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CONVERSION OF 5-IODO-2-PYRIMIDINONE-2′-DEOXYRIBOSE TO 5-IODO-DEOXYURIDINE BY ALDEHYDE OXIDASE

IMPLICATION IN HEPATOTROPIC DRUG DESIGN

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Abstract—5-Iodo-2-pyrimidinone-2′-deoxyribose (IPdR) can be converted into 5-iodo-deoxyuridine (IUdR), a clinical radiosensitizer, by aldehyde oxidase in the liver. This conversion does not require exogenous cofactors and cannot be catalyzed by mixed-function oxidases, xanthine oxidase or many other oxido-reductases. This "IPdR oxidase" activity is enriched in the liver; thus, extensive conversion of IPdR to IUdR could be anticipated in the liver and the therapeutic index of IPdR could be better than that of IUdR as a radiosensitizer for primary liver cancers or tumors metastasized to the liver. Based on structure and activity relationship studies, nucleoside analogues which could be activated by this enzyme to compounds capable of inhibiting DNA synthesis could be designed and should be explored as agents against cancer, viruses or parasites in the liver.

5-Iodo-deoxyuridine (IUdR†) was synthesized as an anti-neoplastic agent in 1959 by Prusoff [1], and was the first thymidine analogue used as an anti-herpes agent in clinic [2]. The toxicities associated with IUdR when used systemically limited its clinical usage. IUdR was also recognized as a potential clinical radiosensitizer for cancer chemotherapy [3]. The radiosensitization is directly dependent on the amount of thymidine replacement in DNA by this analogue [4]. Intrahepatic infusion of IUdR followed by radiation for the treatment of tumor cells in the liver has had some success [5]. In an attempt to develop selective anti-herpes simplex virus agents based on the broader spectrum of substrate specificity of thymidine kinase of herpes simplex virus (HSV) compared to the human kinase, 5-iodo-2-pyrimidinone-2′-deoxyribose (IPdR), which differs from IUdR by a double-bonded oxygen at the 4-position of the base, was synthesized. IPdR was found to have potent activity against HSV-1 and HSV-2 in cell culture and against HSV-2 in mice [6], but it was not toxic to uninfected cells, nor was any toxicity apparent when it was given orally to mice at the dosage employed [6]. Since IPdR and IUdR are structurally related, the possibility of IPdR being converted to IUdR was examined. It was shown previously that IPdR could not be converted to IUdR by xanthine oxidase [6]. In this paper we describe an oxidase in liver that could catalyze the oxidation of IPdR to IUdR and identify this enzyme as aldehyde oxidase. This observation suggests an interesting possibility: that of designing drugs to be metabolized in the liver to target liver cancer and microbes which reside in the liver.

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† Abbreviations: IUdR, 5-iodo-deoxyuridine; IPdR, 5-iodo-2-pyrimidinone-2′-deoxyribose; HSV, herpes simplex virus; IU, iodo-uracil; EPdR, 5-ethynyl-2-pyrimidinone deoxyribose; IP, 5-iodo-2-pyrimidinone; BPdR, 5-bromo-2-pyrimidinone deoxyribose; MPdR, 5-methyl-2-pyrimidinone deoxyribose; EtPdR, 5-ethyl-2-pyrimidinone deoxyribose, and EUdR, 5-ethynyl deoxyuridine.

MATERIALS AND METHODS

Chemicals. IPdR and its analogues were synthesized by Dr. T. J. Bardos and coworkers (State University of New York at Buffalo). Xanthine oxidase and sarcosine oxidase were purchased from Boehringer Mannheim. Alcohol dehydrogenase, alcohol oxidase and N^1-methylnicotinamide were purchased from Sigma and SKF-525A was from Calbiochem.

Tissue preparation. Rat hepatic tissue was washed with ice-cold 1.15% KCl and blotted dry. The tissue was then homogenized with a tissue homogenizer, in a volume of 1.15% KCl that was three times the tissue weight, to form a 25% (w/v) homogenate. The homogenate was then centrifuged at 10,000 *g* for 10 min at 4°. The resulting supernatant was filtered through Miracloth (similar to cheesecloth), then dialyzed overnight against 50 mM Tris–HCl buffer (pH 7.5), and stored at −80° before use. Rat hepatocytes were obtained by a perfusion technique; then the cells were extracted with 10 mM phosphate buffer (pH 7.5), containing 1 M KCl and dialyzed for 4 hr against 50 mM Tris–HCl buffer (pH 7.5).

Assay conditions for the IPdR oxidation. For the standard assay condition, the reaction mixture contained 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 180 μM IPdR (or its analogues) and approximately 0.01 mg protein of a 10,000 *g* supernatant of tissue homogenate in a final volume of 500 μL and incubated for 10 min at 37° unless specified otherwise. Three hundred microliters of reaction mixture was

removed at the end of incubation, mixed with 600 μL acetonitrile and then agitated. The precipitated proteins were removed by centrifugation and the supernatant was lyophilized to dryness. The samples were reconstituted to the original aliquot volume with the HPLC mobile phase buffer and analyzed on an Alltech RP-18 column. IPdR, IUdR and iodouracil (IU) were detected at a UV absorption wavelength of 230 nm, and IPdR was also detected at a UV absorption wavelength of 335 nm. The mobile phase was 10% acetonitrile/90% 100 mM ammonium acetate (pH 6.8), and the flow rate was 1 mL/min. Standard curves of IPdR and IUdR were established from the integration value of known concentrations.

Enzyme assay. Alcohol dehydrogenase activity was determined from the rate at which it catalyzes the NAD reduction by ethanol (procedure supplied by Sigma). Alcohol oxidase and xanthine oxidase activities were determined by a colorimetric method (procedure supplied by Sigma) based on the production of hydrogen peroxide (H_2O_2) to determine the ability of xanthine oxidase and alcohol oxidase to oxidize xanthine or methanol, respectively.

RESULTS

Conversion of IPdR to IUdR. To study the conversion of IPdR to IUdR by liver enzymes, the metabolites of IPdR were analyzed after incubation with a supernatant of rat liver homogenate using a reverse phase HPLC technique. IPdR and IUdR could be detected at an absorption wavelength of 230 nm but only IPdR could be detected at 335 nm. To limit the phosphorylytic cleavage of IUdR to IU by thymidine phosphorylase [3], Tris–HCl buffer was employed in the assay. As shown in Fig. 1, there was a time-dependent conversion of IPdR to IUdR, and IUdR appears to be the only product produced by IPdR. The identification of IUdR was confirmed based on the retention time on a C-18 column (8 min vs 9.5 min for IPdR) and the UV spectrum as well as nuclear magnetic resonance spectroscopy (results not shown).

Properties of "IPdR oxidase" activity. This "IPdR oxidase" activity did not require exogenous cofactors, was much less active in extracts of kidney and spleen than liver (Table 1), and could not be detected in lung or intestine extracts from rats. The human liver contained amounts of this enzyme similar to those found in the rat liver. Extracts of hepatocytes, which represent about 80% of the cell population in liver, had specific activity very similar to that of the whole liver extract, suggesting that the IPdR oxidation is mainly present in this cell population. Differential fractionation centrifugation was used in an attempt to localize this IPdR conversion enzyme activity in the liver homogenate. The enzyme activity assay was performed on each fraction. The only fraction which showed the enzyme activity appeared in the 100,000 g × 60 min supernatant; this suggested that this enzyme is located only in the soluble fraction of cytosol. Further purification was achieved by use of DEAE cellulose column chromatography, blue Sepharose column chromatography, and glycerol gradient centrifugation in that order. This IPdR oxidase activity was purified 380-fold starting from the crude extracts of rat liver. The partially purified enzyme catalyzed IUdR synthesis at a rate of 3.8 μmol/min/mg protein at 37° under these

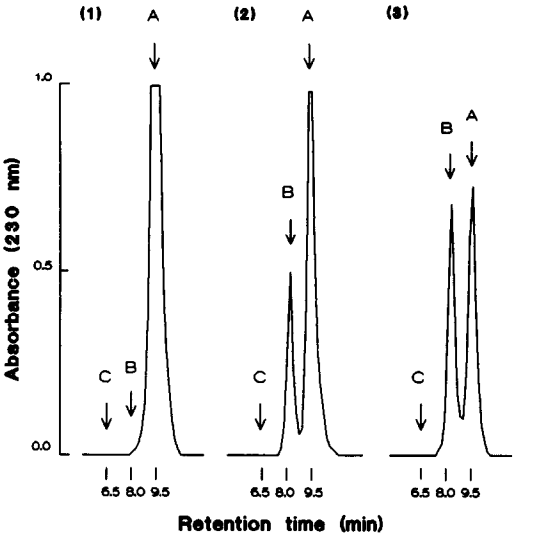


Fig. 1. HPLC profiles of the conversion of IPdR to IUdR by liver homogenate. IPdR conversion was examined by incubating rat liver homogenate in the presence of IPdR. For the control reactions, a portion of the supernatant was boiled for 5 min to inactivate all enzymes before use. The assay conditions were as described under Materials and Methods except that 60 μL of the 100,000 g × 60 min supernatant (equivalent to approximately 0.5 mg protein/mL) was used in a reaction volume of 1,500 μL. Aliquots (300 μL) were removed at various time points [0 min (1), 15 min (2) and 30 min (3)] during the incubation period at 37°. A control reaction which contained the same amount of protein was used as the background and a background subtraction has been applied to these HPLC profiles. The retention times were approximately 9.5 min for IPdR (A), 8.0 min for IUdR (B) and 6.5 min for IU (C), respectively.

Table 1. Tissue specificity of the conversion of IPdR to IUdR by cell extracts

Tissue (or cells)	Specific activity* (nmol/mg protein/min)
Liver (human)	2–7
Liver (rat)	5–15
Hepatocyte (rat)	5–11
Kidney (rat)	0.8–1.6
Spleen (rat)	0.2–0.5
Intestine (rat)	<0.02†
Lung (rat)	<0.02†

Specific activities were obtained from three samples for each tissue (also three samples for rat hepatocyte) except the data for rat liver which were obtained from five samples.

† Conversion of IPdR to IUdR by rat lung and intestine was not detected.

conditions. The apparent molecular weight of this enzyme in both rats and humans, as determined by centrifugation on a 20 to 40% glycerol gradient, was approximately 280,000 Da. Neither cofactors (NAD, NADH, NADP and NADPH) nor divalent cation requirements have been found for the rat liver enzyme or the human liver enzyme.

Identification of "IPdR oxidase". To determine which enzyme is responsible for the catalysis of this IPdR oxidation, a series of known oxido-reductases which have similar capabilities to oxidize a carbon atom with an adjacent amino group into a carbonyl functionality were explored for their abilities to catalyze IPdR oxidation. Xanthine oxidase, isolated from cow milk, which catalyzes the oxidation of hypoxanthine to xanthine failed to convert IPdR to IUdR. A mixed-function oxidase system prepared from the microsomes of rat liver oxidizes a broad spectrum of substrates and is NADPH dependent. However, this microsomal fraction from rat liver showed no IPdR oxidase activity whether NADPH was added or not. Alcohol dehydrogenase and alcohol oxidase both reside in the soluble fraction of liver cell extracts, but the conversion of IPdR to IUdR was not detected with a purified preparation of either enzyme under the same conditions in which they converted their natural substrates effectively. Sarcosine oxidase which catalyzes the conversion of N^1-methylglycine to glycine had no IPdR oxidase activity. Phenylalanine hydroxylase, urocanase, cystathionine γ-lyase, L-glutamate dehydrogenase, cystathionase and several other oxido-reductases were ruled out based on substrate competition assays, their different cofactor specificities or some other characteristic features from literature [7–12].

Since hepatic aldehyde oxidase has the same molecular weight, is in the cytosolic fraction of liver cells and has broad substrate specificity [13], we suspected that it might be the IPdR oxidase enzyme responsible for the conversion of IPdR to IUdR. Hepatic aldehyde oxidase which catalyzes the oxidation of a variety of aldehydes to the corresponding acids also converts N^1-methylnicotinamide to N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide [13–19]. This aldehyde oxidase activity was reported to be stimulated by potassium ferricyanide and Tris buffer but not by $MgCl_2$ [17]. This enzyme could be inhibited by 2-mercaptoethanol, dithiothreitol and other thiol agents [14]. There was no significant inhibition by cysteine at 5 mM; however, at 50 mM a potent inhibition of the enzyme activity was observed [17]. Divalent metal cations such as Cu^{2+}, Zn^{2+} and Fe^{2+} caused strong inhibition [13]. The activity could also be inhibited by acetaldehyde but not by allopurinol ($IC_{50} > 0.5$ mM) or formaldehyde ($IC_{50} > 20$ mM) [14, 16, 19]. Therefore, we examined a series of compounds for their effects on the oxidation of IPdR to IUdR and found that the inhibition profile of compounds with the IPdR oxidase activity (Table 2) was essentially identical to the characteristic pattern of aldehyde oxidase. Furthermore, throughout each step of the purification, aldehyde oxidase could not be separated from "IPdR oxidase" activity.

Substrate specificity. Several 2-pyrimidinone deoxyribose analogues have also been examined for conversion to their deoxyuridine counterparts. The Michaelis constant K_m values for IPdR in the reaction at pH 7.5 and 9.5 were 150 and 87 μM, respectively, and the K_m values for 5-ethynyl-2-pyrimidinone deoxyribose (EPdR) in the reaction at pH 7.5 and pH 9.5 were 77 and 46 μM, respectively. Nevertheless, the relative V_{max} for IPdR in the reaction at pH 7.5 and 9.5 is the same. 5-Iodo-2-pyrimidinone (IP), the aglycose of IPdR, was an excellent substrate for aldehyde oxidase. The synthetic substrates for aldehyde oxidase appear to be better than its natural substrates, N^1-methylnicotinamide and acetaldehyde, as judged by the potency of inhibition of N^1-methylnicotinamide and acetaldehyde to the IPdR oxidation reaction (Table 2). The rate of reactivity of the liver enzyme with different IPdR analogues follows the order EPdR, IP, IPdR, 5-bromo-2-pyrimidinone deoxyribose (BPdR) and 5-methyl-2-pyrimidinone deoxyribose (MPdR) or 5-ethyl-2-pyrimidinone deoxyribose (EtPdR) (Table 3). Electronegative substituents in the 5-position seemed to increase the substrate activity in this oxidation reaction.

Table 2. Effects of inhibitors on the oxidation of IPdR to IUdR by liver homogenate

Compound tested	IC_{50}* (mM)
Mercaptoethanol	1.8 ± 0.4
Dithiothreitol	0.1 ± 0.02
Cysteine†	>50
1-Butane thiol‡	>50
Methanol	>50
Allopurinol	>0.5
SKF-525A	0.03 ± 0.01
Hydroxyl amine	1 ± 0.2§
Hydrogen peroxide	10 ± 3§
Cu^{2+}	0.4 ± 0.08
Zn^{2+}	0.3 ± 0.06
Fe^{2+}	4 ± 0.8
Acetaldehyde	5 ± 1
Formaldehyde	>20
N^1-Methylnicotinamide	1 ± 0.2

* Concentration of each compound that caused 50% inhibition of the conversion of IPdR to IUdR. Values are means ± SEM of at least three experiments unless noted otherwise.

† Approximately 40% product inhibition was observed at 50 mM.

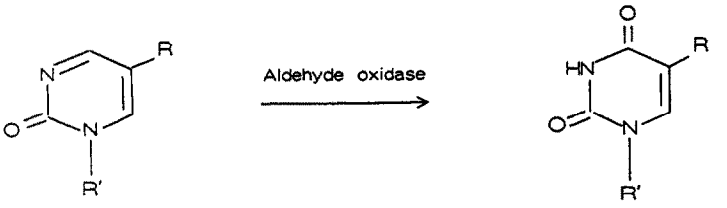
‡ Approximately 25% product inhibition was observed at 50 mM.

§ Average ± range of two experiments.

DISCUSSION

Hepatic aldehyde oxidase is widely spread within the liver of mammals. This enzyme catalyzes the oxidation of a variety of aliphatic and aromatic aldehydes as well as a number of non-aldehyde heterocyclic compounds such as N^1-methylnicotinamide, 4-amino-antifolates and methotrexate and its analogues. The finding of the oxidation of 5′-substituted pyrimidinone to their uracil or uridine

Table 3. Substrate specificity of the conversion of RPdR to RUdR*



R	R'	Substrate Abbreviation	Rate of conversion† (nmol/mg/min)	Product abbreviation
C≡CH	dR‡	EPdR	20 ± 4	EUdR
I	dR	IPdR	11 ± 2	IUdR
I	H	IP	18 ± 3	IU
Br	dR	BPdR	7 ± 2§	BUdR
CH_3	dR	MPdR	<0.1‖	MUdR
CH_2CH_3	dR	EtPdR	<0.1‖	EtUdR

* The amount of substrate used was 180 μM in all cases.
† Values are means ± SEM of at least three experiments, unless noted otherwise.
‡ dR = deoxyribose.
§ Average ± range of two experiments.
‖ Products of the reaction from MPdR and EtPdR were not detected.

counterparts by aldehyde oxidase from human and rats not only creates a completely new category of substrate specificity for this enzyme but also brings up an interesting possibility: that of designing drugs to be metabolized in the liver to target liver cancer and microbes which reside in the liver. Compounds such as IUdR, FUdR and 5-ethynyl-deoxyuridine (EUdR) are toxic to highly proliferating cells, including liver tumor cells, which have high thymidine kinase activity. Since normal liver cells do not have a high activity of thymidine kinase, selectivity in the liver by these drugs can be achieved. The systemic toxicity associated with these drugs, however, limited their clinical usefulness. Our substrate specificity studies suggested that these types of toxic components could be generated *in situ* by aldehyde oxidase using its 5'-substituted pyrimidinone precursors which do not serve as substrate of human thymidine kinase and thymidine phosphorylase. Furthermore, our tissue specificity studies indicated that this conversion is more active in liver extract; other tissues have either no or limited capability of conducting this oxidation. Thus, extensive conversion from pyrimidinone to the toxic component could be anticipated in the liver, and the therapeutic index of IPdR, FPdR or EPdR should be better than that of their UdR counterparts for primary liver cancer or cancer metastasized to liver. Other anticancer agents such as cyclophosphamide and nitrosoureas are also activated in the liver [20]; however, their activated form indiscriminately attacks normal cells. Folic acid analogues, such as methotrexate, can also be oxidized by aldehyde oxidase [21] but its hydroxylation product is much less potent and may even interfere with the action of methotrexate.

Currently IUdR and BUdR are being explored as radiosensitizers; however, their effective use is limited by their cytotoxicity and rapid catabolism to the free base followed by dehalogenation [4]. Since IPdR and its analogues are virtually nontoxic and are not substrates for thymidine phosphorylase*, the use of IPdR and its analogues instead of IUdR or BUdR may circumvent the difficulties of toxicity and degradation related to radiation therapy. Perhaps the most intriguing implication comes from the conversion of IP to IU. This conversion suggests that the deoxyribose moiety is not required for this type of oxidation reaction. It also means that this enzyme may oxidize IPdR analogues with modified sugar structures. Therefore, based on our studies, the action of liver aldehyde oxidase for prodrug activation may serve as a new strategy in hepatotropic drug design and therapy.

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