Inhibition of Nucleoside Transport in Murine Lymphoma L5178Y Cells and Human Erythrocytes by the Uridine Phosphorylase Inhibitors 5-Benzylacyclouridine and 5-Benzylxoxybenzylacyclouridine

Kang-Hyun Lee, Mahmoud H. el Kouni, Shih-Hsi Chu, and Sungman Cha

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

ABSTRACT

The uridine phosphorylase inhibitors, 5-benzyllacyclouridine (BAU) and 5-benzylxoxybenzylacyclouridine (BBAU) (Biochem. Pharmacol., 31: 1857, 1982), inhibited uptake of uridine in L5178Y cells. By a rapid sampling technique, BAU and BBAU were shown to inhibit the transport (zero-trans influx) of uridine, thymidine, and adenosine in human erythrocytes as well as in murine L5178Y cells. In all cases, competitive inhibitions were observed. Km values for the transport of adenosine, uridine, and thymidine in erythrocytes were 2.2, 195, and 199 μM, while Vmax were 2.9, 118, and 96.5 pmol/min/10⁶ cells, respectively. In L5178Y cells, Km values of 14.8 and 23.1 μM and Vmax of 389 and 176 pmol/min/10⁶ cells were obtained for adenosine and uridine, respectively. For erythrocytes, the Km values of BAU were 127, 124, and 198 μM using adenosine, uridine, and thymidine as the substrate; and those of BBAU, 14.1 and 19.2 μM for adenosine and uridine, respectively. In L5178Y, the Km values of BAU were 202 and 234 μM, and those of BBAU, 39.8 and 27.9 μM for adenosine and uridine, respectively. These data indicate that, in two cell types, Km values for BAU and BBAU did not vary regardless of the substrate used; that the values of Km are different for the erythrocytes and L5178Y cells; and that BBAU is at least 5-fold more potent than BAU as an inhibitor of nucleoside transport. The inhibitory effects on the efflux of preloaded uridine indicate that BAU and BBAU are inhibitors, rather than permeants, of the nucleoside transport system.

INTRODUCTION

The recently developed analogues of uridine, BAU and BBAU, are potent and specific inhibitors of uridine phosphorylase (24). Km values were estimated at 98 and 32 nm for BAU and BBAU, respectively, for uridine phosphorylase from mouse sarcoma 180 sarcoma cells (24). These 2 compounds do not affect the activities of thymidine phosphorylase, uridine kinases, and thymidine kinase (24), or orotate phosphoribosyltransferase (26). It was speculated that the inhibition of uridine phosphorylase might potentiate the cytotoxicity of chemotherapeutic pyrimidine nucleosides analogues (e.g., FUrd and FdUrd) by preventing their cleavage to the less effective bases (1). On the basis of the observations that certain tumors have little or no thymidine phosphorylase (14, 15, 19, 25, 38-40), Niedzwicki et al. (24, 25) proposed that uridine phosphorylase inhibitors might potentiate FUrd and FdUrd effects on those tumors, but not on normal cells. We have studied the effects of BAU and BBAU on the cytotoxicity of FUrd and FdUrd in cultured L5178Y cells. Contrary to expectations (1, 24, 25), BAU and BBAU did not potentiate the cytotoxicity of the FUra nucleosides but inhibited the total cellular incorporation of radiolabeled uridine in exponentially growing L5178Y cells.

These unexpected findings prompted us to investigate the effects of these drugs on the nucleoside transport system of L5178Y cells and human erythrocytes. In our earlier studies (16), we observed that BAU and BBAU inhibit long-term uptake of nucleoside (cellular uptake during 5-min intervals), which is known to reflect both the rate of membrane transport and the subsequent metabolism of the permeant (Refs. 10, 20, 28, and 36). In the present studies, a modified version of the rapid sampling technique of Wohlueter et al. (35) was used to measure the initial rates of nucleoside transport. Human erythrocytes were also studied because they lack both uridine and thymidine kinase activities (27, 33); hence, the rate of uptake is not likely affected by the intracellular phosphorylation of pyrimidine nucleosides. In these studies, we found that BAU and BBAU inhibit transport of nucleosides in both types of cells.

MATERIALS AND METHODS

Chemicals. BAU and BBAU were synthesized according to the procedure published previously (24). [8-14C]Adenosine (56 mCi/mmol), [2-14C]uridine (58 mCi/mmol), and [2-14C]thymidine (57 mCi/mmol) were purchased from Moravek Biochemicals, Brea, CA; unlabeled nucleosides, NBMPR, and other chemicals were from Sigma Chemical Co., St. Louis, MO.

Media. Medium A consists of Fischer's medium (Grand Island Biological Co., Grand Island, NY) supplemented with 13.4 mm N-2-hydroxyethyl-1-piperazine-N'-2-ethanesulfonic acid, pH 7.4.

Cell Preparations for Rapid Sampling Procedure. L5178Y cells were maintained in the peritoneal cavities of male C57BL/6 x DBA/2 F1 mice (Cumberland Farm, Clinton, TN). Mice were killed by cervical dislocation and ascitic fluid containing L5178Y cells was collected in Puck's saline aurate thio-9-[3-ß-ribofuranosylpurine.

© 1984 American Association for Cancer Research.
obtained from a local hospital and used within 7 days. The cells were washed 3 times in Medium B according to the method of Oliver and Paterson (27). Erythrocytes were incubated in 2 volumes of Medium B for 30 min at 37°C in a shaking water bath, washed 3 more times, and finally suspended in Medium B to give about 2% hematocrit. The actual number of cells (1 × 10^8 cells/ml) was determined using a Coulter Counter (Model B; Coulter Electronics, Hialeah, FL).

Zero-trans Influx. The rapid sampling procedure of Wohlhueter et al. (35) as modified by Dr. S.F. Chen was used. A Y-bored Plexiglas mixing chamber, designed to fit 2 Eppendorf Multipettes (Model 4780; Brinkmann Instruments, Inc., Westbury, NY) was used.8 After withdrawing the cell suspension and the substrate solution, each into a separate Combitip, the mixing chamber was attached to the ends of the Combitips. Eppendorf microcentrifuge tubes (1.5-ml capacity) containing 150 μl of the oil mixture (84% Dow Corning 550 silicone fluid-16% paraffin oil) were placed in a Fisher Scientific Co. Model 235A microcentrifuge. Aliquots of 100 μl (50 μl of cell suspension and 50 μl of substrate solution) were dispensed through the mixing chamber at desired time intervals (usually 2 sec) into the centrifuge tubes. Because of its poor water solubility, BBAU was dissolved in dimethyl sulfoxide so that the final concentration of the solvent was 1%, a concentration which did not affect the uptake process. The transport process was terminated by a 30-sec centrifugation. The lag period of about 2 sec between the onset of the centrifugation and the complete entrance of cells into the oil mixture allowed the shortest incubation period of about 3 sec. After removal of the supernatant fluids, the tube walls were rinsed with water, which was then subsequently removed along with most of the oil mixture. L5178Y cell pellets were mixed with 0.5 ml of 0.4 N KOH and heated for 20 min at 90°C to hydrolyze RNA. After cooling to room temperature, the mixtures were centrifuged, and 0.4-ml aliquots of the supernatant fluids were transferred to scintillation vials containing 10 ml of ACS scintillator (Amer sham/Searle Corp., Arlington Heights, IL), and the radioactivities were counted in a Packard Tri-Carb 460 scintillation counter. RBC pellets were solubilized by the addition of 0.75 ml of M-74 solution (2% ammonium bicarbonate-1% Triton X-100-1% trypsin) (21) and vigorous vortexing, followed by an overnight incubation at room temperature. The tubes were then placed in scintillation vials. To decolorize the erythrocytes samples, 1 ml of toluene and 0.5 ml of 30% hydrogen peroxide were added. After a 1-hr incubation at 37°C and 3 to 6 hr of cooling to ensure the complete decomposition of hydrogen peroxide, 10 ml of ACS fluid were added, and the radioactivity was counted. A control experiment was carried out in the presence of 5 μM NBMPR in order to correct data for non-carrier-mediated influx and for radioactivity in the extracellular fluids entrapped in the packed cells. Initial slopes of the plot of cpm versus time, after the control values (usually negligibly small) were subtracted, were defined as the initial influx velocities, i.e., as transport rates.

Uridine Efflux. Preparation of uridine-loaded erythrocytes and efflux experiments were done according to the method of Cass and Paterson (3). Washed and packed erythrocytes were loaded by incubating for 40 min at 37°C in an equal volume of Medium B containing 4.53 μM [14C]-uridine (1.1 μCi/mmol). After centrifugation and removal of the supernatant fluid, the packed cells were taken up in one Combitip of the dual dispenser and used for the efflux study. The concentration of intracellular uridine was calculated from the difference of absorbance of the incubation media at 262 nm before and after the loading incubation and was estimated to be 2.43 μM. A rapid sampling procedure, using the one-hand dual-syringe apparatus described in the zero-trans influx, was also used. The hematocrit of the final efflux assay mixture was made to be less than 10%, by using 2 different sizes of Combitip (one to deliver 25 μl of loaded packed cells, the other to deliver 250 μl of Medium B containing various compounds in each single injection). If one assumes that 10% of the packed cell volume is the extracellular space, then the radioactive uridine present in the extracellular space of packed-loaded cells was diluted to approximately 20 μM, a concentration far below the Km value reported previously of 140 μM for zero-trans influx (11). The rate of non-carrier-mediated efflux was estimated in the presence of 30 μM NBMPR and used to correct the experimental data. The efflux of radicelabeled uridine from loaded cells was terminated by a 30-sec centrifugation through the oil layer, and the radioactivity released into the medium was counted.

RESULTS

Preliminary studies showed that BAU and BBAU inhibit the long-term (5-min) uptake of uridine in cultured L5178Y cells (16). Results of uptake experiments such as these were undoubtedly affected by the metabolism of substrate. Therefore, in the present investigation, we used the initial velocities determined by a rapid sampling procedure to estimate the transport component of the uptake as independently as possible from the metabolic component (e.g., phosphorylation).

Time Course for [14C]-Uridine Uptake (Zero-trans Influx). Chart 1 shows that the time course of [14C]-uridine uptake by erythrocytes, in the absence of BAU, is linear for at least the first 5 sec, until about 5 pmol/10^6 cells have accumulated in the cell. The result is typical of that obtained for all uridine concentrations (50, 63, 83, 125, and 250 μM) used. It should be noted that the initial portions of all lines, which were obtained after correcting for the radioactive counts entrapped in the extracellular fluids, extrapolate to the origin. This strongly indicates that the initial velocities measured represent transport velocity. Similar results were obtained with all substrates at all concentrations used in both erythrocytes and L5178Y cells (data not shown). The progressive decline of the rate of net uptake after the initial phase is probably attributable in part to efflux of uridine. Chart 1 also shows that BAU caused a decrease in the rate of uptake in all cases and that the uptake rates were linear until the intracel-

![Chart 1. Time courses of [14C]-uridine uptake (zero-trans influx) in human erythrocytes in the presence of different concentrations of BAU. The uridine concentration was 125 μM. All points were corrected for the radioactivity trapped in the extracellular space by subtracting the radioactivity counts of the control experiments with 5 μM NBMPR extrapolated to zero time values.](chart1.png)

---

8 Dr. S.F. Chen and G. Panal of Brown University were responsible for the design of the rapid sampling procedure and the construction of the mixing chamber.
Kinetic Studies. Chart 2 shows, as an example of kinetic studies of zero-trans influx, a double-reciprocal plot of initial rate of [\(^{14}\text{C}\)]uridine uptake (i.e., velocities estimated from the early time points falling on a straight line) at the substrate concentration range of 50 to 250 \(\mu\text{M}\). Kinetic parameters were calculated by a least-squares fitting of the Michaelis-Menten equation (6, 34). Chart 2 shows that the uptake was a saturable process and that BAU competitively inhibited uridine transport. The results of similar experiments using thymidine and adenosine are summarized in Table 1. In all cases, competitive inhibitions were observed. These data indicate that in a given cell type, \(K_m\) values for BAU and BBAU did not vary regardless of the substrate used but were different between erythrocytes and L5178Y cells, suggesting that the \(K_m\) values may indeed be the dissociation constants of the carrier-inhibitor complex of each cell type. BBAU was at least 5-fold more potent than BAU as an inhibitor of nucleoside transport.

Uridine Efflux. In order to determine whether these competitive inhibitors of nucleoside transport, BAU and BBAU, belong to the class of permeants or inhibitors of the nucleoside transport system, we have studied the exchange diffusion in [\(^{14}\text{C}\)]uridine-loaded erythrocytes, using the rapid sampling procedure. The rates of uridine efflux from uridine-loaded cells were measured in the absence and presence of added uridine, BAU or BBAU, in external medium. Table 2 shows that the presence of external uridine accelerated uridine efflux ("trans-acceleration") as shown by other investigators (3, 37). On the other hand, BAU and BBAU inhibited uridine efflux. As was the case with zero-trans influx, BBAU showed higher inhibitory potency than did BAU. These results indicate that BAU and BBAU should be classified as "inhibitors" rather than "permeants" of the nucleoside transport system.

DISCUSSION

The present studies, using a rapid sampling procedure, clearly demonstrate that BAU and BBAU competitively inhibit the transport of uridine, thymidine, and adenosine into human erythrocytes and murine L5178Y cells. These compounds also inhibited uridine efflux from uridine-loaded erythrocytes. In all cases, BBAU was at least 5 times more potent than BAU as an inhibitor of nucleoside transport.

The observed initial velocities of adenosine uptake, however, may not be independent of intracellular metabolism. A recent report by Plagemann and Wohlhueter (30) showed that, at low concentrations of adenosine (2 \(\mu\text{M}\)), a significant amount of phosphorylation occurred during the first 3 sec of incubation. Thus, the exact nature of the so-called "initial velocity" (within 3 sec) of adenosine influx may still be debatable. Despite this controversy, which is beyond the scope of the present study, there seems to be little question about adenosine being transported by a common nucleoside transport system. Thus, the observed inhibition of adenosine "uptake" by BAU and BBAU could be due to inhibition of metabolism or transport or both. It should be noted, however, that, in a given cell type, the \(K_m\) values of BAU and BBAU did not vary regardless of the substance used.
This fact favors strongly the possibility that these compounds indeed inhibit adenosine transport.

In addition to the inhibition of uridine phosphorylase, these benzylcyclouridines, particularly BAU, may have another pharmacological effect for which the inhibition of the nucleoside transport may be a responsible factor. Recently, 2 different laboratories (7, 23) reported that circulating plasma uridine in rats is regulated by the liver which degrades most of all incoming uridine from the portal vein and releases about the same amount of newly synthesized uridine into the hepatic vein, and they suggested that the salvage pathway may be of primary importance in some cells. BAU caused a profound increase in the plasma uridine level in rats (9, 22) and in the salvage of circulating uridine (9). It was suggested that this increase in plasma uridine level by BAU may rescue animals from cytotoxic pyrimidines, and human lung carcinoma (LX-1).

In conclusion, inhibitors of uridine phosphorylase, BAU and BBAU, have an additional property of inhibiting nucleoside transport. Both of these properties may be responsible for the effect of BAU in increasing the level and the reutilization of circulating uridine, and they must be taken into account in considering the mechanism of pharmacological actions of BAU and BBAU.

ACKNOWLEDGMENTS

Authors are indebted to B. S. Robison for her technical assistance; Dr. S. F. Chen and Dr. J. D. Stoeckler for many useful discussions and for sharing with us their newly developed techniques; Dr. D. L. Duster for helpful advice; Dr. C. Y. Jung for critical reading of the manuscript; and G. Parnell for fabricating the rapid mixing chamber.

REFERENCES


Inhibition of Nucleoside Transport in Murine Lymphoma L5178Y Cells and Human Erythrocytes by the Uridine Phosphorylase Inhibitors 5-Benzylacyclouridine and 5-Benzyloxybenzylacyclouridine


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/44/9/3744

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.