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NUCLEOSIDE-CATABOLIZING ENZYME ACTIVITIES IN PRIMARY RABBIT KIDNEY CELLS AND HUMAN SKIN FIBROBLASTS

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Abstract—Primary rabbit kidney (PRK) and human skin fibroblast (HSF) cell cultures, two cell systems which are regularly employed in our laboratory to explore the antiviral properties of nucleoside analogues, were analyzed for the specific activities of the following nucleoside catabolizing enzymes: cytidine deaminase (EC 3.5.4.5), pyrimidine nucleoside phosphorylases (uridine phosphorylase: EC 2.4.2.3, and 2′-deoxythymidine phosphorylase: EC 2.4.2.4), purine nucleoside phosphorylase (EC 2.4.2.1) and adenosine deaminase (EC 3.5.4.4). No cytidine deaminase activity was detected in either PRK or HSF cells. Likewise, no 2′-deoxythymidine phosphorylase activity could be demonstrated in PRK and HSF cells. PRK cells contained low levels of uridine phosphorylase (3–5 U/mg protein) and high levels of purine nucleoside phosphorylase (~100 U/mg protein). For both PRK and HSF cells, relatively high adenosine deaminase activities were recorded (at an average 20 and 36 U/mg protein, respectively). Infection of the PRK or HSF cell cultures with either vaccinia virus, herpes simplex virus (type 1 or 2) or vesicular stomatitis virus at high multiplicity of infection (MOI ~ 1) did not bring about marked changes in any of the enzymatic activities tested. For uridine phosphorylase (from PRK cells) and adenosine deaminase (from both PRK and HSF cells) the substrate specificities were determined with various uridine and adenosine analogues.

Many of the substances currently investigated as potential antiviral agents are structural analogues of purine and pyrimidine nucleosides. These may undergo cellular uptake by means of facilitated diffusion [1, 2]. Clearly the antiviral potency exhibited by a given analogue will be determined by its metabolism within the infected cell, which also may differ significantly from that in the uninfected cell. Such differences may profitably be applied to the development of specific antiviral drugs, e.g. the efficacy of analogues of 2′-deoxythymidine (TdR) and 2′-deoxycytidine (CdR) is closely associated with the ability of the virus to induce its own kinase(s) [3–7].

Activity will also be affected by the presence of catabolizing enzymes within the cell. It is therefore pertinent to inquire what are the relative substrate specificities of different nucleoside analogues for nucleoside catabolizing enzymes, and to what extent, if any, these activities are affected by viral infection. In the present study we explore these two points with primary rabbit kidney (PRK) and human skin fibroblasts (HSF) cultures, both of which are employed in our laboratories for testing *in vitro* antiviral properties of nucleoside analogues. Extracts of both these cellular systems were examined for the following enzyme levels: cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5), pyrimidine nucleoside phosphorylase (uridine: orthophosphate ribosyltransferase, EC 2.4.2.3 and 2′-deoxythymidine: orthophosphate deoxyribosyltransferase, EC 2.4.2.4), adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) and purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1).

The significance of these enzymes in clinical medicine is well established, e.g. pyrimidine nucleoside phosphorylases are responsible for activation of 5-fluorouracil, an effective antitumor agent [8]. Responses of human leukemias to 1-β-D-arabinofuranosylcytosine (ara-C) have been reported to be inversely correlated with intracellular levels of cytidine deaminase activity [9]. This enzyme converts ara-C to the therapeutically inactive ara-U [10]. For other analogues such as 5-methyl-ara-C [11], and 5-iodo-2′-deoxycytidine [12], deamination appears necessary for their conversion into biologically active products, ara-T (1β-D-arabinofuranosylthymine- and IUdR (5-iodo-2′-deoxyuridine), respectively. Another catabolizing enzyme is adenosine deaminase, which reduces the chemotherapeutic activity of analogues of adenine nucleosides, e.g. ara-A (9-β-D-arabinofuranosyladenine) [13, 14]. Ara-A, which is relatively non-toxic to mammalian cells and not immunosuppressive, has demonstrated *in vitro* and *in vivo* activity against several DNA viruses (e.g. herpes simplex, vaccinia, varicella–zoster and cytomegalovirus) and retraviruses (e.g. Moloney sarcoma and Rauscher leukemia virus [15]. Ara-Hx (9-β-D-arabinofuranosylhypoxanthine), the product of ara-A deamination, is approximately ten times less active than ara-A, at least in cell culture [14]. The promise of ara-A as an antiviral agent has recently been attested to by the finding that in a placebo-controlled study of twenty-eight patients with biopsy-proved herpes simplex encephalitis, ara-A treatment caused a significant reduction in mortality (from 70 to 28 per cent) [16].

Considerable effort has been devoted to define the

functional groups of adenosine, which determine its interaction with adenosine deaminase [17–21], using the commercial enzyme from calf intestinal mucosa. Since we had at our disposal a series of new adenosine analogs, we examined their substrate specificities for adenosine deaminase from PRK and HSF cells. In addition, a series of modified uracil nucleosides were evaluated as substrates for uridine phosphorylase from PRK cells.

MATERIALS AND METHODS

Chemicals. The origin of the nucleoside analogues was as follows: 5-methyl-, 5-ethyl-, 6-methyl, 2′-*O*-methyl-, 3′-*O*-methyl- and 5′-*O*-methyl-uridine, pseudouridine (5-ribosyluracil), 5-methyl- and 5-ethyl-2′-deoxyuridine: cf. [22, 23]; 5-fluoro-2′-deoxy-uridine: Aldrich Chemical Company; ara-U and ara-T: Terra-Marine Bioresearch, La Jolla; ara-A: Parke–Davis, courtesy of Dr R. Wolf, Parke–Davis Clinical Research Western Europe, München; 2′-*O*-methyl-, 3′-*O*-methyl-, 2′,3′-di-*O*-methyl and 5′-*O*-methyl derivatives of adenosine and ara-A: cf. [24, 25]; N_1- and N_2-methyl-formycin: cf. [26]; 7-deazaadenosine: cf. [27]; 5-propynyloxyuridine and 5-propynyloxy-2′-deoxyuridine: cf. [28]; 2′-deoxyadenosine, 3′-deoxyadenosine (cordycepin) and xanthine oxidase were obtained from Sigma Chemical Co.

Cells. All experiments were carried out with PRK and HSF (VGS strain) cells, grown to confluency in Roux bottles in Eagle's MEM (minimal essential medium), supplemented with 10% fetal calf serum.

Viruses. Vesicular stomatitis virus (VSV) (Indiana strain) was propagated in BSC-1 (African green monkey kidney) cells, whereas herpes simplex virus type 1 (strain KOS) and herpes simplex virus type 2 (strain 333) were maintained in PRK cells. Vaccinia virus was multiplied on the chorioallantoic membrane of embryonated eggs.

Preparation of cell extracts. Cell homogenates were prepared from normal uninfected cell cultures or cultures which had been inoculated with either vaccinia, vesicular stomatitis or herpes simplex virus. Virus input was approximately 1.5 $CCID_{50}$ per cell for vesicular stomatitis virus and herpes simplex virus and 0.3 $CCID_{50}$ per cell for vaccinia virus (where $CCID_{50}$ is equal to virus dose needed to infect 50% of the cell cultures). After 1 hr adsorption at 37° residual virus was removed, fresh medium (Eagle's MEM plus 10% fetal calf serum) was added, and the cells were further incubated at 37°: vaccinia and herpes virus–infected cultures for 4, 8 or 12 hr, and VSV-infected cultures for 2, 4, 6 and 8 hr. Cell monolayers were washed four times with PBS (phosphate buffered saline), and the cells were scraped off with a rubber policeman into 2 ml (per Roux bottle) of a buffer containing 20 mM Tris–HCl (pH 7.3), 1 mM EDTA and 3–4 mM 2-mercaptoethanol. The cells were disrupted by sonication two to three times for 10 sec at 0° and the cell homogenates cleared by centrifugation (30 min at 16.500 rpm). The supernatants served as sources of enzyme. Aliquots of these supernatants were stored at −70° until use.

Spectrophotometric determinations were done with a Zeiss PMQ II manual instrument, using semi-micro cuvettes of 1 cm pathlength.

Protein determinations were carried out according to the method described by Lowry *et al.* [29] with bovine serum albumin as standard.

Enzyme assays. Unless stated otherwise, all enzymatic activities were determined at 37° and pH 7.3. The assays were performed under conditions where the reaction was linear with time and cell protein, and the appropriate times of incubation and cell protein concentrations were chosen from separate pilot experiments. All enzyme assays were run in duplicate or triplicate. *Cytidine deaminase* assay was carried out as described previously [23]. *Pyrimidine nucleoside phosphorylase assay* was performed as described previously [22]. The release of free bases was monitored at 290, 300 and 304 nm for uracil, 5-alkyluracils and 5-fluorouracil, respectively. The millimolar absorbance changes resulting from the transformation of nucleosides to bases were assumed to be $\Delta\epsilon_{290} = 5.41$, $\Delta\epsilon_{300} = 3.61$ [30], and $\Delta\epsilon_{304} = 2.3$ (as calculated from data of Berens and Shugar [31]. The standard incubation mixture contained, in a total volume of 0.2 ml, 50 mM Tris–HCl (pH 7.3), 5–10 mM sodium phosphate (or sodium arsenate) (pH 7.3), 5 mM substrate, and cell extract. Zero time blanks contained all reagents, except the substrate, and were submitted to the same treatment as the other samples. Substrate was added to the cold reaction mixtures immediately prior to acidification.

Purine nucleoside phosphorylase. Activity was assayed in a thermostated cuvette compartment of the Zeiss PMQ II spectrophotometer. The method employed was the spectrophotometric assay coupled with xanthine oxidase, based on the measurement of the increase of absorbancy at 293 nm, due to the formation of uric acid [32]. The standard incubation mixture contained, in a total volume of 1 ml, 180 μmoles of sodium phosphate buffer (pH 7.3), 0.01 to 0.02 units of xanthine oxidase, 2 μmoles of inosine, and cell extract. The reaction was started by addition of the substrate. The latter was added as soon as the incubation mixture with the enzyme reached equilibrium.

Adenosine deaminase activity was assayed as described by Kalckar [33] and modified by Trams and Lauter [34]. The standard incubation mixture contained, in a total volume of 0.5 ml, 50 mM Tris–HCl (pH 7.3), 1 mM $MgCl_2$, 0.1 mM substrate and cell extract. The reaction was started by addition of the substrate and terminated by addition of ice-cold $HClO_4$ (to a final concentration of 0.25 M $HClO_4$). After centrifugation, absorbance of the supernatant was read at 265 nm. The millimolar absorbance change resulting from transformation of adenosine to inosine was assumed to be $\Delta\epsilon_{265} = 6.5$ [34]. Since there is practically no difference in the u.v. spectrum of the ribo- and deoxyribonucleoside in the acid and neutral pH region [35], an identical $\Delta\epsilon_{265}$ value was assumed for 2′-deoxyadenosine, 3′-deoxyadenosine and ara-A, and for the *O*′-methyl derivatives of adenosine and ara-A. The deamination of formycin and its N_1- and N_2-methyl analogues was monitored at 310 nm, assuming the millimolar absorbance change to be $\Delta\epsilon_{310} = 5.0$ (as calculated from spectral

data reported for formycin A and formycin B [21, 36].

Specific enzymatic activities. These are expressed as units per mg protein, one unit of enzymatic activity being the amount of enzyme required to transform 1 nmole of substrate per min at 37°. Apparent Michaelis–Menten constants (K_m) and maximal velocity values (V_{max}) were obtained graphically from the linear portions of double-reciprocal plots of substrate concentrations vs initial velocities.

RESULTS AND DISCUSSION

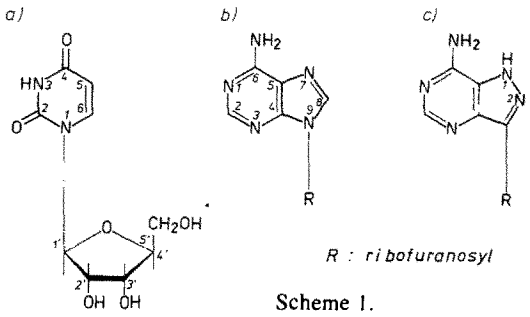
Cytidine deaminase activity. Unlike extracts from *Salmonella typhimurium* [22], peripheral human leukocytes, and a continuous line of human kidney cells (unpublished data, 1976), extracts of HSF and PRK cells exhibited no cytidine deaminase activity detectable by our procedure. This situation was unaltered following infection with herpes simplex, vaccinia or vesicular stomatitis viruses, although both cell types exhibited appreciable levels of dCMP deaminase (unpublished data, 1976). A similar observation has been recorded for HEp-2 cells, where infection with HSV-2 (strain G) does not affect the rate of deamination of [^{3}H]CdR [3]. More recently Chan [37], using radioactive substrates, demonstrated induction of deoxycytidine deaminase activity in several mammalian cell lines by HSV-1 (strain C1–101). The induced activity was postulated to be virus coded. The apparent discrepancy between our findings and those of Chan may be due to the fact that the level of such activity in both normal and virus infected HSF and PRK cells was below the sensitivity of our assay procedure. It should, however, be noted, that expression of enzyme activities induced by the virus depends not only on the type of infected cell, but equally on as yet ill defined physiological and environmental factors [7].

Since both CdR and ara-C are substrates for cytidine deaminase, the absence (or very low level) of the latter in PRK and HSF cells may, in part, explain why (1) an analogue such as 5-ethyl-2′-deoxycytidine shows little antiviral activity relative to 5-ethyl-2′-deoxyuridine in PRK cells (38) and (2) that ara-C exhibits pronounced antiviral activity in both PRK and HSF cells and, at low concentrations, inhibits PRK cell DNA synthesis [39].

Pyrimidine nucleoside phosphorylase activities. No pyrimidine ribo- or deoxyribonucleoside cleaving activities were detected in extracts of normal or infected HSF cells, whether these were challenged with vaccinia, herpes simplex type 1 or 2, or vesicular stomatitis virus. Since our assay is based on spectral determination of the free base (uracil or thymine) released during cleavage, one might argue that these bases, even if released, might rapidly be degraded to products not absorbing in the u.v. This appears unlikely since our procedure did not show the appearance of free bases from analogues such as 5-ethyl and 5-propynyloxy ribo- and deoxyribonucleosides; the aglycones of the latter would hardly suffer the same fate as uracil or thymine.

In contrast to HSF, PRK cell extracts displayed marked pyrimidine ribonucleoside phosphorylase activity, with virtually identical results when phosphate was replaced by arsenate in the incubation medium. With uridine as substrate, the specific activity of the extracts was 4 ± 1 U/mg protein. Following infection with vaccinia, herpes simplex or vesicular stomatitis virus, the activity tended to decrease somewhat, but this decrease did not exceed 1 U/mg protein. It was previously shown that infection of PRK cells with any of the foregoing viruses led to a significant increase in pyrimidine nucleoside kinase activities [40]. The fact that, in virus-infected cells, an increase in pyrimidine nucleoside kinase levels is accompanied by a decrease in phosphorylase activity, suggests that in the infected cells intact pyrimidine nucleosides are preferentially utilized for biosynthesis of nucleic acids.

The relative susceptibilities of various uridine analogues (scheme 1a) towards pyrimidine nucleoside phosphorylase(s) of PRK cells are presented in Table 1. It will be seen that the PRK extracts preferentially cleaved uracil nucleosides, activity vs deoxyuridine being quite low, but appreciable in the case of ara-U. In contrast to the *Salmonella thyphimurium* enzyme, which was inactive vs 2′-*O*-methyluridine [22], the PRK enzyme exhibited a level of activity with this analogue comparable to that vs deoxyuridine (Table 1). But, as in the case of the bacterial enzyme, the mammalian extract was inactive against 3′-*O*-methyl and 5′-*O*-methyl uridines, and 5-ribosyluracil (pseudouridine). The activity against 5-fluorouridine and 5-fluorodeoxyuridine is comparable to that for uridine and deoxyuridine, respectively.



Scheme 1.

It is, however, of interest that alkylation of the C-5 of the pyrimidine ring led to appreciable decreases in substrate susceptibility, although it is somewhat surprising that 5-propynyloxyuridine is a better substrate than 5-ethyluridine (Table 1).

As in the case of the bacterial enzyme [22], the rather odd observation was made that 6-methyluridine, a nucleoside presumed to be in the *syn* conformation about the glycosodic bond [41], proved to be a good substrate (Table 1).

Summing up, PRK cells seem to contain uridine phosphorylase with some activity vs deoxyuridine, an ability common to the enzyme from other mammalian sources [42]. The enzyme also recognizes 5-fluorodeoxyuridine, but not 5-alkyldeoxyribonucleosides, including thymidine. The latter observation supports the contention that uridine and thymidine phosphorylase activities are linked to different molecular entities [43].

Table 1. Pyrimidine nucleoside analogues as substrates for pyrimidine phosphorylases of PRK cells

Nucleoside	Relative substrate activity (%)
Uridine	100*
5-Methyl-	51
5-Ethyl-	12
5-Fluoro-	78
5-Propynyloxy-	32
6-Methyl-	63
2'-*O*-Methyl-	5
3'-*O*-Methyl-	<1
5'-*O*-Methyl-	<1
2'-Deoxyuridine	6
5-Methyl-	<1
5-Ethyl-	<1
5-Fluoro-	7
5-Propynyloxy-	<1
Ara-U (arabinofuranosyluracil)	18
Ara-T (arabinofuranosylthymine)	<1
Pseudouridine (5-ribosyluracil)	<1

* Specific activity of pyrimidine nucleoside phosphorylase, with uridine as substrate, was 5.2 units per mg protein.

Table 2. Adenosine analogues as substrates for adenosine deaminase of PRK and HSF cells

Nucleoside	Relative substrate activity (%) PRK	HSF
Adenosine	100*	100*
2'-*O*-Methyl-	61	58
3'-*O*-Methyl-	33	34
5'-*O*-Methyl-	<1	<1
2'-Deoxyadenosine	79	112
3'-Deoxyadenosine (cordycepin)	68	88
7-Deazaadenosine (tubercidin)	<1	<1
Ara-A (arabinofuranosyladenine)	28	22
2'-*O*-Methyl-	2	4
3'-*O*-Methyl-	4	7
2', 3'-di-*O*-Methyl-	<1	<1
5'-*O*-Methyl-	<1	<1
Formycin	104	93
N_1-Methyl-	<1	<1
N_2-Methyl-	11	8

* Specific activities of the adenosine deaminases of PRK and HSF cells, with adenosine as substrate, were 21.6 and 40.1 units per mg protein, respectively.

The previously noted lack of cytotoxic effect and/or antiviral activity of some pyrimidine nucleoside analogues in HSF and PRK systems [38, 39], cannot therefore, be ascribed to degradation of these analogues by the cellular pyrimidine nucleoside catabolizing enzymes.

Purine nucleoside phosphorylase activity. The level of purine nucleoside activity in PRK cell extracts, with inosine as substrate, fell within the range 110 ± 20 U/mg protein, hence approximately 25-fold higher than that for uridine phosphorylase. Inoculation of the cells with vaccinia, herpes simplex or vesicular stomatitis virus led to no significant change in activity levels. The enzyme levels of HSF cells were not determined.

Adenosine deaminase activity. Under the same experimental conditions as used to measure the other enzymatic activities, the level of adenosine deaminase activity of HSF extracts was in the range of 36 ± 120 U/mg protein. For PRK cell extracts it was in the range of 20 ± 8 U/mg protein. These values were essentially unaltered on infection of either cell system with vaccinia, herpes simplex or vesicular stomatitis virus. It should be noted that Sweetman *et al.* [13] also found no changes in adenosine deaminase activity in LLC-MK2 cells following infection with vaccinia virus.

The relative susceptibilities of various adenosine analogues (scheme 1b) to adenosine deaminase from both PRK and HSF cells are listed in Table 2. No significant differences were noted in the behavior of the enzymes of HSF and PRK cells, except, perhaps, that 2'-deoxyadenosine and 3'-deoxyadenosine (cordycepin) were recognized slightly better as substrates (as compared to adenosine) by the HSF than by the PRK cell enzyme(s). For the enzyme(s) of both sources, methylation of the 2'- and 3'-hydroxyls led to similar decreases in substrate activity while 5'-*O*-methylation completely suppressed susceptibility to the enzymes (Table 2).

Ara-A proved to be a reasonably efficient substrate for adenosine deaminase of both PRK and HSF cells. It was deaminated at about 20–25 per cent the rate for adenosine (Table 2). The 2'-*O*- and 3'-*O*-methyl derivatives of ara-A were deaminated at 10–30 per cent the rate for ara-A. Substitution of 5'-methoxy for 5'-OH and simultaneous substitution of both 2'- and 3'-methoxy for 2'- and 3'-OH completely abolished the susceptibility of ara-A for the HSF and PRK enzymes (Table 2). The relative susceptibilities of ara-A and its *O*'-methyl derivatives to deamination by PRK and HSF cell extracts, as reported herein, are remarkably similar to those reported previously with adenosine deaminase from calf intestinal mucosa [25].

Competition experiments indicated only a small inhibitory effect of 5'-*O*-methyl ara-A on deamination of ara-A by HSF and PRK cell extracts. When 5'-*O*-methyl ara-A was used at twice the concentration of ara-A, the relative substrate activity of the latter was reduced by 30 per cent. When both substrate and analogue were used at equimolar concentrations, the inhibition was not greater than 10 per cent (data not shown).

In accord with previous results obtained with the intestinal mucosa enzyme [44], the HSF and PRK enzymes did not recognize 7-deazaadenosine (tubercidin) as substrate (Table 2). In addition, tubercidin did not interfere with the deamination of adenosine, when both substrate and analogue were used at equimolar concentrations.

Formycin, a pyrazolopyrimidine C-nucleoside antibiotic (scheme 1c) was readily deaminated by both HSF and PRK cell extracts (Table 2). It has also been shown to function as substrate for adenosine deaminase from calf intestinal mucosa [21], blood parasites (*Shistosoma mansoni*) [45] and human erythrocytes [46]. Substitution of a methyl group at N_2 reduced the substrate activity by about 90 per cent, whereas substitution of a methyl at N_1 completely abolished the activity (Table 2). The

Table 3. Apparent kinetic constants of adenosine analogs as substrates for adenosine deaminase of PRK and HSF cells

Nucleoside	PRK K_m (μM)	PRK V_{max} (relative)	HSF K_m (μM)	HSF V_{max} (relative)
Adenosine	58	100	52	100
2′-*O*-Methyl-	—*	—*	164	97
3′-*O*-Methyl-	—*	—*	258	81
2′-Deoxyadenosine	66	75	68	113
3′-Deoxyadenosine (cordycepin)	74	68	64	84
Ara-A (arabinofuranosyladenine)	157	52	227	49

* Not determined.

latter observations are in good agreement with results obtained previously [26], for the relative susceptibilities of formycin and its N_1- and N_2-methyl derivatives to deamination by calf intestinal adenosine deaminase.

The apparent kinetic constants for the rates of deamination of the different adenosine analogues by HSF and PRK cell extracts are presented in Table 3. The K_m and V_{max} values obtained in this study for the common adenine nucleosides are comparable to those obtained with enzymes of other mammalian sources: calf intestinal mucosa [47], rat cerebral cortex [48], human erythrocytes [46], and human skin fibroblasts [13].

The lack of activity of both 7-deazaadenosine and N_1-methylformycin (Table 2) points to the critical role of the purine *N*-7 in the recognition and hydrolysis of adenine nucleosides by adenosine deaminase [cf. 20, 45]. In contrast with *N*-7, the 2′- and 3′-hydroxyl groups appear to play a minor role in the substrate activity of adenosine (Table 2) [18, 19, 47]. That 2′-*O*-methyl- and 3′-*O*-methyladenosine displayed a partially reduced substrate activity, as compared to the unsubstituted adenosine (Table 2), may be attributed to steric hindrance of the relatively bulky methyl group. The presence of the free 5′-hydroxyl appears to be a stringent requirement for the catalytic activity of the enzyme, as *O*-methylation at C-5′ abolishes the affinity of adenosine (and ara-A) for adenosine deaminase, irrespective of the source of the enzyme (Table 2) [18–20, 25, 26].

The importance of adenosine deaminase in the biological activities of ara-A and other adenosine analogues is obvious. Adenosine deaminase contributes to a diminution in the antiviral activity of ara-A by virtue of its conversion to the less active ara-Hx [14]. On the other hand, tubercidin (7-deazaadenosine) owes its potent antimetabolic properties to its resistance to deamination, as its deaminated derivative, 7-deazainosine, is considerably less effective as an antimetabolic agent [27]. While the antiviral and antimetabolic activities of several adenosine analogues at least partially depend on their resistance to deamination, such resistance does not necessarily guarantee biological activity. For example, 5′-*O*-methyl-ara-A and N_1-methylformycin proved completely resistant to the action of adenosine deaminase (Table 2) [25, 26]. Yet, both analogues were devoid of any antiviral or antimetabolic properties [24, 26].

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