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Benzylacyclouridines as Nucleoside Transport Inhibitors in Human Erythrocytes[1]

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ABSTRACT

Various benzylacyclouridines (BAU, HM-BAU, suc-BAU, BBAU, HMBBAU, suc-BBAU, and BBBAU) developed as specifc inhibitors of uridine phosphorylase (UrdPase), inhibit transport (zero-trans influx) of ridine (Urd) in human erythrocytes. The inhibition pattern of these compounds is competitive, though suc-BBAU and BBBAU show a slight noncompetivieness. The order of potency as an inhibitor of the nucleoside transport system is BBAU ~ HM-BBAU ~ suc-BBAU > BBBAU > BAU ~ suc-BAU ~ HM-BAU (K_i values of 19, 23, 38, 112, 124, 174 and 176 μM, respectively). These data indicate that there is a differnece in potency of Urd transport inhibition between the analogs of BAU and BBAU. Further, the potency correlates with the hydrophobicity of the compound, but it has a limit in the size of C_5 substitution.

Abbreviation: BAU (5-benzylacyclouridine), 5-benzyl-1-(2'-hydroxyethoxymethyl) uracil; HM-BAU (3'-hydroxymethyl-BAU), 5-benzyl-1-[(1',3'-dihydroxy-2-propoxy) methyl]uracil; suc-BAU, 3'-succinyl-BAU; BBAU (benzyloxybenzylacyclouridine), 5-(*m*-benzyloxybenzyl)-1-(2'-hydroxyethoxymethyl)uracil; HM-BBAU (3'-hydroxymethyl-BBAU), 5-(*m*-benzyloxybenzyl)-1-[(1'3'-dihydroxy-2-propoxy)methyl]uracil; suc-BAU, 3'-succinyl-BBAU; BBBAU, 5-[3-(4-benzyloxybenzyl)benzyl]-1-(2'-hydroxyethoxymethyl)uracil; UrdPase, uridine phosphorylase; Urd, Uridine; Fd-Urd, 5-fluoro-2'-deoxyuridine; AcThd, acyclothymidine; AcUrd, acyclouridine; dThd, thymidine; NBMPR, nitrobenzylthioisone; FUra, 5-fluorouracil

Key Words: Benzylacyclouridine, Nucleoside transport, Human erythrocytes

INTRODUCTION

Benzylacyclouridines (BAU and BBAU), specific UrdPase inhibitors (Niedzwicki *et al.*, 1982), were shown to potentiate the antitumor activity of FdUrd against human colon cancer and human lung cancer *in vivo* as well as *in vitro*(Chu *et al.*, 1984). However, we could not observe the potentiative effect of BAU or BBAU against L5178Y cells in either the *in vivo* or the *in vitro* systems[1]. In pursuing the reason of this discrepancy, we have shown that BAU and BBAU competitively inhibit the transport of nucleosides both in human erythrocytes and in L5178Y cells (Lee *et al.*, 1984).

Recently, several derivatives of BAU and BBAU (Fig. 1) were synthesized to increase either their water solubility (*i.e.*, HM-BAU and suc-BAU *vs* BAU; HM-BBAU and suc-BBAU *vs* BBAU) or hydrophobicity (BBBAU *vs* BBAU) in our laboratory. We found that the hydrophilic derivatives of

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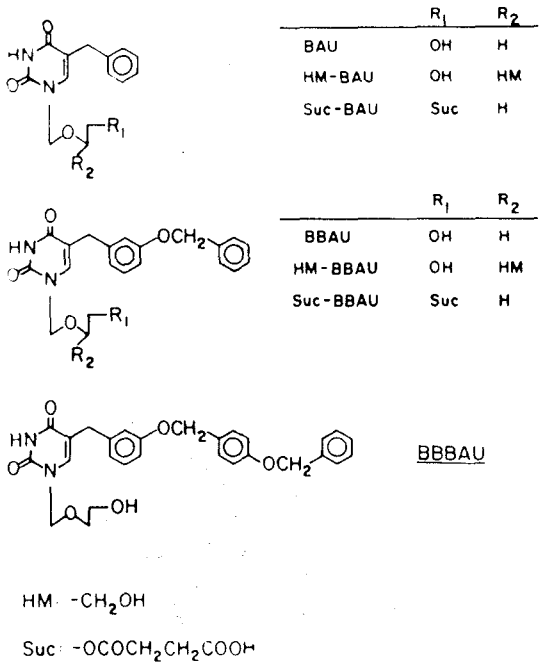

Fig. 1. The chemical structures of 5-benzylacyclouridines.

BAU and BBAU were also as good inhibitors of cytosolic UrdPase from human and mouse liver as their parent compounds (Naguib *et al.*, 1987). However, a further increase in hydrophobicity at C_5 position greatly reduced the potency of the inhibition. In the present study, we investigated the effects of the above compounds on the transport of Urd into human erythrocytes.

MATERIALS AND METHODS

Chemicals

HM-BAU, suc-BAU, HM-BBAU, suc-BBAU, and BBBAU were synthesized according to the procedure published elsewhere (Chu *et al.*, 1984; 1986). [2-^{14}C]Urd (58 mCi/mmol) was purchased from Moravek Biochemicals, Brea, CA. Unlabeled Urd and other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Transport Assay

Zero-trans influx of [^{14}C]Urd into human erythrocytes was determined as previously described (Lee *et al.*, 1984). Human erythrocytes were obtained from healthy individuals in our laboratory and washed 3 times in buffer A containing 140 mM NaCl, 5 mM glucose, 1.4 mM $MgSO_4$, and 18 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.4, and suspended in the same buffer to give about 2% hematocrit. A rapid sampling procedure employing an one-hand dual syringe apparatus equipped with a Y-bored mixing chamber was used to determine the initial velocity of Urd transport. Eppendorf microcentrifuge tube (1.5 ml capacity) containing 0.15 ml of an oil mixture (84% Dow Corning 550 silicone fluid-16 % paraffin oil) were placed in a microcentrifuge (Fisher Scientific Co, Model 235A). Aliquots of 0.1 ml (0.05 ml of cell suspension and 0.05 ml of substrate solution) were dispensed through a mixing chamber at 1.5 to 2 sec intervals into the centrifuge tubes and followed by immediate centrifugation for 30 sec to terminate the transport process. Thus, the shortest incubation period was about 2.5 sec which included the amount of time elapsed before the initiation of centifugation (0.5 sec) and the actual centrifugation time (2 sec) for the complete emergence of cells into the oil mixture. After removal of supernatant, the tubes were rinsed with water and subsequently removed along with most of the oil mixture. The erythrocyte pelets were solubilized in 0.75 ml of M-74 solution (2% ammonium bicarbonate/1% Triton X-100/1% trypsin) (Michaels *et al.*, 1979). The erythrocyte pellets incubated in 1 ml of toluene and 0.5 ml of 30% hydrogen peroxide. This was followed by one hour incubation at 37℃ and a cooling period of 3 hours at room temperature to ensure a complete decomposition of hydrogen peroxide. Radioactivity of the sample was determined in a Packard Tri-Carb 460 scintillation counter after adding 10 ml of ACS fluid. Initial slopes of the plot of cpm *vs* time were defined as the "initial influx velocities", since the noncarrier-mediated influx was negligible (Lee *et al.*, 1984). Kinetic parameters were calculated by a least-square fitting of Michaelis-Menten equation (Wilkinson, 1961; Cleland, 1961).

RESULTS

We have previously reported that BAU and BBAU competitively inhibited nucleoside transport in L5178Y cells and in human erythrocytes

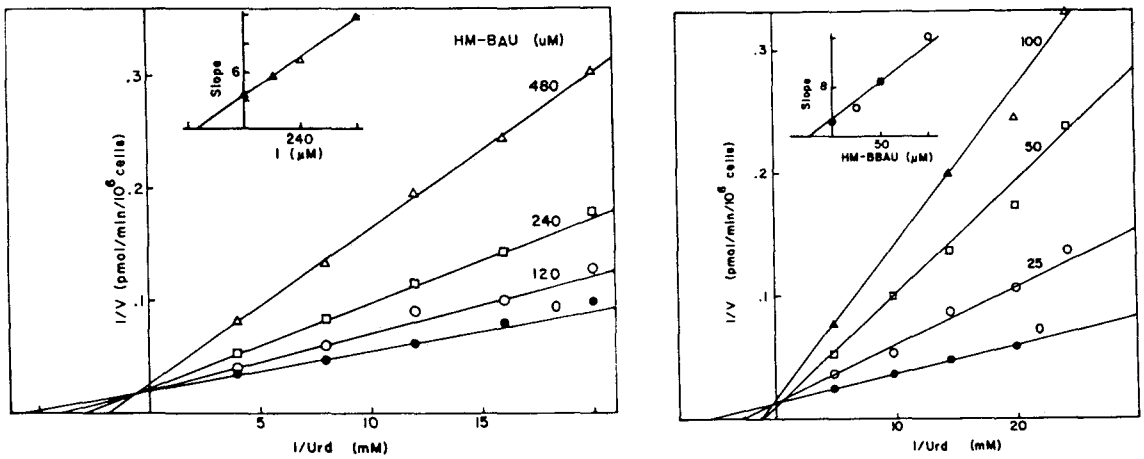

Fig. 2. Double-reciprocal plots of initial velocities of [^{14}C] Urd zero-*trans* influx into human erythrocytes at various concentrations of HM-BAU or HM-BBAU. Inset is a replot of the slopes of the double-reciprocal plot vs inhibitor contrations. The K_i values are 176 and 23 uM for HM-BAU and HM-BBAU, respectively.

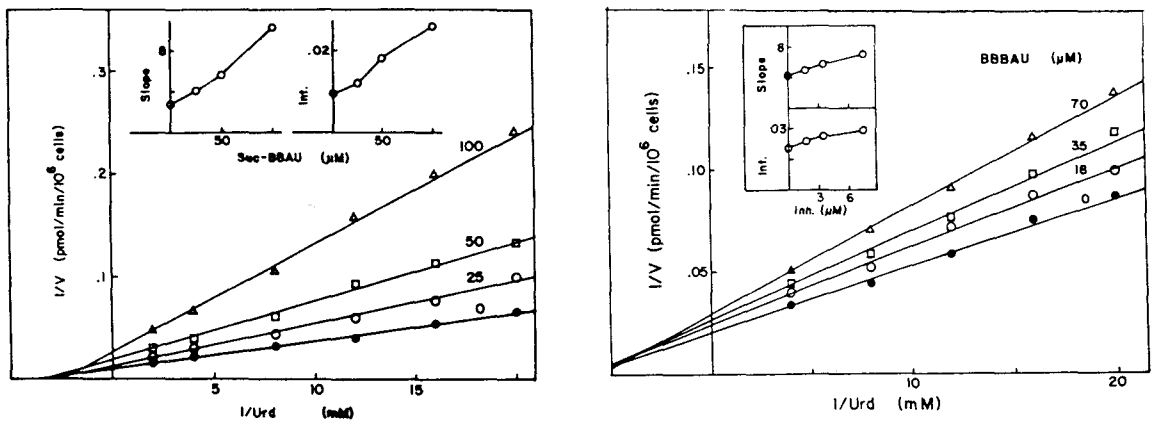

Fig. 3. Double-reciprocal plots of initial velocities of [^{14}C] Urd zero-*trans* influx into human erythrocytes at various concentrations of suc-BBAU or BBBAU. Insets are replots of the slopes and intersections of the double-reciprocal plot vs inhibitor concentrations. The K_i values are 38 and 112 uM for suc-BBAU and BBBAU, respectively.

(Lee *et al.*, 1984). The kinetics of [^{14}C]Urd zero-*trans* influx into human erythrocytes was studied by incubating the cells with a variety of benzylacyclouridines at [^{14}C]Urd concentrations ranging from 50 to 250 μM (Figures. 2 and 3). Double-reciprocal plot of initial velocities *vs* [^{14}C]Urd influx in Fig. 2 show that the transport is a saturable process and is competitively inhibited by both HM-BAU and HM-BBAU. In contrat, suc-BBAU and BBBAU (Fig. 3) exhibits a pattern of non-competitive inhibition. The results of similar experiments with other benzylacyclouridines are summerized in Table 1. The values of K_m and V_{max} from seven experiments are $191 \pm 65 \mu$M and 81 ± 33 pmol/min/10^6 cells. The K_i values of the derivative of BAU and BBAU are similar to those of their parent compounds (*i.e.*, BAU, 124; HM-BAU, 176; suc-BAU, 174 *vs* BBAU, 19; HM-BBAU, 23, suc-BBAU, 38μM). In most cases, the values of K_{ii} are musch greater than the values of K_{is}, which indicates a pattern of competitive inhibition. However, the similar values of K_{ii} and

Table 1. Inhibition constants of 5-benzylacyclouridines for the uridine[a, b] transport in human erythrocytes

Inhibitors	K_{is} (μM)	K_{ii} (μM)
BAU	124	1587
HM-BAU	176	1312
suc-BAU	174	1287
BBAU	19	364
HM-BBAU	23	1581
suc-BBAU	38	58
BBBAU	112	182

[a] Urine concentration range of 50 to 250 uM was used.

[b] The average value ± S.D. of K_m and V_{max} for uridine from 7 experiments are 191 ± 65 μM and 81 ± 33 pmol/min/10^6 cells.

K_{is} in suc-BBAU (38 *vs* 58μM) and BBBAU (112 *vs* 183 μM) suggest that the inhibitory pattern of these compounds contains some noncompetitive components. The potency of uridine transport inhibition of BBAU analogs is about 5 times greater than that of BAU analogs, while that of BBBAU fell in between the values of the above two groups.

DISCUSSION

The present studies demonstrated that the analogs of BAU and BBAU are competitive inhibitors of Urd transport in human erythrocytes. The analogs of BBAU are about 5 times more potent than those of BAU, although the potency of BBBAU is close to the value of BAU. These findings confirm the previous report that the potency of the nucleoside transport inhibitory activity of an N-6-(RCH_2S)-purine nucleoside is related to the number of carbon atoms in the substituent within bulk tolerance (Brajeswar *et al.*, 1975). A similar structure activity relatioship has been obtained with benzylacyclouridines in this study. Increasing hydrophobicity by substituting benzyl group at C_5 to benzyloxybenzyl group in 5-benzylacyclouridines decreases the K_i values by 5- to 8-folds. However, BBBAU which has an additional benzyloxy-group on th *para* position of the benzyloxybenzyl-group of BBAU exhibits a substatially higher K_i value (112μlM) as compared to that of BBAU (19μlM) or its analogs (23 and 38μM for HM-BBAU and suc-BBAU, respectively). These data suggest that there is a limit in the size at C_5 position.

Recently, Gati *et al.* (1984) reported that AcThd and AcUrd had very high K_i values (7.5 and 11 mM, respectively) as compared to the K_m value (98μlM) for zero-*trans* influx of dThd in mouse erythrocytes. In their studies, the substitution for a hydrogen in AcUrd by a methyl group at C_5 position to AcThd increased the affinity by 1. 5-folds. However, The substitution for a methyl group with a benzyl group at C_5 changes the K_i value from 7.5 mM to 198μM (Lee *et al.*, 1984). Since the K_m value fo 98μM for dThd falls within the reported ranges of 51 (Gati *et al.*, 1983) to 199μM (Lee *et al.*, 1984), it appears that there is no significant differnece in the nucleoside carrier systems between human and mouse erythrocytes. From these results, it is speculated that the strength of the hydrophilic interaction between 3'-OH group of dThd and the nucleoside transport carrier system is comparble to that of a hydrophobic interaction between C_5-benzyl group and the carrier system. The decrease in affinity to the carrier due to the absence of 3'-OH group in AcThd (*i.e.* 199μM to 7.5 mM) is roughly euqual to the increase caused by the presence of the benzyl group at C_5 position in BAU (7.5 mM to 198μM).

In addition to the inhibition of UrdPase, these benzylacyclouridines have been shown to increase the circulation plasma level of uridine (Handschumacher *et al.*, 1983; Monks *et al.*, 1983) and the salvage of the circulating uridine by various tissues (Handschumacher *et al.*, 1983). A similar increase in the plasma uridine level has been also observed after the administation of NBMPR-phosphate, a prodrug form of a specific inhibitor of nucleoside transport (Handschumacher *et al.*, 1983). Thus, we have proposed that those effects of BAU on the level of circulating Urd may be the combined results of the inhibition of UrdPase and the Urd transport (Lee *et al.*, 1984). Darnowski and Handschumacher (1984) have also demonstrated that BAU administered alone or with low doses of Urd can increase the therapeutic effectiveness of FUra in mice bearing colon tumor 38. They have found that the increase in the therapeutic effectivenss is due to elevated Urd nucleotide pools in host tissues, while BAU decreases uridine nucleotide pools in tumor tissues (Darnowski and Handschumacher, 1985). However, FUra+BAU have not increased the life span of mice bearing

L5178Y cells[1].

It is difficult at the present ime to assess the relative degree of contribution of BAU (*i.e.,* the inhibition of UrdPase and the inhibition of Urd transport) to the increased level of circulating Urd level (Handschumacher *et al.,* 1983; Monks *et al.,* 1983; Levy *et al.,* 1982; Moyer *et al.,* 1985) and its half life (Handschmacher *et al.,* 1983; Moyer *et al.,* 1985). The benzylacyclouridines have roughly equal ratios of UrdPase inhibitor/nucleoside transport inhibitor potency. Therefore, it would be interesting to test the effect of AcThd, a UrdPase inhibitor (Niedzwicki *et al.,* 1981) with no effect on the nucleoside system (Gati *et al.,* 1984), on the level of circulating Urd.

In conclusion, the results obtained in the present study have demonstated that benzylacyclouridines competitively inhibit the transport of Urd in human erythrocytes. The degree of inhibition correlates with the increase in hydrophobicity within the benzyloxybenzyl group size at C_5 position. It needs to be further investigated what is the precise mechanism by which benzylacyclouridines increase the concentration of circulating Urd as well as the spectrum of tumors responding to the combination therapy with FUra or FdUrd.

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═국문초록═

Benzylacyclouridines의 적혈구에 있어서 Nucleoside 수송 억제

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이 강 현, 차 승 만

Uridine phosphorylase 효소의 강력한 억제제로 개발된 유리딘 비고리핵산들(Benzylacyclouridine: BAU, HM-BAU, suc-BAU, BBAU, HM-BBAU, suc-BBAU, and BBBAU)이 uridine의 사람 적혈구 내로의 수송(Zero-*trans* influx)에 미치는 영향에 관하여 rapid sampling technique을 이용하여 고찰하였다.

이 실험에서 유리딘-비고리핵산들은 parent compounds인 BAU, BBAU와 같이 uridine 수송에 상경적인 억제작용을 보였다. 그러나 suc-BBAU와 BBBAU는 비상경적인 억제요소도 나타냈다. Uridine 수송억제제로서 효력의 크기는 BBAU～HM-BBAU～suc-BBAU>BBBAU>BAU～suc-BAU～HM-BAU이었으며, 그 억제상수는 각각 19, 23, 38, 112, 124, 174 그리고 176 μM 이었다.

본 실험 결과에서는 uridine 수송에 있어서 BAU의 C_5 자리의 친지방성 치환 group의 크기에 따라 억제효력이 다른 것이 시사되었다.