitatively related to the "transportability" of these agents by the nucleoside transport mechanism, that is, to their acceptance as substrates for that membrane transport system. Azomycin riboside, but not the aglycone, was shown to be a substrate for the nucleiocide transport mechanism in human erythrocytes by demonstrating that exposure to azomycin riboside (a) induced inward countertransport of thymidine and (b) competitively inhibited thymidine influx in these cells. That V79 cells possess a functional nucleoside transport mechanism was evident in the demonstration of the binding of nitrobenzylthioinosine, a potent inhibitor of nucleoside transport, to high-affinity cellular sites under oxic and hypoxic conditions. Nitrobenzylthioinosine did not impair radiosensitization by azomycin riboside, suggesting that the transporter-mediated entry of azomycin riboside into cells was not a determinant of the radiosensitizing effect under conditions of the assay for that effect. The basis of the contribution of the ribosyl group to the radiosensitizing effect remains uncertain.

INTRODUCTION

Clinical evaluations of hypoxic cell radiosensitizing agents are in progress in several countries (9, 25, 26), and misonidazole [1-(2-nitroimidazol-1-yl)-3-methoxypropan-2-ol] (28) has become a standard against which comparisons of radiosensitizing activity may be made. The evaluation of misonidazole as a potentiator of the antineoplastic effects of alkylating agents is also in progress (23). Limiting the clinical dosages of misonidazole in radiosensitization or chemosensitization are peripheral neuropathies which confine dosages to 12 g/sq m or less in protocols thus far reported (27). The search for effective, less toxic radiosensitizing agents is a significant component of current sensitizer research.

AZR, synthesized by Rousseau et al. (24), did not show significant therapeutic activity against murine leukemia in screening tests. AZR is a congener of misonidazole in which the 1-(3-methoxypropan-2-ol) group of the latter is replaced by the ß-D-ribofuranosyl group. Because 2-nitromidazoles other than misonidazole are known to be effective radiosensitizers, AZR was considered to have this potential. This study demonstrated (a) that AZR is an hypoxic cell radiosensitizer with effectiveness comparable to that of misonidazole when this property was evaluated in an in vitro test system using cultured V79 cells and (b) that AZR is a more effective radiosensitizer than is AZ. We have attempted to relate the latter difference to the acceptability of these 2 agents as substrates for the nucleoside transport mechanism.

The entry into animal cells of physiological nucleosides and of a variety of synthetic nucleoside derivatives is mediated by nucleoside-specific transport elements of the plasma membrane. This transport mechanism has broad specificity with respect to the base (i.e., aglycone) portion of the nucleoside permeants which are accepted as substrates (8, 20, 29). Rates of transporter-mediated entry of physiological nucleosides and of some nucleoside analogs into cultured cells are clearly much greater than those of diffusional entry at permeant concentrations in the vicinity of the influx K_m (2, 8, 20). Accordingly, an object of this study was to determine whether AZR and AZ were substrates for the nucleoside transporter and whether their transportability was related to their relative efficacies as radiosensitizing agents. It is well established that permeability is a determinant of the cytotoxicity of various nucleoside analogs toward cultured cells and that genetic (2, 8) or chemical (20) impairment of nucleoside transporter function imparts resistance to such cytotoxic effects.

While we have demonstrated that AZR was a substrate for the nucleoside transport mechanism and that AZ was not so accepted, the idea that the acceptability of AZR as a substrate by the nucleoside transport mechanism was a determinant of their transportability was related to their relative efficacies as radiosensitizing agents.

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the radiosensitizing activity was challenged by the result that the latter activity was not impaired in the presence of NBMPR, a potent, tightly bound inhibitor of nucleoside transport (12, 13, 16, 19).

MATERIALS AND METHODS

[1-3H]NBMPR (16 Ci/mmole), [2-3H]adenosine (16 Ci/mmole), and [methyl-3H]thymidine (50 Ci/mmole) were purchased from Moravek Biochemicals, City of Industry, Calif., and, just before use, they were repurified to >98% radiochemical purity by high-performance liquid chromatography using a C18-Bondapak column (Waters) eluted with methanol-water solutions. AZ was generously provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and AZR was synthesized by the method of Prisbe et al. (22).

Radiosensitizing Activity. The radiosensitizing activities of AZR and AZ were assayed with V79 Chinese hamster lung fibroblasts which were cultured and maintained as monolayers in MEM supplemented with 10.5% fetal calf serum and antibiotics. To assay for radiosensitivity, cells in logarithmic growth were trypsinized from their culture flasks and resuspended in spinner medium (Ca2+- and Mg2+-free MEM supplemented with 6.2% fetal calf serum and antibiotics) in irradiation flasks, which were then gassed with mixtures of air plus 5% CO2 for aerobic conditions or ultrapure N2 (<5 ppm O2) plus 5% CO2 for hypoxic conditions at 22 and 37°C. The gas mixtures were flowed through the culture vessels at the rate of 1 liter/min for 60 min prior to and during irradiation intervals. Cells were irradiated at 22 and 37°C with or without drugs in stirred suspensions (105 cells/ml) with γ-rays from a cesium irradiator. The average dose rate in the liquid volume of the cell irradiator as determined by Frickle dosimetry was 140 centigrays/min. Portions of the cell suspensions, removed through vessel-sampling ports after each dose fraction, were diluted in MEM and plated into 5 ml of MEM in 60- x 15-mm plastic Petri dishes for determination of viability by colony-forming ability. Such cultures were incubated at 37°C for 6 days in a humidified air plus 5% CO2 atmosphere and then stained with methylene blue prior to enumeration of colonies. Radiation survival curves such as those shown in Chart 1 were constructed from such data.

Cellular Toxicity. Both cytostatic and cytoidal effects of AZR and AZ toward V79 cells were measured using procedures utilized previously (5, 6). Replicate culture samples containing about 106 cells were plated in 60- x 15-mm plastic dishes and incubated overnight, a procedure that resulted in exponentially proliferating cultures. The medium from such cultures was replaced with warmed medium containing graded concentrations of the agents under test, and cultures were incubated for a further 24 hr, at which time cultures were trypsinized, and cells were enumerated to determine the net change in cell numbers during that interval. Cell proliferation data were reduced to a fraction of maximum cell increase according to the equation described previously (7).

Cytoidal toxicity was measured by exposing stirred suspensions of V79 cells in the irradiation chambers to graded concentrations of AZR and AZ for various times under both aerobic and hypoxic conditions. Portions of these suspensions were diluted in fresh medium and plated in Petri dishes for determination of viability by colony-forming ability. Cell survival curves such as those shown in Chart 4 were constructed from these data.

Nucleoside Transport. Experiments that evaluated the interaction of AZR and AZ with the nucleoside transport mechanism used human erythrocytes. Blood from healthy human volunteers was collected into heparinized evacuated tubes. Erythrocytes were washed 3 times with 20 volumes of a medium containing 140 mM NaCl, 5 mM KCl, 20 mM Tris-HCl (pH 7.4 at 22°C), 2 mM MgCl2, 0.1 mM EDTA (disodium salt), and 5 mM glucose. Hemoglobin-free "ghosts" were prepared as described previously (12).

Rates of inward transport of nucleosides into erythrocytes (2 × 108 cells/incubation) were measured as initial rates of cellular uptake at 22°C by the method of Jarvis et al. (14). Incubation times (2 to 5 sec) and other experimental conditions were such that maximum cellular concentrations of permeant did not exceed 30% of the corresponding extracellular concentration.

To determine if the test compounds were substrates for the nucleoside transport mechanism, their abilities to cause the inward countertransport of [3H]thymidine in human erythrocytes were measured at 22°C. Washed erythrocytes were first "loaded" with test permeant by incubating 0.6 ml of a suspension of washed cells (40% hematocrit) with an equal volume of medium containing the test compound (final concentration, 5 mM). After 30 min at 22°C, cells were pelleted (10 sec, 15,000 x g) in an Eppendorf Model 5412 microcentrifuge. To remove extracellular test permeant, the cells were then rapidly washed 3 times with 1-ml portions of ice-cold medium, and without delay, 0.7 ml of medium (22°C) containing 0.2 mM [3H]thymidine was added to the pellet with concurrent vortex mixing. At measured time intervals after addition of thymidine, 0.1-ml samples of the incubation mixture were added to 0.8-ml portions of ice-cold stopping solution (medium containing 10 μM NBMPR) layered on 0.5 ml of n-dibutylphthalate in 1.5-ml microcentrifuge tubes. Tubes were immediately spun for 10 sec in a microcentrifuge to pellet cells under the dibutylphthalate layer. Radioactivity associated with the cell pellets was determined as described previously (32). Sample H activities were corrected for blank values obtained by subjecting to the foregoing procedures erythrocytes that had been pretreated with 10 μM NBMPR.

Nitrobenzythioinosine Binding. Equilibrium binding of [3H]NBMPR to V79 cells was determined by an oil centrifugation method (2).

RESULTS

Radiosensitizing Effectiveness of AZR and AZ. The effects of AZR and AZ at various concentrations on the radiosensitivity of V79 cells irradiated in both aerobic and hypoxic suspensions were measured at 22 and 37°C. Cell suspensions of the asynchronous cultures were divided into portions so that up to 6 radiation survival curves were generated with the same cell population. Typical survival data for cells irradiated at 22°C are shown in Chart 1, which shows that the presence of AZR in the culture medium at concentrations of 0.2 to 5.0 mM resulted in significant hypoxic cell radiosensitization. At similar concentrations, AZ was a less effective radiosensitizer. "Sensitizing enhancement ratios" were derived from experimental plots such as those of Chart 1. This parameter is defined as the ratio of the radiation dosages required to reduce cell survival to 0.1 under (a) hypoxic conditions and (b) sensitizing conditions. Enhancement ratios from several independent experiments are shown in Chart 2. The dashed line refers to sensitizing enhancement ratios obtained when V79 cells were irradiated under hypoxic conditions in the presence of misonidazole at 22°C. It is apparent in these data that AZR was about as effective in radiosensitization as misonidazole when culture media contained concentrations of either agent in the 0.1 to 1.0 mM range. Furthermore, AZR is approximately 5-fold more effective than AZ as a radiosensitizer. The time and temperature dependence of a component of misonidazole radiosensitization has been considered to be metabolism dependent (4, 31). The experiments summarized in Chart 2 also demonstrate marked

7 Countertransport is a process in which the flux of Permeant A from one face of the plasma membrane to the other generates a net flux of B, a related permeant, in the opposite direction.
Radiation Dose (cGy)

Chart 1. a. effect of AZR on the survival of Chinese hamster V79 cells after irradiation at 22°. Cells were irradiated in air (x), nitrogen (O), and nitrogen preincubated with 5.0 (A), 2.0 (O), and 0.2 (•) mM AZR for 1 hr. The surviving fraction (log scale) is plotted against the radiation dose [centigrays (cGy)]. b. effect of AZ on the survival of Chinese hamster V79 cells after irradiation at 22°. Cells were irradiated in air (x), nitrogen (O), and nitrogen preincubated with 5.0 (A) and 2.0 (O) mM AZ for 1 hr. The fractional survival of cell clonogenicity (S/So, log scale) is plotted against the radiation dose (centigrays).

Chart 2. Radiosensitizer enhancement ratios for AZR and AZ at 22 and 37°. The sensitizing enhancement ratios (ratios of radiation doses under hypoxia alone and hypoxia plus sensitizer that produce 90% cell kill) were determined for graded initial concentrations of AZ (D, •) and AZR (O, •) following preincubation for 1 hr at 22° (D, O) and 37° (•, •) data obtained previously for misonidazole at 22°. The oxygen enhancement ratio (at 90% cell kill) for cells irradiated under conditions used for these studies is 2.8.

Chart 3. Effect of AZ, AZR, and misonidazole on proliferation of Chinese hamster V79 cells under aerobic conditions. Exponentially proliferating cultures were exposed to graded concentrations of AZR (O), AZ (A), and misonidazole (•) for 24 hr, at which time the net increase in cell numbers was determined. Cell proliferation data were reduced to a fraction of maximum cell increase and plotted against the initial concentration of sensitizer.

Chart 4. Cytocidal activity of AZ and AZR toward Chinese hamster V79 cells under hypoxic conditions. V79 cells were exposed to graded concentrations of AZ (O, A, O) and AZR (•, •, •) at 0 (x), 0.5 (O, •), 1.0 (A, A), 2.0 (•, O), and 5.0 (•) mM for various times under hypoxic conditions at 37°. The fractional survival of V79 cells, as measured by the colony-forming ability of surviving cells, (S/So, log scale) is plotted against the time of incubation.

Transport Studies. Because isotopically labeled AZR and
AZ were not available, indirect methods were used to determine whether these compounds were substrates for the broadly specific nucleoside transport system which has been demonstrated in a variety of cell types (for reviews, see Refs. 19, 21, 30). These studies were conducted with human erythrocytes under aerobic conditions. Human erythrocytes were used, because the general features of nucleoside transport in these cells are well defined (13, 19, 21, 22) and typical of various types of cultured cells, and, accordingly, activity of AZR or AZ as substrates for the erythrocyte transporter would mean that similar activity was probable in other cell types such as V79 cells. As well, the inability of human erythrocytes to metabolize thymidine (17) permitted use of thymidine counter-transport as an indicator of the acceptance of a test permeant by the nucleoside transport mechanism. In the counter-transport experiments, enhancement of cellular uptake of [3H]thymidine which resulted from prior loading of erythrocytes with thymidine (positive control) or test substances signified that the test substance was a substrate for the transport mechanism.

Chart 5 shows the effect of preloading human erythrocytes with high concentrations of thymidine, AZR, or AZ on cellular uptake of [3H]thymidine. Preincubation with 5 mM thymidine or 5 mM AZR resulted in a transient accumulation of [3H]thymidine in which the maximum intracellular concentration of [3H]thymidine (0.45 mmol/liter water) was approximately 3-fold higher than the extracellular [3H]thymidine concentration (0.15 mmol/liter water). In contrast, the time courses of [3H]thymidine uptake by cells incubated previously with AZ were indistinguishable from those of cells with no exposure to AZ. AZR was also an apparently competitive inhibitor of the inward transport of thymidine with an apparent Kᵦ of 0.43 mM (Chart 6).

The data of Chart 6 demonstrate that AZR competitively inhibited the inward transport of thymidine in erythrocytes. This interaction of AZR with the nucleoside transport mechanism implies substrate activity. A Lineweaver-Burk plot of these data was also consistent with competitive inhibition by AZR. However, AZ (initial extracellular concentration, 2 mM) had no effect on thymidine transport by human erythrocytes when presented simultaneously with thymidine or when cells were incubated with AZ at 22° for 30 min prior to the assay of thymidine transport.

Nitrobenzylthionoisine Binding Studies. A substantial body of evidence (see “Discussion”) indicates that the site-specific, high-affinity binding of NBMPR to cell surfaces is quantitatively related to nucleoside transport capability. The determination of site-specific binding of [3H]NBMPR to aerobic V79 cells is illustrated in Chart 7, in which cell-associated NBMPR is plotted against the equilibrium concentration of the inhibitor in the medium. NBMPR binding was resolved into 2 components: (a) a saturable association of NBMPR with cells in which 5.4 X \(10^5\) molecules of NBMPR were bound per cell with an apparent Kᵦ of 0.19 mM; and (b) a nonsaturable association. The presence of 5 μM NBTGR in the binding assay system had no effect on the nonsaturable component of binding but abolished the saturable NBMPR binding (Chart 7). AZR was an apparent competitive inhibitor of high-affinity NBMPR binding to V79 cells (apparent Kᵦ, 1.6 mM; data not shown).

The idea that the radiosensitizing effectiveness of AZR molecules may be related to their “transportability” (by the nucleoside transport mechanism) was tested in experiments similar to those of Chart 8. Aerobic V79 cells were preincubated for 10 min with or without 10 μM NBMPR in the presence and absence of AZR (0.5 mM), followed by a 60-min interval of gassing with N₂ before irradiation. Under these conditions, the radiosensitization of hypoxic V79 cells by AZR was not significantly affected when the culture media contained 10 μM NBMPR (Chart 8). Control experiments established that NBMPR alone had no effect on the fraction of cells surviving irradiation. Further control experiments established that the site-specific binding of [3H]NBMPR to hypoxic V79 cells was similar to that of NBMPR binding to aerobic V79 cells (data not shown).
DISCUSSION

These experiments showed that AZR sensitized hypoxic V79 hamster lung fibroblast cells to radiation and that this effect did not occur under aerobic conditions. A component of this radiosensitization was apparently metabolism related. On a molar basis, AZR was about as effective as misonidazole in radiosensitization of hypoxic V79 cells. In analogy with misonidazole, AZR showed little toxicity toward cells in oxygenated culture but was toxic toward anaerobic cells. As a radiosensitizer, AZR was about 5-fold more effective than the aglycone, AZ.

Interactions of AZR with the nucleoside transport mechanism in erythrocytes indicated that AZR was a substrate for the nucleoside transporter. The entry of thymidine into human erythrocytes was competitively inhibited by AZR, which also caused the counter-transport of thymidine in these cells. AZR also inhibited thymidine influx into S49 mouse lymphoma cells (data not shown). The inactivity of AZ in these assays supports the conclusion that AZR is a substrate for the same mechanism that mediates the permeation of thymidine and other physiological nucleosides.

A number of S-substituted 6-thiopurine nucleosides related to NBMPR are reversible, potent inhibitors of nucleoside transport. Loss of transport activity in human erythrocytes and HeLa cells exposed to NBMPR is associated with high-affinity binding of the inhibitor to the plasma membrane (1, 12, 13, 16, 19). Recently, it has been demonstrated that high-affinity binding of NBMPR to erythrocyte membranes represents a specific interaction of NBMPR with functional nucleoside transport sites. Erythrocytes from 'nucleoside-permeable type' sheep transport nucleosides and bind NBMPR, whereas erythrocytes from 'nucleoside-impermeable type' sheep lack functional nucleoside carrier elements and do not bind the inhibitor (11–13, 32). Similarly, cultured AE1 cells [a clone isolated from a mutagenized population of mouse lymphoma S49 cells (8)] neither transport nucleoside nor exhibit site-specific binding of NBMPR (2). However, cells of the parental line (S49) bind NBMPR and transport nucleosides, in agreement with the suggestion that NBMPR binding may be regarded as a quantitative assay for nucleoside transport function (12, 13). Therefore, our demonstration that high-affinity NBMPR binding sites are present on aerobic and hypoxic V79 cells indicates the presence of functional nucleoside transport sites. Our observations that AZR inhibited the high-affinity, site-specific binding of NBMPR to V79 cells and to human erythrocyte membranes (data not shown) provide additional evidence that AZR interacts with the nucleoside transport mechanism.

It has been shown that, in NBMPR-containing medium, cultured cells proliferate in the presence of otherwise toxic concentrations of various nucleoside analogs (18). Such NBMPR-protective effects demonstrate clearly the importance of transporter-mediated cellular uptake in the mechanism of the cytotoxic effects of these agents. The present study demonstrates (a) that the ribosyl group contributes importantly to the efficacy of AZR as a radiosensitizer and (b) that AZR is a substrate for the nucleoside transport mechanism (evidently present in V79 cells). However, the 'transportability' of AZR does not appear to account for the contribution of the ribosyl group to the radiosensitizing activity of AZR, because the failure of NBMPR to perturb radiosensitization by AZR (Chart 8) suggests that effective cellular concentrations of AZR were acquired without
a functioning nucleoside transport mechanism. In these experiments, V79 cells were exposed to AZR for over 60 min, a period sufficient for substantial entry of AZR by simple diffusion. Also, Krupka and Deves (15) have shown that inhibitors of transport acting asymmetrically at the outer surface of the cell may inhibit permeant exit more than entry. It has been proposed recently that the NBMPR binding site for nucleoside-permeable sheep erythrocytes is located largely on the outer surface of the cell membrane (10, 13). Experimental definition of the relationships between cell content of the nitroimidazole sensitizers and the sensitization response will be an important part of understanding the basis for the contribution of the ribosyl group to the radiosensitizing efficacy of AZR.

AZR appears to have potential as an in vivo radiation sensitizer. In an assay system in which tumor growth delay is measured, early studies have shown that AZR sensitized i.m. implants of the Lewis lung carcinoma to radiation with a molar dose-response relationship similar to but less potent than that of misonidazole.6 We suggest that AZR has clinical potential, but whether AZR toxicology will afford advantage over the nitroimidazole sensitizers presently in clinical use remains to be seen.

In summary, we have shown that AZR is both a substrate for the nucleoside transport mechanism of animal cells and a potent radiosensitizer. That the ribosyl group contributes to the radiosensitizing efficacy of AZR is evident in the severalfold greater potency of AZR relative to AZ in this property. However, the failure of the inhibitor of nucleoside transport, NBMPR, to block radiosensitization of V79 by AZR indicates that the transporter-mediated entry of AZR into cells was not a determinant of radiosensitizer efficacy in this instance. We suggest that the nucleoside transport mechanism, by virtue of its remarkably broad specificity (20), is a potential route for the contribution of the ribosyl group to the radiosensitizing efficacy of AZR.

REFERENCES


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