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☐ Review Article ☐

Development of Inhibitors of Pyrimidine Metabolism

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Benzylacyclouridines were developed as specific and potent competitive inhibitors of uridine phosphorylase with K_i values in the nanomolar range. These compounds have no activity against thymidine phosphorylase, uridine kinase, thymidine kinase and orotate phosphoribosyltransferase. Benzylacyclouridines potentiate the chemotherapeutic effect of FdUrd. Coadministration of uridine phosphorylase inhibitor with FdUrd caused selective toxicity against tumors with low or no thymidine phosphorylase, but not against the host tissues which have thymidine phosphorylase, and thus retain the capacity to cleave FdUrd, and hence overcome its toxicity. There are distinct differences between uridine phosphorylase and thymidine phosphorylase. Benzylacyclouridines competitively inhibit the nucleoside transport of mammalian cells. The structure-activity relationship of inhibitors of uridine phosphorylase showed that a large hydrophobic pocket exists where C-5 of uracil binds, and that it is necessary to have the 3'-hydroxyl group and syn-configuration around the N-glycosidic bond for the nucleosides or their analogs to bind. Dihydrouracil dehydrogenase was found to be widely distributed among mammalian cells, where it was previously believed to be present only in the liver and the kidney. The structure-activity relationship of its inhibitors revealed benzyloxybenzyluracil and 2,6-pyridinediol as most potent. Also identified for orotate phosphoribosyltransferase was 2,4-pyridinediol.

Key Words: Uridine phosphorylase inhibitors, benzylacyclouridine, dihydrouracil dehydrogenase inhibitors, chemotherapy, FUra, FdUrd, benzyloxybenzyluracil, 2,6-pyridinediol, 2,4-pyridinediol

All cells maintain adequate supplies of nucleotides of pyrimidines along with purines by a *de novo* synthetic pathway or a salvage pathway, or both, and mammals maintain their homeostasis by controlling synthesis and degradation of these nucleosides. Pyrimidine biosynthesis may be represented by Fig. 1. Starting from aspartate, ammonia and ATP to UMP represents the *de novo* synthetic pathway. The conversion of nucleosides and bases to UMP represents the salvage pathway (Fig. 2). In mammalian cells, uracil, uridine, cytidine, and thymine as well are known to incorporate into nucleic acids via the salvage pathway, but not thymine under normal conditions, cytosine, or deoxycytosine. Among reported pyrimidine salvage enzyme deficiencies are the reduced dThdPase activity in leukemic lymphocytes (Lazarus *et al.* 1974; Fox *et al.* 1979; Kufe *et al.* 1980) and in colon tumors (Niedzwicki *et al.* 1981), the deficiency of pyrimidine-5'-nucleotidase in hemolytic anemia (Valentine *et al.* 1974), the deficiency of 5'-nucleotidase in lymphocytic leukemia (Fox *et al.* 1980), and the deficiency of UMP kinase in immune defective individuals (Giblett *et al.* 1974; Petersen *et al.* 1985). The pathway from UMP to nucleic acids is common to both *de novo* and salvage pathways. The catabolic pathway consisting of three enzymes, dihydrouracil dehydrogenase (DHUDase[1]), dihydropyrimidinase, and β-ureidopropionase is responsible in mammals for degradation of the pyrimidine bases, uracil or thymine to their β-amins acids. (Fig. 2). Absence of the first enzyme, DHUDase, is associated with severe neurological disorders including autism (Wadman *et al.* 1984; Tuckman *et al.* 1985). Neurological disorders also result from hyper-β-alaninemia (Scriver *et al.* 1983). β-Alanine is also implicated in the regulation of body temperature (Scriver *et al.* 1983; Peters *et al.* 1987; Griffith 1986). It should be noted that uracil is

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[1]Abbreviations: BAU, 5-benzylacyclouridine; BBAU, 5-(*m*-benzyloxybenzyl) acyclouridine; BU, 5-benzyluracil; BBU, 5-benzyloxybenzyluracil; DHUDase, dihydrouracil dehydrogenase (Ec 1.3.1.2); dThdPase, thymidine phosphorylase (EC 2.4.2.4); FdUrd, 5-fluoro-2'-deoxyuridine; 5'-FdUrd, 5-fluoro-5'-deoxyuridine; FUra, 5-fluorouracil; HMBAU, hydroxymethyl-BAU or 5-benzyl-1-[(1, 3-dihydroxy-2-propoxy) methyl] uracil; HM-BBAU, hydroxymethyl-BBAU; OPRTase, Orotate phosphoribosyltransferase (EC 2.4.2.10); ODCase, Orotidine-5'-phosphate decarboxylase (EC 4.1.1.23); UrdPase, uridine phosphorylase (EC 2.4.2.3).

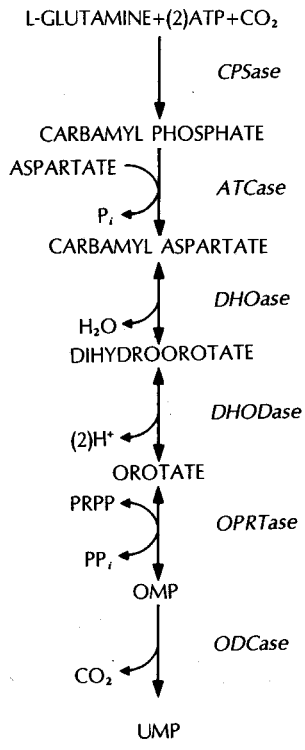

Fig. 1. *The de novo synthetic pathway of pyrimidine nucleotide.*

the sole source of newly synthesized endogenous β-alanine, the precursor of the neurotransmitters, carnosine and anserine. These enzyme deficiency syndromes suggest that temporary interference using various inhibitors of these enzymes is likely to result in cytotoxicity, and therefore could be exploitable for chemotherapy.

There are two similar enzymes for pyrimidine nucleoside phosphorolysis which are the major subject of this article. In the normal mammalian cells, usually uridine and thymidine serve as substrates for UrdPase and dThdPase respectively, and FdUrd serves as a substrate for both enzymes. Although the two phosphorylases share in substrate specificity, they exhibit striking differences in their preferences for the nucleoside substrate (Niedzwicki *et al.* 1981; Niedzwicki *et al.* 1983). Moreover, it became apparent from our work and others that there are distinct differences between these two enzymes besides differential substrate specificity. Most analogs which inhibit UrdPase from all species tested so far do not affect dThdPase, and the kinetic mechanisms of the two enzymes isolated from the same tissue are not identical. Therefore the two enzymes must have fundamental structural differences (Niedzwicki *et al.* 1983). Also it was concluded that, under physiological conditions, UrdPase acts in both catabolic and anabolic directions, but dThdPase always acts toward catabolism (Naguib *et al.* 1987a).

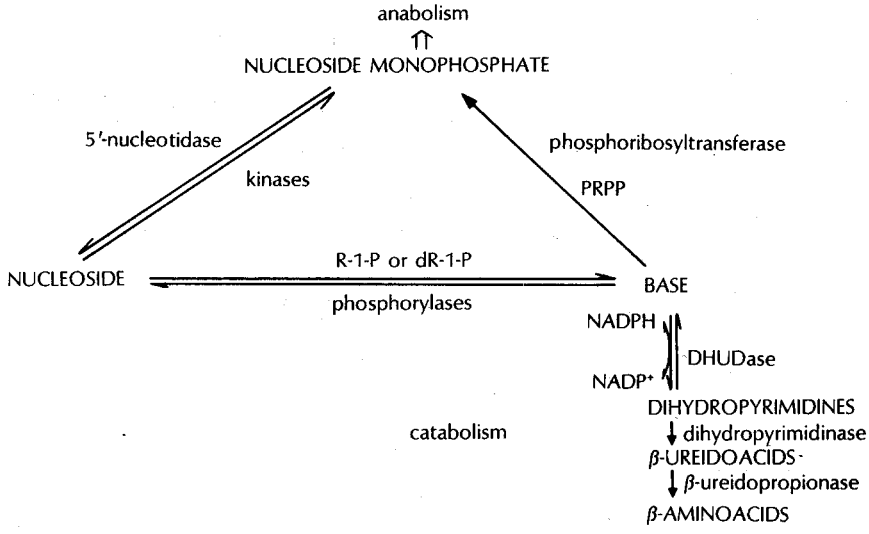

Fig. 2. *The salvage and degradation pathways of pyrimidine nucleotides.*

Most cancer chemotherapeutic pyrimidine analogs must be administered as nucleosides or bases to facilitate transport into cells. However, almost all must be converted to the nucleoside 5′-monophosphate level by the salvage enzymes before their anticancer activity can be realized. These drugs are also catabolized by salvage enzymes, usually resulting in a loss of effectiveness. Hence, the balance between the anabolic (activation) and catabolic (inactivation) functions of the salvage enzymes must be carefully considered when designing or choosing a chemotherapeutic regimen for various malignancies. Many of the neoplastic tissues are deficient in dThdPase, especially human colon tumors (Niedzwicki *et al.* 1981). Therefore, in most of the tumors, FdUrd is degraded by UrdPase but not dThdPase, thus providing the rationale for the enhanced cytotoxicity of FdUrd in the presence of a specific UrdPase inhibitor. In the following, the highlights of the investigations carried out in my own and other colleagues' laboratories will be presented.

DEVELOPMENT OF BAU AND CONGENERS

History

On the afternoon of March 13th, 1980, Shih Hsi Chu, the organic chemist of our Section, casually mentioned to me that he had synthesized acyclo-analogues of pyrimidines. Acycloguanine, synthesized by the Burroughs-Wellcome group, has become famous for its antiherpetic action and was renamed as acyclovir (Schaffer *et al.* 1978; Elion, 1988). Therefore, it was logical for Chu to synthesize similar compounds where the guanine part was replaced by various pyrimidines and to test against various viruses. But this initial guess proved to be wrong. They had no antiviral activity. Chu gave me some acyclo-compounds and asked me if I could find some use for them. I asked John G. Niedzwicki to test whether or not the acyclo-compounds inhibit the pyrimidine phosphorylases. Niedzwicki was a graduate student who was working on various pyrimidine metabolizing enzymes, particularly nucleoside phosphorylases. Next morning, tired but excited, Niedzwicki reported to me that the compounds were good inhibitors of uridine phosphorylase but not of thymidine phosphorylase. He must have worked all night to be able to make this report. Thus, the search for inhibitors of uridine phosphorylase and other pyrimidine metabolizing enzymes in my laboratory started.

Pyrimidine Acyclonucleosides

The most potent of the pyrimidine acyclonucleosides tested against UrdPase from S-180 cells was acyclothymidine (5-methyl-1-(2′-hydroxyethoxymethyl) uracil) with a K_i value of 3μM. K_i values of less than 30μM were estimated for the acyclo series of uridine, fluorouridine, bromouridine and iodouridine, but replacement of the hydroxyl group on the acyclo-tail abolished the inhibitory activity, suggesting the importance of this group. These compounds have no inhibitory activity toward dThdPase, uridine kinase, or thymidine kinase. This lack of activity was found to be true with all UrdPase inhibitors of the BAU series which were developed later. Extracts of xenografts from six human tumors were assayed for tissue levels of UrdPase and dThdPase, and for inhibition of FUrd phosphorolysis by acyclouridine. FUrd cleavage was inhibited more in those tissues in which the ratio of dThdPase to UrdPase was low (Niedzwicki *et al.* 1981).

BAU and BBAU

Having proved that the pyrimidine acyclonucleosides are much more potent than the normal sugar conjugates, Niedzwicki speculated that he might make better inhibitors if he could combine the acyclo-moiety and already known inhibitory bases such as BU and BBU (Baker and Kelly 1970; Woodman *et al.* 1981). This prediction proved correct when Chu synthesized and Niedzwicki tested such compounds. The structures of BAU and BBAU are shown in Fig. 3. Uracil was modified by attaching the 2′-hydroxyethoxymethyl group at the 1-position, and a benzyl or a benzyloxybenzyl group to the 5-position. BAU (K_i=98nM) was derived from BU (K_i=1575nM), as a result, an enhancement of potency of about 15-fold was achieved. Similar results were obtained with BBAU (K_i=32nM) derived from BBU (K_i=270μM). The inhibition patterns were competitive with respect to uridine. Mouse liver cytosol enzymes and the enzymes in S-180 cells were identical with regard to the lack of activity toward thymidine and the complete inhibition of UrdPase activity (Niedzwicki *et al.* 1982).

Other Congeners

HM-BAU (hydroxymethyl BAU) and HM-BBAU are the compounds that have one extra hydroxymethyl group on the acyclo portion of the parent compounds BAU and BBAU. They were synthesized by Chu's laboratory and their properties were tested. Apparent

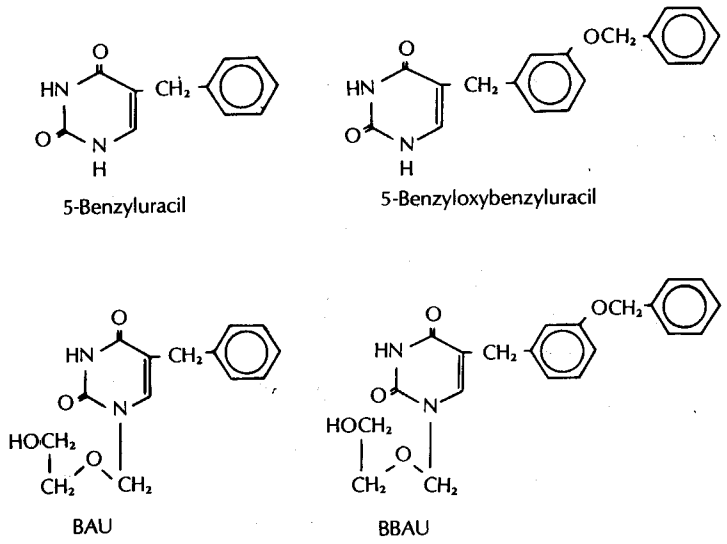

Fig. 3. *The chemical structures of some inhibitors.*

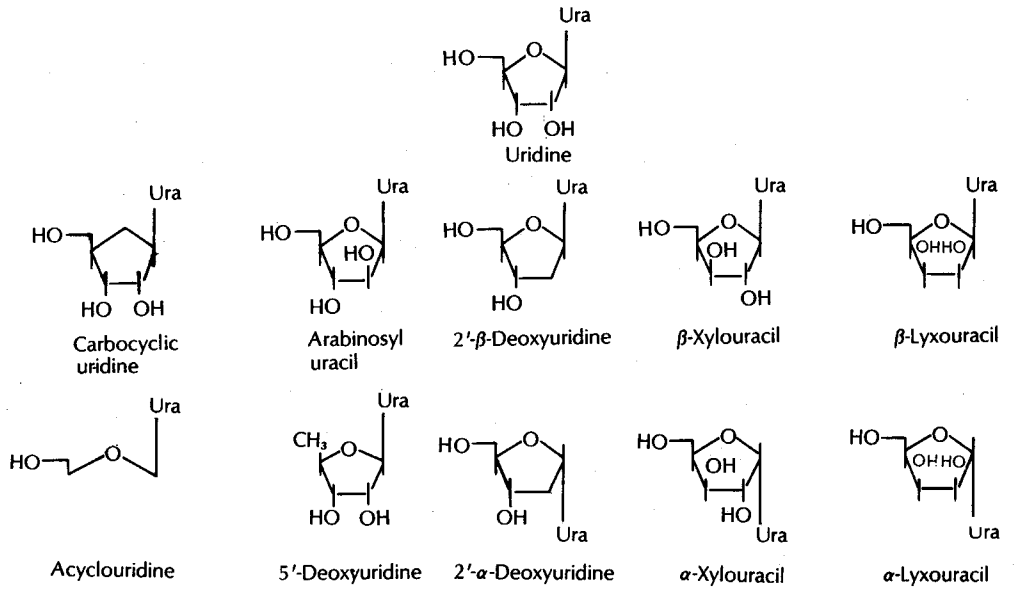

Fig. 4. *The chemical structures of uridine and various types of analogues modified in the pentose moiety. Reproduced by permission of American Society for Pharmacology and Experimental Therapeutics, el Kouni MH, Naguib FNM, Chu SH, Cha S, Ueda T, Gosselin G, Imbach J-L, Shealy F, and Otter BA: Mol Pharmacol 34:104-110, 1988.*

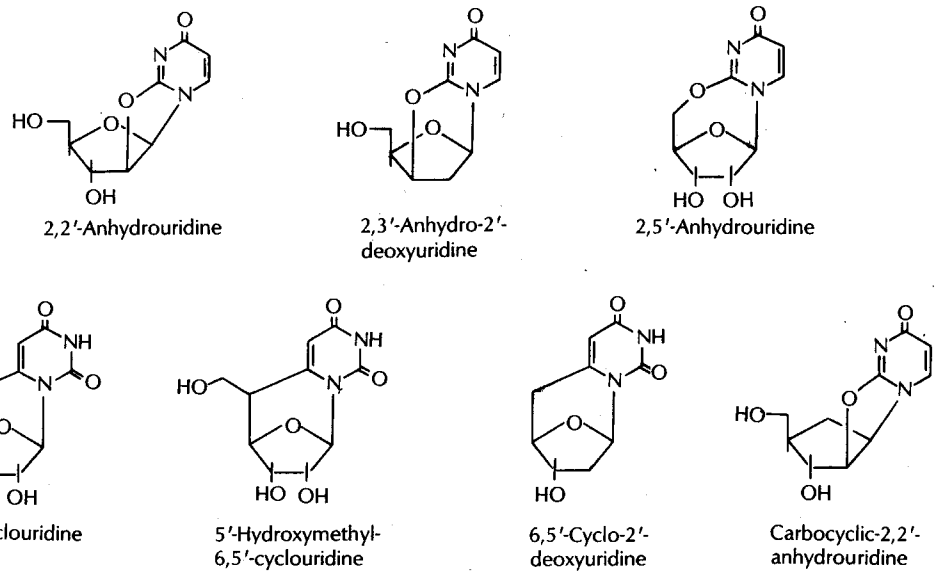

Fig. 5. *The chemical structures of various types of anhydrouridines, cyclouridines, and carbocyclic anhydrouridines. Reproduced by permission of American Society for Pharmacology and Experimental Therapeutics, el Kouni MH, Naguib FNM, Chu SH, Cha S, Ueda T, Gosselin G, Imbach J-L, Shealy F, and Otter BA: Mol Pharmacol 34:104-110, 1988.*

K_i values of 1.6 and 0.32μM respectively for the enzyme from mouse liver, and 2.5 and 0.65μM respectively for the human liver were obtained (Chu *et al.*, 1984). Fifteen new compounds in addition to BAU, BBAU, HM-BAU and HM-BBAU were synthesized and tested for UrdPase inhibitory activity, from which much of the structure-activity relationship of acyclouridines was learned. Aminomethyl-BBAU (5-(3'-benzyloxybenzyl)-1-[(1'-aminomethyl-2'-hydroxyethoxy) ethyl] uracil) with a K_i value of 18nM with UrdPase from mouse liver was the most potent, and all inhibitors were competitive (Naguib *et al.* 1987). Some, notably aminomethyl-BAU, have a much better water solubility hence less problems of handling, and others, like succinyl derviatives, have potential use as affinity chromatography material for UrdPase. A Hungarian group (Veres *et al.* 1985) reported that 2,2'-anhydrouridine and its derviatives are potent inhibitors of UrdPase. el Kouni thought that if we could combine the features from BU or BBU and 2,2'-anhydro uridine, we might have better inhibitors of UrdPase. Two compounds, 5-benzyl and 5-benzyloxybenzyl analogs of 2,2'-anhydro-uridine (See Fig. 5) were synthesized. When the analogs were tested, they were found to inhibit slightly better than the acyclo-compounds but less than the best anhydro compound, 2,2'-anhydro-5-ethyluridine (Chu *et al.* 1988; el Kouni *et al.* 1988).

PROPERTY OF BAU AND CONGENERS

Activities against pyrimidine nucleoside phosphorylases from various sources

Acyclopyrimidines were tested against enzymes from S-180 and L-5178Y cells (Niedzwicki *et al.* 1981), mouse liver (Niedzwicki *et al.* 1981; Naguib *et al.* 1987; Chu *et al.* 1988), human liver (MYW Chu *et al.* 1984; Chu *et al.* 1984; Chu *et al.* 1988), xenografts of human tumor in mouse (DLD-1, DLD-1D, DLD-2, HCT-15, DLD-1A, RWP-1, XL-1 and DAN) (Niedzwicki *et al.* 1981; MYW Chu *et al.* 1984), E. coli (Park *et al.* 1986), and *Schistosoma mansoni* (el Kouni *et al.* 1988). Competition with uridine as the substrate of UrdPase was always observed, and no inhibitory activity against dThdPase, thymidine kinase nor uridine kinase was found (Niedzwicki *et al.* 1981; Niedzwicki *et al.* 1982). In *S. mansoni,* BAU and congeners have no effect on the phosphorolysis of adenosine, inosine, and guanosine, and the phosphorolysis of thymidine was carried out by UrdPase because it can be completely inhibited by BAU, thus there is no thymidine phosphorylase (el Kouni *et al.* 1988).

Table 1. Selected K_i values with Mouse Enzymes, μM

Apparent K_i values are estimated by Dixon's method (Dixon and Webb 1979) at 0.15mM ^{14}C-uridine for UrdPase, 0.15mM ^{14}C-thymine for dThdPase, 0.1mM ^{14}C-FUra for dThdPase, and 0.025mM [6-^{14}C]-uracil for DHUDase. Those items marked with [a] designate the true K_i values, * not inhibitory, and ND not determined.

	UrdPase	dThdPase	OPRTase	DHUDase
BAU	0.42±0.04[a]	*	*	*
HM-BAU	0.09±0.004[a]	*	ND	ND
BBAU	0.17±0.00[a]	*	ND	ND
Succinyl-BBAU	0.38±0.01[a]	*	ND	ND
BBU	1.5 ±0.3	*	ND	0.2±0.005
BU	3.7 ±0.4	*	ND	3.4±0.042
6-Benzyl-2-thiouracil	*	181±95	*	378±261
1-Deazauracil (2,6-Pyridinediol)	3.4 ±0.4	433±77	2640±1300	0.5±0.005
3-Deazauracil (2,4-Pyridinediol)	*	*	261± 82	2.1±0.2
5-Nitrobarbituric acid	ND	ND	ND	3.8±0.3
5-Azaorotic acid (Oxonic a.)	ND	ND	45± 16	ND

UrdPase vs. dThdPase

These two enzymes are similar in that they both catalyze the phosphorolysis of pyrimidine nucleosides, uridine and thymidine, and their substrate specificities overlap. UrdPase primarily cleaves uridine, but is relatively non-specific as it also cleaves dUrd, dThd, FdUrd (Krenitsky *et al.* 1964; Pontis *et al.* 1961; Niedzwicki *et al.* 1983) and 5'-FdUrd (Ishitsuka *et al.* 1980). Moreover, in tissues such as S-180, L1210 and L5178Y cells (Niedzwicki *et al.* 1981), *S. mansoni* (el Kouni *et al.* 1988) and *Giardia lamblia* (Jiménez *et al.* 1989), UrdPase is the sole pyrimidine nucleoside phosphorylase. dThdPase primarily cleaves thymidine, and is specific for deoxyribonucleosides, as it also cleaves deoxyuridine, FdUrd and 5'-FdUrd, but not uridine (Birnie *et al.* 1963; Prusoff 1963; Zimmerman and Seidenberg 1964; Nakayama *et al.* 1980; Kono *et al.* 1983; Kono *et al.* 1984; Meynial *et al.* 1986).

Although UrdPase and dThdpase are quite similar, there are distinct differences between these two enzymes. They are easily separable from each other in mouse liver extracts by DEAE-cellulose column chromatography, dThdPase eluting in 20mM phosphate buffer wash and UrdPase appearing approximately after a 0.5M NaCl gradient (Niedzwicki *et al.* 1981). Their kinetic properties are different. Initial velocity and product inhibition studies of thymidine phosphorylase from mouse liver revealed that the basic mechanism of this enzyme is a rapid equilibrium random bi-bi mechanism with an enzyme-phosphate-thymine dead-end complex (Iltzsch *et al.* 1985), and Urdpase from the same source exhibited a different mechanism (Park 1988). The properties toward inhibitors are distinctively different. Most inhibitors of UrdPase have no activity on dThdPase. This may mean that dThdPase does not possess as large a hydrophobic pocket as the one postulated to exist adjacent to the binding site of *C*-5 of uracil in UrdPase. In any case, one of the most useful properties of BAU is its use in determining by which enzyme, UrdPase or dThdPase or both, a tissue degrades uridine, thymidine or FdUrd. Metabolic roles of these two enzymes in the cell seem to be different. Although equilibrium constants favor nucleoside synthesis *in vitro* under physiological conditions, the phosphorylase reactions are generally considered unidirectional toward formation of the bases due to the relatively high inorganic phosphate and low pentose-1-phosphate concentrations in the cell. However, there are some exceptions where anabolism of uracil and FUra proceeds mainly through the phosphorylase step (Sköld 1960; Cantarow *et al.* 1961; Pontis *et al.* 1961; Reichard *et al.* 1962; Laskin *et al.* 1979; Ardalan *et al.* 1980; Ikenaka *et al.* 1981; Raisonnier *et al.* 1981; Naguib *et al.* 1987). Unlike UrdPase, however, dThdPase seems to function as a catabolic enzyme under the physiological conditions (Naguib *et al.* 1987; Breitman *et al.* 1966).

Nucleoside transport inhibition

Lee studied the effect of BAU and BBAU on the cytotoxicity of FUrd or FdUrd in cultured L5178Y cells. Contrary to expectations, BAU and BBAU did not potentiate the cytotoxicity of the fluorinated nucleosides but inhibited the total cellular incorpora-

tion of radiolabeled uridine in exponentially growing L5158Y cells. Using a rapid sampling procedure, Lee could demonstrate that BAU and BBAU competitively inhibit the transport of uridine, thymidine, and adenosine into human erythrocytes and murine L5178Y cells. The K_i values of BAU and BBAU were approximately 130μM and 15μM respectively for human erythrocytes, and 230μM and 35μM respectively for L5178Y cells (Lee *et al.* 1984). The data were negative when BAU was tested in *S. mansoni* with tubercidin and nebularine to see whether or not it protects the host as other nucleoside transport inhibitors like nitrobenzylthioinosine 5'-monophosphate, dilazep, and dipyridamole did (el Kouni *et al.*, 1987).

Circulating uridine concentration

The work on the effect of BAU on plasma uridine concentrations was primarily done by Handschumacher and his colleagues at Yale University. UrdPase has been implicated in the maintenance of the fairly high concentration (~1.4μM) of plasma uridine. The liver of rat and mouse takes up and catabolizes more than 70% of plasma uridine entering the organ, yet at the same time, it contributes an equivalent amount of newly synthesized uridine to the blood, thus maintaining the steady level of plasma uridine (Gasser *et al.* 1981; Moyer *et al.* 1981; Monks and Cysyk 1982). Therefore, the liver is the important regulator as well as the key producer of plasma uridine. UrdPase in the hepatic nonparenchymal endothelial and Kupffer's cells is the first step in elimination of plasma uridine. Administration of UrdPase inhibitors, BAU or BBAU, caused a marked increase in the level of plasma uridine (Monks *et al.* 1983; Darnowski and Handschumacher 1985) as well as the salvage of uridine by various tissues (Monks *et al.* 1983; Darnowski and Handschumacher 1989). This in turn implies that there must be certain tissues that rely primarily on the salvage of circulatory uridine. Indeed rat intestinal mucosa (and by inference, cancer cells derived from colon) appears to be one of those tissues because it has been reported to have little or no *de novo* pyrimidine synthesis (Ikenaka *et al.* 1981; Raisonnier *et al.* 1981) and very high UrdPase activity (Ikenaka *et al.* 1981; Maehara *et al.* 1981).

Structure-activity relationship of inhibitors of pyrimidine nucleoside phosphorylases

Eighty-seven pyrimidine bases and nucleoside analogs were evaluated as inhibitors of UrdPase and dThdPase. These findings, together with an extensive literature review, have allowed construction of structure-activity relationships for the binding of ligands to UrdPase and dThdPase. Generally the intact *C-2, N-3* and *C-4* positions of the uracil ring are required for the binding, modifications at *C-1* and *C-5* positions give the most versatile diversification in the binding of ligands, substrates or competitive inhibitors, and *C-6* position can accommodate some alterations but not as much as *C-1* or *C-5*. It is believed that UrdPase has a large hydrophobic pocket adjacent where *C-5* carbon binds, whereas dThdPase has such a pocket but much smaller in size. Aside from the BAU series, 2,6-pyridinediol (1-deazapyrimidine) has been identified as being a potent inhibitor of UrdPase (K_i=3.4μM), and 6-benzyl-2-thiouracil as a specific inhibitor of dThdPase (K_i=180μM). The 5-halogenated or 5-nitro-uracils have K_i values of 2-60μM against UrdPase which are about 1/3 of the K_i against dThdPase respectively, but these are, although not tested, most likely alternative substrates (Niedzwicki *et al.* 1983). el Kouni investigated the modifications in the pentose moiety on the binding of nucleoside ligands to UrdPase. In order to do this, he gathered pyrimidine nucleoside analogs from all over the world (Fig. 4 and Fig. 5). and compared their effects. As a result, he could draw two important conclusions that, in order for any nucleoside analog to bind to UrdPase, the 3'-hydroxyl group of the would-be sugar moiety of uridine must be present, and that the *syn*-conformation around the *N*-glycosidic bond is required (el Kouni *et al.* 1988). The importance of the 3'-hydroxy group was suspected from earlier work on acyclopyrimidines (Etzhold *et al.* 1968; Niedzwicki *et al.* 1981) but was never proven before.

CANCER CHEMOTHERAPY

Potentiation of the effect of fluorinated uridines

UrdPase inhibitors potentiate the chemotherapeutic effects of fluorinated uracil compounds. The results of treatment with the human pancreatic carcinoma (DAN) line have shown that BBAU at up to 10μM, added 5 minutes prior to the addition of FdUrd (0.1 and 1μM), enhanced FdUrd cytotoxicity as evaluated by both counting cultured cell numbers and by the soft agar cloning method. The capacity to enhance cytotoxicity was much greater with DAN than LX-1, a human small cell lung carcinoma line. BBAU also potentiated the effects of FdUrd on reducing the tumor weights of DAN xenograft in mice immunosuppressed by antithymocyte serum. Approximately one-third of the dose of FdUrd was needed to have the same effect

as FdUrd alone. The pyrimidine enzymes measured in DAN and LX-1 revealed some significant differences. When BBAU is added, cleavage of FdUrd will solely depend on dThdPase. DAN has almost a two-fold lower dThdPase activity than LX-1. Therefore, BBAU is more effective in DAN than in LX-1 (MY Chu *et al.* 1984). This is consistent with the earlier results that showed the higher the dThdPase/UrdPase ratio, the lower the percent inhibition of FdUrd phosphorolysis by acyclouridine (Niedzwicki *et al.* 1981). Furthermore, since levels of thymidine kinase have been shown to be elevated in most tumors, as opposed to non-dividing tissues which virtually lack this enzyme (Kit 1976), selective toxicity of FdUrd may be enhanced by coadministration of UrdPase inhibitors. Subsequently the human pancreatic carcinoma cell line, DAN, was used to test the effectiveness of BAU congeners HM-BAU and HM-BBAU (Chu *et al.* 1984), and 5-benzyl- and 5-benzyloxybenzyl-2,2'-anhydrouridines (Chu *et al.* 1988).

Uridine rescue of FUra toxicity

BAU was shown to "rescue" animals from FUra toxicity, and the combination was selectively toxic against a colon tumor (Klubes *et al.* 1982; Klubes *et al.* 1983; Martin *et al.* 1982: Darnowski and Handschumacher 1985). This effect was attributed to the elevation of plasma uridine by BAU and to the less efficient salvage of uridine by colon tumor-38 than by normal tissues (Darnowski and Handschumacher 1985).

Direct effect of UrdPase inhibition *vs.* indirect effect through uridine rescue

As one may see, UrdPase inhibitors prevent the degradation of FdUrd and FUrd, hence the better the chance of fluorinated nucleosides to be converted to nucleotides, thus leading to more cytotoxicity. On the other hand, these inhibitors cause increased circulating uridine, thus interfering with the formation of fluorinated uridine nucleosides, resulting in less cytotoxicity. These two facts are seemingly paradoxical for chemotherapy. Perhaps the reason that both interpretations are correct is as follows. When FdUrd or FUrd was used in combination with benzylacyclouridines, the retention of more uncleaved fluorinated uridines, hence more conversion to nucleotides in tumor cells, caused the potentiation because most of the tumor cells have no or little dThdPase (Niedzwicki *et al.* 1981), but normal cells having dThdPase can degrade fluorinated uridines in the presence of one of the benzylacyclouridines. On the other hand, when FUra and one of the BAU congeners were administered, as in the case of colon tumor-38 (Darnowski and Handschumacher 1985), the high concentration of circulating uridine due to BAU rescued the normal tissues more than the tumor cells. In one case, the increase of cytotoxicity to the cancer cells, and in the other, lowering of toxicity to the normal cells may be involved, and in either case an increase of therapeutic index will be realized. However, formulation of the exact mechanisms by which UrdPase inhibitors potentiate the chemotherapeutic effect of fluorinated uracils and fluorinated uridines must await further investigation. Among the factors to be considered are the dosage, the temporal relationship of administration of the two drugs, the role of inhibition of nucleoside transport, and of course the nature of the tumor itself. Also to be considered is the possibility that human tumor xenografts react differently from the host mouse tissues. For instance, the enzymes in xenografts being those of human origin, may react in a different way from those of the host.

Toxicity, pharmacokinetics and metabolism

The BAU series of compounds seem to be remarkably nontoxic drugs. However, there has never been a systematic survey of toxicity in small animals or Phase-I studies in humans. The closest to the description of toxicity was what was described in a publication of MYW Chu *et al.* (1984) that LD50 by inference was much greater than 100 mg/kg/day for 2 days in $B6D2F_1$ mice.A thorough study of pharmacokinetics was done by Darnowski and Handschumacher (1988) in C57B1/6 female mice. They found various pharmacokinetic parameters after i.v. injection and oral administration. The plasma clearance of BAU after i.v. administration followed first-order kinetics with a half life of approximately 36 min. They also recovered 41% of the injected dose of BAU intact in urine within 24 hr. and another 27% appeared as a more polar metabolite which has lost the UrdPase inhibitory activity (Darnowski and Handschumacher 1988).

Interests in the UrdPase inhibitors in other laboratories

Interests in BAU and BBAU in other laboratories may be illustrated by the literature on the synthesis of related compounds by Chu *et al.* (1986, 1987), Lin *et al.* (1985) and Siegel *et al.* (1985), on X-ray and H^1nmr analysis of BBAU by Birnbaum (*et al.* 1986), on UrdPase inhibition by Siegel *et al.* (1985) and Drabikowska *et al.* (1987), on the effect on circulating uridine by Monks *et al.* (1983), on the pharmacokinetics by Peters *et al.* (1987), on the effect of

BAU in uridine rescue of FUra therapy (Martin *et al.* 1989), and on inhibition of UrdPase from *Giardia lamblia* (Jiménez *et al.* 1989).

OPRTase AND STRUCTURE ACTIVITY RELATIONSHIP OF ITS INHIBITORS

In *S. mansoni,* the major product of orotate metabolism was OMP, whereas in mouse liver it was UMP, suggesting that OPRTase is not tightly coupled to ODCase in *S. mansoni* (Iltzsch *et al.* 1984; Jones 1980). Eighty pyrimidine base analogs were evaluated as inhibitors of mouse liver OPRTase. 4,6-Dihydroxypyrimidine has been found to be a potent OPRTase inhibitor with an apparent K_i value of 15μM for the mouse liver enzyme (Niedzwicki *et al.* 1984a). In *S. mansoni,* two distinct OPRTases were found. One OPRTase isozyme, having the higher molecular weight, utilized orotate, 5-fluorouracil and uracil as substrates, while the other only utilized orotate. Both enzymes were inhibited by 5-azaorotic acid (oxonic acid), with the composite apparent K_i value of 0.9μM for *S. mansoni* OPRTase isozymes. This value is approximately 50-fold of that for OPRTase from mouse (K_i=45μM). Therefore, when azaorotate is used to treat schistosomiasis, the therapeutic index of 50 is a possibility. In contrast, only the orotate-specific isozyme was inhibited by 4,6-dihydroxypyrimidine with the apparent K_i value of 8000μM, while the latter compound inhibits the enzyme from mouse with an apparent K_i value of 15μM, giving a ratio of less than 0.002. Thus, it can not be used in the treatment of schistosomiasis.

DHUDase AND STRUCTURE ACTIVITY RELATIONSHIP OF ITS INHIBITORS

Naguib *et al.* (1985) demonstrated the activity of DHUDase in several human extrahepatic tissues and tumors. The existence of DHUDase other than in the liver and the kidney was in dispute for a long time. Part of the reasons for this controversy was the use of [2-^{14}C]-uracil as the substrate and the measurement of formation of ^{14}C-CO_2 as the product. The use of [6-^{14}C]-uracil in thin-layer chromatography in our laboratory revealed that all the tissues examined indeed have the DHUDase activity but some tissues, e.g., lymphocytes and intestinal mucosa, lack the next enzyme, dihydropyrimidinase, therefore it was not possible to produce ^{14}C-CO_2 with [2-^{14}C]-uracil as substrate. Interestingly enough, all solid tumors have dihydropyrimidinase activity whereas the normal counterpart may not. Lymphocytes are the exception as both the normal tissues and tumors have no dihydropyrimidinase activity. DHUDase is rate limiting in extrahepatic solid tumors but not in their normal counterparts. And some of these solid tumors contain greater amounts of activity than do their normal equivalents, which encourages the use of inhibitors of this enzyme in conjunction with treatment of these tumors by FUra. The importance of DHUDase inhibitor for chemotherapy stems from the fact that the widely used anticancer drug FUra or the radiosensitizing drugs 5-iodouracil and 5-bromouracil are better substrates for DHUDase than the naturally occurring nucleobases, uracil and thymine, and hence are inactivated by this enzyme (Naguib *et al.* 1985). A recent report associated the host toxicity of FUra, including cholestasis (Sweeney *et al.* 1988) and neurological disorders, to its catabolite α-fluoro-β-alanine (Hull *et al.* 1988). One hundred five analogs were evaluated for their inhibitory activities. 5-Benzyloxybenzyluracil, 1-deazauracil (2,6-pyridinediol), 3-deazauracil (2,4-pyridinediol), 5-benzyluracil, 5-nitrobarbituric acid and 5,6-dioxyuracil (alloxan) were identified as potent inhibitors (Naguib *et al.* 1989). It is interesting to note that BU, BBU, 2,6-pyridinediol, etc. are both good inhibitors of DHUDase and UrdPase. Thus, *in vivo,* they will give a double blockade of pyrimidine degradation.

SUMMARY

Among the pyrimidine metabolizing enzymes, UrdPase and DHUDase were identified as target enzymes for the enhancement of the chemotherapeutic effects of fluorinated uracils and uridines. No potent inhibitor was found for dThdPase. The most potent inhibitor of dThdPase found to date, 6-benzyl-2-thiouracil (K_i=*180μM*) is 1000-to 2000-fold less inhibitory than any of the BAU series against UrdPase (HM-BAU K_i=0.09μM). A few inhibitors of OPRTase were found which are potential chemotherapeutic agents by themselves rather than adjuncts to fluorinated uracils and uridines. BAU and its congeners seem to be remarkably nontoxic, although more work is necessary before definitive judgments can be made. BAU also increases circulatory uridine concentrations. This suggests its use for the so-called "uridine rescue" of FUra toxicity. DHUDase was a difficult enzyme to assay, but once optimum conditions were set, its activity was found in various mouse tissues and human tumors. BBU and 2,6-pyridinediol are potent inhibitors of DHUDase.

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