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C(2′)-SUBSTITUTED PURINE NUCLEOSIDE ANALOGS

INTERACTIONS WITH ADENOSINE DEAMINASE AND PURINE NUCLEOSIDE PHOSPHORYLASE AND FORMATION OF ANALOG NUCLEOTIDES

JOHANNA D. STOECKLER,* CLAIRE A. BELL and ROBERT E. PARKS, JR.
Section of Biochemical Pharmacology, Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

and

CHUNG K. CHU and JACK J. FOX
Laboratory of Organic Chemistry, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, Sloan-Kettering Division of Graduate School of Medical Sciences, Cornell University, New York, NY 10021, U.S.A.

and

MORIO IKEHARA
Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan

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Abstract—Four C(2′)-substituted 2′-deoxyadenosines were examined as substrates for human erythrocytic adenosine deaminase and for formation of intracellular nucleotide analogs in human erythrocytes, lymphocytes and murine Sarcoma 180 cells: 9-(2′-deoxy-2′-fluoro-β-D-ribofuranosyl)adenine, 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine, 9-(2′-azido-2′-deoxy-β-D-ribofuranosyl)adenine (2′-N_3-riboA) and 9-(2′-azido-2′-deoxy-β-D-arabinofuranosyl)adenine. All four adenosine analogs were substrates of human erythrocytic adenosine deaminase, but the corresponding inosine analogs (synthesized by the adenosine deaminase reaction) were highly resistant to cleavage by human erythrocytic purine nucleoside phosphorylase. Only 9-(2′-deoxy-2′-fluoro-β-D-ribofuranosyl)hypoxanthine underwent very slow phosphorolysis, and no inhibition of inosine phosphorolysis was detected when a 30 μM concentration of any studied inosine analog was added to a reaction mixture containing 30 μM inosine (the K_m concentration). Kinetic parameters were determined for the deamination of the adenosine analogs. The greatest affinity for adenosine deaminase was found with 2′-N_3-riboA (K_i = 2 μM), but the reaction velocity was highest with the F-substituted analogs. All four adenosine analogs formed triphosphate nucleotides after incubation with human erythrocytes, murine Sarcoma 180 cells, or human lymphocytes (tested only with the F analogs) in the presence of deoxycoformycin.

The C(2′) of the pentose moiety is assuming increased importance for the rational design of chemotherapeutic nucleoside analogs. The arabinosides of cytosine and adenine are established as clinically useful agents for the treatment of certain neoplastic and viral diseases [1, 2]. More recently, it has been learned that substitution on C(2′) of 2′-deoxycytidine or 2′-deoxy-5-iodocytidine by azide (N_3) or fluorine (F) moieties in the arabinose configuration yields potent agents against murine leukemias [3] and certain DNA viruses, e.g. HSV-1, HSV-2 and Varicella-Zoster [4, 5]. Preliminary reports indicate that 2′-azido-2′-deoxyarabinofuranosyladenine is active against L1210 and KB cells in culture [6, 7] and P388 leukemia in mice [8]. These activities were potentiated by inhibition of adenosine deaminase [6, 8].

These promising results indicate the need for detailed biochemical analyses of the interactions of C(2′)-modified nucleosides with key catabolic and anabolic enzymes, i.e. the relevant nucleoside deaminases, phosphorylases and kinases. A family of 2′-deoxyadenosine analogs has become available with substitutions by F or N_3 in both the arabinose (up) and ribose (down) configurations. We have studied them as substrates for human erythrocytic adenosine deaminase (ADA) and for incorporation into the nucleotide pools of human erythrocytes, lymphocytes, and murine Sarcoma 180 cells. Deamination by ADA produced the respective 2′-deoxyinosine analogs, which were examined as substrates and inhibitors of human erythrocytic purine nucleoside phosphorylase (PNP). The results of these studies offer encouragement that related analogs of 2′-deoxyguanosine may have potential as chemotherapeutic agents. Preliminary reports of these studies have been presented [9, 10]. An earlier study showed 2′-deoxy-2′-fluoroarabinofuranosyl adenine to be a good substrate of calf intestinal ADA [11]. Deamination of 2′-azido-2′-deoxyarabinofuranosyladenine by ADA from calf intestine and L1210 murine leukemia cells was much slower than that of arabinosyladenine [6, 7].

* Author to whom correspondence should be addressed.

MATERIALS AND METHODS

The syntheses of 9-(2′-azido-2′-deoxy-β-D-ribofuranosyl)adenine (2′-N_3-riboA), 9-(2′-deoxy-2′-fluoro-β-D-ribofuranosyl)adenine (2′-F-riboA), and 9-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)adenine (2′-F-araA) have been reported [12–14]. The 9-(2′-azido-2′-deoxy-β-D-arabinofuranosyl)adenine (2′-N_3-araA, product of Sandoz) was provided by Dr. A. C. Sartorelli; 8-azaadenosine was synthesized by Dr. S-H. Chu by a known procedure [15]. The Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, provided 2′-deoxycoformycin (Pentostatin, dCF). Xanthine oxidase (grade III), calf intestinal ADA (type III), and calf intestinal alkaline phosphatase attached to agarose were purchased from the Sigma Chemical Co., St. Louis, MO. Human erythrocytic ADA (0.037 unit/mg protein) and PNP (1.5 units/mg protein) were purified as previously described [16].

Enzymatic assays. The activity of ADA with adenosine and the C(2′)-substituted adenosine analogs was determined spectrophotometrically at 265 nm [17]. When 8-azaadenosine was used as the substrate in determining the K_i value of 2′-N_3-araA, its deamination was monitored at 290 nm. Reaction conditions and analysis of the kinetic data have been described earlier [18].

Preparation and testing of inosine analogs. The C(2′)-substituted adenosine analogs were deaminated to their respective inosine analogs by preincubating reaction mixtures with 1 unit calf ADA until no further decrease in absorbance was observed at 265 nm. The reactions of PNP with both inosine and the inosine analogs were analyzed by the coupled xanthine oxidase assay [19]. The inosine analogs were tested as inhibitors of PNP by observing their effect on the phosphorolysis of inosine (30 μM) in the spectrophotometric assay. The inosine analogs were also incubated overnight at room temperature with purified PNP, extracted, and chromatographed by reversed-phase high-pressure liquid chromatography (HPLC) on a Waters μBondapak C_{18} analytical column as described earlier [18].

Studies with intact cells. Lymphocytes were isolated from human blood by centrifugation with Ficoll-Paque (Pharmacia, Piscataway, NJ) and were washed with, and incubated in, Puck's saline G without phenol red. The isolation of human erythrocytes and murine Sarcoma 180 cells, the incubation procedures, the preparation of cell extracts, and the analysis by anion-exchange HPLC have all been described elsewhere [20]. Analog concentration in the incubation mixtures was 1 mM. To prevent reaction with adenosine deaminase, all experimental and control incubations were performed in the presence of dCF (1 μg/ml). Solutions containing 2′-N_3-araA were protected from light.

Separation of 2′-substituted-2′-deoxynucleotides from natural ribonucleotides by boronate column chromatography. A column (1 × 10 cm) of phenylboronic acid coupled to polyacrylamide gel (Pierce Chemical Co., Rockford, IL, or Bio-Rad Laboratories, Rockville Centre, NY) was equilibrated with 0.1 M triethylammonium bicarbonate (TEAB) containing 10 mM $MgCl_2$ at pH 8.5 to 8.7. Neutralized perchloric acid extracts of erythrocytes that had been incubated with the adenosine analogs were mixed with equal volumes of the equilibration buffer and applied to the column. The column was eluted with the same buffer at a rate of 1 ml/min, and 1.2 ml fractions were collected. The effluents were monitored at 254 nm, and the tubes containing the first peak of u.v.-absorbing material (the 2′-substituted 2′-deoxynucleotides) were pooled and subjected to anion-exchange HPLC [20]. The concentration of starting buffer did not appear to be critical since similar results were obtained with TEAB concentrations ranging from 0.1 to 1.0 M. The ribonucleotides were eluted with 200 mM sorbitol, and the gel was regenerated by washing with 1 mM HCl.

RESULTS

Reactivity with ADA and PNP. All four 2′-deoxyadenosine analogs were substrates for human and calf ADA. Table 1 lists the kinetic parameters determined with human erythrocytic ADA. The substitution of fluorine at C(2′) resulted in V_{max} values closer to that of adenosine than of 2′-deoxyadenosine. With fluorine in the ribose (down) configuration, the K_m value was comparable to that of 2′-deoxyadenosine, whereas with fluorine in the ara (up) configuration, the K_m value was comparable to that of adenosine. Substitution by an azido group significantly lowered the V_{max} values. With the azido group in the ribose configuration, the affinity was increased so greatly that, although the relative V_{max} was easily determined, it was not possible by the spectrophotometric assay to monitor the reaction at

Table 1. Kinetic parameters of C(2′)-modified adenosines with human erythrocytic ADA*

	K_m or K_i (μM)	Relative V_{max}
Adenosine	21, 25†	100
2′-Deoxyadenosine	7†	69†
Arabinofuranosyladenine	100†	47†
2′-F-riboA	9	89
2′-F-araA	19	97
2′-N_3-riboA	2 (K_i)	14
2′-N_3-araA	35	6

* Results are averages of two separate experiments.
† Published values [21].

Table 2. Formation of triphosphate nucleotides from C(2')-modified adenosines*

	Relative HPLC peak area			
	Sarcoma 180	Lymphocytes	Erythrocytes	
	3 hr	4 hr	4 hr	18–24 hr
2'-F-riboA	2	4	2	4
2'-F-araA	2	1	4	5
2'-N_3-riboA†	1	ND‡	1	4
2'-N_3-araA†	1	ND‡	2	4

* The area of the analog peak was compared to that of ATP and rated as follows: 1 = <½ ATP area; 2 = >½ ATP; 3 = ATP; 4 = >ATP; 5 = > twice ATP. The incubations were performed in duplicate.

† Only the analog peak which eluted after ATP was quantitated.

‡ Not done.

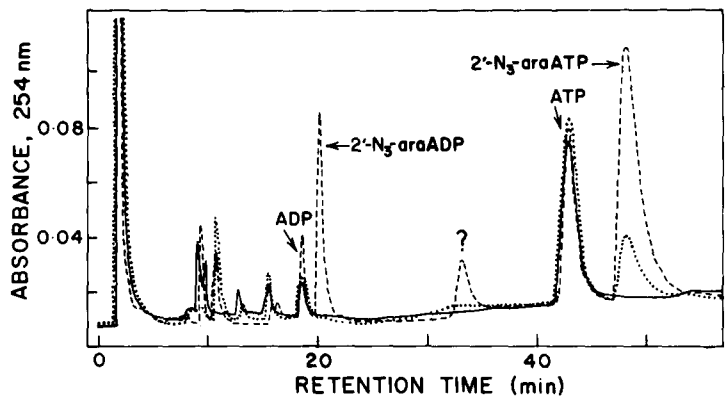

Fig. 1. High-pressure liquid chromatographic profiles of human erythrocytes incubated for 0 (——), 4 (. . . .) and 24 (– – –) hr with 2'-N_3-araA.

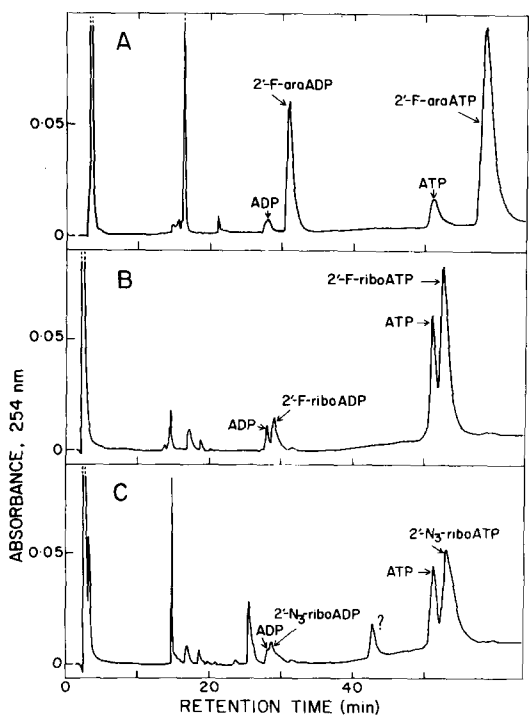

Fig. 2. High-pressure liquid chromatographic profiles of analog nucleotides formed during 18- to 22-hr incubations with human erythrocytes from: (A) 2'-F-araA, (B) 2'-F-riboA, and (C) 2'-N_3-riboA.

sufficiently low substrate concentrations to make accurate estimates of the K_m value. The K_i value of 2'-N_3-riboA, determined at concentrations below 3 μM, was 2.0 μM.

When the four 2'-deoxyinosine analogs (prepared by incubation of the respective adenosines with ADA) were incubated overnight with 1.2 units of PNP and examined by reversed-phase HPLC, only 2'-F-riboHx was cleaved to hypoxanthine by PNP. This compound displayed a relative V_{max} of <0.2% and a K_m value of 490 μM. None of the 2'-deoxyinosine analogs inhibited inosine phosphorolysis when substrate and analog were each added at 30 μM concentration, i.e. approximately the K_m value for inosine [18].

Incorporation of adenosine analogs into intracellular nucleotide pools. All four adenosine analogs formed triphosphate nucleotides when incubated with human erythrocytes, human lymphocytes, or murine Sarcoma 180 cells in the presence of dCF, as shown in Table 2. Cellular breakdown precluded longer incubations with lymphocytes and Sarcoma 180 cells. Figure 1 depicts the time-dependent accumulation of analog nucleotides from 2'-N_3-araA in erythrocytes. The HPLC profiles in Fig. 2 show the positions of analog nucleotide peaks formed from the three other compounds. Both azido analogs gave rise to small extra peaks which eluted before ATP in the triphosphate region. Reversed-phase HPLC of each 2'-azido nucleoside revealed a single peak. This suggests that the unidentified peaks were

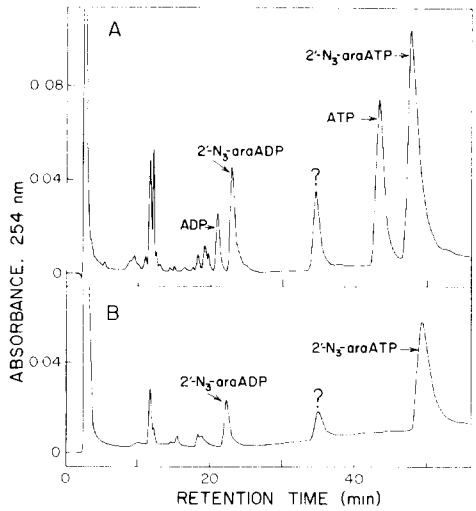

Fig. 3. High-pressure liquid chromatographic profiles of 2′-N_3-araA incubated 24 hr with human erythrocytes: (A) before and (B) after phenylboronate column chromatography. The smaller analog peaks in profile B resulted from dilution of the sample.

derived from the original analog nucleosides during incubation. The highest concentrations of analog di- and triphosphate nucleotides were formed from 2′-F-araA and accompanied a 70% reduction in the ATP (relative to the control), whereas the accumulation of large amounts of 2′-N_3-araATP caused only slight decreases in the concentrations of the natural adenine nucleotides (Fig. 1). Also, in contrast to the results with the other analog adenosines, the accumulation of nucleotides of 2′-F-araA caused a decreased ratio of ATP:ADP and of 2′-F-araATP:2′-F-araADP, i.e. about 2:1. The ATP:ADP ratio of untreated normal erythrocytes is usually 6–8:1. This suggests that the accumulation of large amounts of 2′-F-araA nucleotides results in an impairment of the energy metabolism of these cells.

Separation of natural ribonucleotides from 2′-substituted-2′-deoxyadenosine nucleotides. Since *cis* hydroxyls on C(2′) and C(3′) are required for the binding of nucleotides to borate, the technique of phenylboronate column chromatography as used to separate natural from 2′-deoxy analog nucleotides. This method is especially useful for separating merged or "buried" peaks and for offering evidence for the identify of unknown peaks. Figure 3 presents HPLC nucleotide profiles of extracts of erythrocytes incubated for 24 hr with 2′-N_3-araA before and after phenylboronate chromatography. Here it is seen that the natural adenine nucleotides were removed cleanly from the extract. Furthermore, the small peak of questionable identity which emerged before ATP at 35 min was not removed, indicating that it did not contain a ribose moiety with *cis* hydroxyls.

Attempts were made to identify the analog triphosphates generated from 2′-N_3-riboA. The neutralized extract (3 ml) from a 22-hr incubation of erythrocytes with 2′-N_3-riboA (see Fig. 2C for nucleotide profile) was adjusted to pH 8.5 with 0.1 M TEAB containing 10 mM $MgCl_2$ and subjected to boronate column chromatography. The fraction not retained by the phenylboronate gel was lyophilized to reduce the volume to 1 ml and rechromatographed on anion-exchange HPLC. The profile showed complete removal of ribonucleotides; all peaks were collected. Normal adenosine-type spectra with a λ_{max} of 260 nm were obtained on a Perkin–Elmer recording spectrophotometer for both peaks from the triphosphate region and for the nucleoside peak that was not retained by the anion-exchange resin (other peaks were not sufficiently concentrated to give useful spectra). This indicates that the extra triphosphate peak did not result from deamination of the adenine moiety of the analog. Each of the peaks from the triphosphate region was concentrated by lyophilization, desalted by passage through a 1 × 11 cm Sephadex G-10 column (Pharmacia) and incubated for 24 hr with 12.5 units of alkaline phosphatase. Reversed-phase HPLC profiles showed that the larger triphosphate peak that emerged just after ATP (Fig. 2C), had the same retention time (22 min) as the original 2′-N_3-riboA. The smaller triphosphate peak yielded a very small peak with a retention time of 15.5 min, but the bulk of its u.v.-absorbing material was not retained by the reversed-phase column, even when the incubation with alkaline phosphatase was extended for 48 hr. In addition, reversed-phase chromatography of the nucleoside peak that had been collected from the anion-exchange HPLC column also showed a small peak at 15.5 min although >95% of the u.v. absorbance appeared in the 22-min peak. The peak at 15.5 min was not found in the reversed-phase profiles of erythrocytes incubated for 22 hr without the analog or in samples of the 2′-N_3-riboA incubation mixture that were extracted at 0 time.

DISCUSSION

These findings have important implications that affect the chemotherapeutic potential of the C(2′)-substituted analogs studied. Both C(2′)F-substituted adenosines are excellent substrates for ADA, rivaling in activities those of the natural substrates, adenosine and 2′-deoxyadenosine. The C(2′)N_3-adenosines also have good to excellent affinity for ADA but lowered reaction velocities. Calf intestinal ADA was recently reported to have both higher K_m (19 μM) and V_{max} (27%) values with 2′-N_3-riboA [22] than those reported here for the human enzyme. In the presence of the ADA inhibitor, 2′-deoxycoformycin, all four analogs formed polyphosphate nucleotides in intact cells, demonstrating activity with the respective kinases for nucleosides, nucleoside 5′-monophosphates and nucleoside diphosphates in the three cell types studied. The depletion of ATP that was seen with 2′-F-araA has been observed previously with base-modified analogs, e.g. 2-fluoroadenosine, that formed very high concentrations of analog nucleoside triphosphate [23]. As with other adenosine and 2′-deoxyadenosine analogs, it is probable that chemotherapeutic or cytostatic activity seen with any of these compounds will be enhanced by concurrent inhibition of ADA, as shown earlier for 2′-N_3-araA [6, 8]. Since 2-fluoroadenosine has negligible activity with ADA but is

a good substrate for adenosine kinase [21, 23], it is likely that analogs related to those examined here, in which the adenine moiety is replaced by 2-fluoroadenine, would also resist deamination and display increased cytotoxic or antiviral activity.

The results obtained with PNP are especially intriguing. Of the four C(2')-substituted 2'-deoxyinosine analogs examined, only 2'-F-riboHx displayed either substrate or inhibitor activity and did so with very unfavorable kinetics, i.e. $K_m = 490\ \mu M$, $V_{max} < 0.2\%$. Thus, these C(2') substitutions drastically decrease the ability of the 2'-deoxyinosine analogs to bind to the catalytic site of PNP. The van der Waals radius of fluorine is similar to that of hydrogen and, with many biologically active molecules, replacement of an H atom by F does not block the binding to macromolecules such as enzymes or hormone receptors. Therefore, it seems unlikely that the slightly greater bulk of the F atom can explain this marked decrease in substrate binding. Studies of the effects of C(2')N_3 and C(2')F substitutions on the thermal stabilities of polynucleotide complexes have also indicated that the size of the substituent is not the main determinant of structure. Poly 2'-N_3- and 2'-F-adenylic acids resemble polyadenylic acid rather than polydeoxyadenylic acid in some of their properties [24, 25]. The F substituent also appears to have a direct or indirect effect on the base. The thermal stability of polynucleotide complexes made from amino-substituted pyrimidine or purine bases (adenine and cytosine) was decreased by C(2')F whereas that of polynucleotide complexes containing C(6)-hydroxyl bases (hypoxanthine or uracil) was increased [26]. A 2'-N_3-inosinic acid polynucleotide complex has been synthesized which mimics interferon-inducing actions of RNA [27]. The extreme polarizability of N_3 and the strongly electron-withdrawing character of F make both of these substituents good hydrogen bond acceptors that may be highly associated with solvent. NMR studies of 2'-F-riboA have indicated an exceptionally high preference for the C(3')-endo sugar conformation [28]. X-Ray analysis of 2'-F-riboHx monohydrate crystals showed an unusual C(3')-endo-C(4')-exo conformation [29]. Thus, the ribose might be predominantly in a conformation unacceptable to PNP. The findings that several C(2')-substituted inosines resist phosphorolysis by PNP, whereas the related adenosine analogs readily form adenine analog nucleotides, suggest the prospect, as discussed elsewhere [9], that C(2')-substituted guanosine analogs will also be inactive with PNP while retaining activity with the nucleoside and nucleotide kinases that form dGTP from 2'-deoxyguanosine. Such analogs would bypass degradation by PNP and directly form analogs of dGTP.

Recent studies on the interaction between 2'-deoxy-2'-chlorocytidine 5'-diphosphate and the enzyme, ribonucleotide diphosphate reductase, have yielded insights into the cytotoxic action of 2'-halogenated nucleosides and have offered important clues on the reaction mechanism of this enzyme. This interaction results in the inactivation of the enzyme (presumably through a free radical mechanism) and disintegration of the analog nucleotide with liberation of the pyrimidine base, cytosine, Cl^-, 2-deoxyribose and pyrophosphate. These and related findings have led to the proposal that the ribonucleotide diphosphate reductase reaction involves formation of a 3'-keto ribonucleotide diphosphate intermediate [30, 31]. The question of whether this phenomenon, i.e. ribonucleotide diphosphate reductase inactivation and nucleotide disintegration, is responsible for the cytotoxic actions of 2'-substituted nucleoside analogs is an important one for future study. The use of dCF-treated human erythrocytes and boronate gel chromatography to produce analog polyphosphate nucleosides may provide a useful alternative to chemical synthesis [24, 25] for the preparation of these interesting biochemical tools.

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