Changes in Sarcoma 180 Cells Associated with Drug-induced Resistance to Adenosine Analogs

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SUMMARY

A subline of mouse Sarcoma 180 (S-180) cells, resistant to adenosine analogs, was selected in cell culture by serial passage in the presence of increasing concentrations of \( \text{N}^6 \)-furfuryladenosine (IPAR). These cells (S-180/KR) were cross-resistant to a number of other adenosine analogs, including \( \text{N}^6-(\Delta^2\text{-isopentenyl}) \)-adenosine (IPAR). The resistance was unaltered after 5 months maintenance in absence of the drug. S-180/KR cells showed increased sensitivity to certain purine and pyrimidine nucleoside analogs such as 6-thiopurine ribonucleoside and 5-fluorodeoxyuridine.

The activity of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), an enzyme that provides an alternate metabolic pathway for utilization of adenosine (Km 29 \( \mu \)M) was increased 45% in extracts of resistant cells. No cleavage of IPAR by this enzyme in extracts of resistant cells was observed, but IPAR was a competitive inhibitor of adenosine deamination with a Km of 1.3 mM. The change that appears to be solely responsible for resistance to adenosine analogs was the 20,000-fold reduction in the activity of adenosine kinase (adenosine 5'-triphosphate:adenosine 5'-monophosphate ribosyltransferase, EC 2.7.1.20). This enzyme converts adenosine and its analogs to their 5'-monophosphates. The elution pattern of adenosine kinase of both cells on to otherwise metabolize IPAR, the rate of uptake of IPAR was greatly reduced; \( V_{\text{max}} \) 3.3 and 0.08 nmoles/hr/mg cells in S-180 (Km 24 \( \mu \)M) and S-180/KR (Km 14 \( \mu \)M), respectively.

INTRODUCTION

Cellular resistance to analogs of purine and pyrimidine nucleosides is generally associated with a reduced conversion to nucleotides or increased breakdown to less cytotoxic derivatives. Thus, resistance to MMPR\(^2\) in human H. Ep. cells and Ehrlich carcinoma cells of mouse has been shown to be associated with a lack of conversion of the nucleoside to its 5'-monophosphate (2, 5, 30).

IPAR and KR are cytotoxic to mammalian cells in vitro while the corresponding free bases are not (11, 12, 15, 25). In addition, IPAR has shown some antitumor activity in rodents (38) and has produced clinical remission in a child with promyelocytic leukemia (26). Both of these nucleosides are potent inhibitors of cellular uptake of purine and pyrimidine nucleosides (21, 22) and have been shown to be converted to 5'-monophosphates by adenosine kinase, partially purified from mouse S-180 cells grown in vitro (7). Also, in crude extracts of L1210 cells a conversion of IPAR to IPAMP has been demonstrated (16). No further phosphorylation of IPAMP by AMP kinase was observed (7), but IPAMP was shown to be a competitive inhibitor of this enzyme (37).

The present study concerns changes associated with drug-induced resistance of S-180 cells to analogs of adenosine. These include: (a) response to drugs in terms of growth, (b) activity of anabolic and catabolic enzymes concerned with IPAR metabolism, (c) cellular uptake of IPAR and adenosine, and (d) cellular metabolism of IPAR. Abstracts on this subject have been published (8, 19, 37).

MATERIALS AND METHODS

Compounds. The sources of most of the compounds used in the present study have already been described (7). Further chemicals were obtained as follows: purine ribonucleoside, 6-thioguanosine, 2,6-diaminopurine deoxyribonucleoside, and 6-thiopurine ribonucleoside from Sigma Chemical Co., St.

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Resistance to Adenosine Analogs

Louis, Mo.; 6-chloropurine ribonucleoside, K and K Laboratories, Plainview, N. Y.; sangivamycin, Charles Pfizer & Co., New York, N. Y.; puromycin aminonucleoside, Nutritional Biochemicals Corp., Cleveland, Ohio; 6-thiopurine deoxyribonucleoside and 6-thioguanine deoxyribonucleoside, Schwarz/Mann, Orangeburg, N. Y.; 2-fluoroadenosine (NSC 30605), tubercidin, 7-deazainosine, and formycin were obtained through the courtesy of Dr. A. Bloch at this Institute.

Preparation of IPAR-8-14C. The method involved direct alkylation of adenosine-8-14C (50 µCi/µmole) which was followed by conversion of N6- to N9-isomer according to a published procedure (32) modified for the present purpose (10).

The final product contained 99.7% IPAR, 0.2% adenosine, and 0.1% adenine, all with specific activities of 50 µCi/µmole. Total recovery of IPAR-8-14C was 45% of theoretical. When stored in absolute ethanol at —70°, the product was stable. In dilute solutions (3 to 300 µm) in experimental media, no breakdown of IPAR-8-14C was observed even during 2 hr of incubation at 36°.

Cells. S-180 cells were maintained as described previously (7). The subline (S-180/KR), resistant to KR, was developed by serial passages of S-180 over a period of 4 months in the presence of increasing concentrations of KR (from 5 to 50 µm). Before being used for enzyme assays or uptake studies, S-180/KR cells were grown for at least 7 days in the absence of KR in order to free them of any possible intracellular KR or its metabolites.

Growth Inhibition Studies. These were conducted as described previously (7).

Cell Extracts. Extracts of S-180 and S-180/KR cells were prepared in 5 mM Tris-chloride buffer, pH 7.0, containing 1 mM EDTA and 5 mM NaCl as described earlier (7). Protein was determined according to the method of Lowry et al. (31) with bovine serum albumin as the standard.

Adenosine Kinase Assay. The method has been described previously (7). The reaction was carried out at pH 7 and 35° in the presence of 2.5 mM ATP, 0.25 mM MgCl2, 50 mM potassium phosphate, and the enzyme.

Adenosine Deaminase Assay. The assay was based on the conversion of adenosine-8-14C to inosine-8-14C and hypoxanthine-8-14C which were estimated after chromatographic separation. The reaction mixture (0.2 ml) contained 1 mM adenosine (1 µCi/µmole), 0.05 M potassium phosphate buffer (pH 7.0), and crude cell extract (1 to 6 µg protein). The reaction was started by adding the enzyme to the assay mixture prewarmed to 30°. After 10 min at 30°, the reaction was stopped by immersing the assay tubes in boiling water bath for 2 min. An aliquot (50 µl) was subjected to descending chromatography on a Whatman No. 3MM paper strip with Solvent A when substrate was inosine and Solvent B (ethanol:0.1 M ammonium borate, 1:9) (32) when substrate was IPAR.

Cellular Uptake Studies. Two methods of measuring the uptake of adenosine and IPAR were used. Both involved the utilization of monolayer cultures incubated for 15 min.

The earlier method involved the use of Roux bottle cultures as previously described (20). An improved method, adopted later, involved the use of T-15 flask cultures, which had been inoculated 2 days previously with 400,000 to 500,000 cells in 2 ml of medium. The cells were refed 1 day before use and incubated at 36° with 2 ml of fresh medium supplemented with the 14C-labeled nucleoside (150,000 to 200,000 cpm/ml). At the end of incubation, the medium was poured off, the flask was set on ice, and the cell layer was rinsed 3 times with 2 ml of ice-cold, serum-free medium and drained. The cell layer was dissolved into 3 ml of 0.2 N NaOH by incubating for 1 hr at 36°. Aliquots (0.5 ml each) of the solution were measured into counting vials, acidified with 0.2 ml of 1 N HCl, and counted. Appropriate aliquots were used for determination of total protein, which varied from 0.6 to 1.0 mg/T-15 flask. One mg of protein is equal to 10.4 mg of cells (minus extracellular H2O) (18). The uptake data determined per mg of protein, when converted to be expressed per mg of cells (minus extracellular H2O), gave results in good agreement with the earlier method based on weight measurement.

Cellular Metabolism of Labeled Nucleosides. Monolayer cultures of cells were prepared and incubated in T-15 flasks as described for the uptake studies. At the end of 1 hr incubation at 36°, the medium was poured off and centrifuged for 10 min at 2000 rpm; then 0.1-ml samples were taken for counting. The flask was set on ice and rinsed as above; the cells then were extracted with 2 ml of ice-cold 5% TCA for 10 min. This extract contained about 90% of the total TCA-soluble 14C. The extraction was repeated 2 more times and the 2nd and 3rd extract were combined in a separate tube. These solutions were extracted 5 times with 3 ml of ethyl ether to remove TCA, and 0.1-ml samples were counted for 14C. The remaining cell layer was dissolved in 3 ml of 0.2 N NaOH and analyzed as above.

Chromatographic Analysis of TCA-soluble Material. An aliquot of the 1st TCA extract, corresponding to about 3500 cpm in case of IPAR, together with nonlabeled marker compounds, was subjected to descending chromatography on Whatman No. 3MM paper (1 inch wide) in Solvent A. The strips were dried at room temperature, marked for UV-absorbing spots, and cut into 1-cm segments for counting. The percentage of distribution of 14C in the various products was determined. This permitted estimation of the total amount of each compound in the TCA-soluble pool. Recovery of the original counts was 90 to 95%.

Counting of 14C. A Packard Tri-Carb liquid scintillation counter was used. Samples containing water were counted in
Divekar, Fleysher, Slocum, Kenny, and Hakala

10 ml of dioxane:PPO:POPOP scintillation solution (4). The counting efficiency of cell and medium samples was 70%, NaOH cell solutions 60%. The 1-cm segments of dry paper strips were counted in 10 ml of toluene:PPO:POPOP as described earlier (7).

RESULTS

Cross-resistance of S-180/KR. The S-180 subline (S-180/KR) developed for resistance to KR was also resistant to several other N°-substituted adenosines, among them IPAR (Table 1). These cells were also resistant to purine ribonucleosides with H, Cl, or a methylmercapto group at position 6. Similarly, several adenosine analogs with a modified imidazole ring were known to be substrates of adenosine kinase (7, 36). It was somewhat unexpected that 7-deazainosine and puromycin aminonucleoside, known not to be substrates of adenosine kinase (13, 36), were also less inhibitory for S-180/KR than for S-180 cells. Resistance to 7-deazainosine has also been observed in H. Ep cells deficient in adenosine kinase and resistant to adenosine analogs (2). It was not surprising that the sensitivity of S-180/KR towards analogs of inosine and guanosine was unaltered as is seen in Table 2. The unaltered sensitivity to 2-fluoroadenosine most probably is explained by an alternate route of utilization, namely, through cleavage to the free base prior to nucleotide formation. Indirect evidence for such pathway has been previously provided (2, 33).

Collateral Sensitivity of S-180/KR. Four purine and pyrimidine nucleosides, which were more potent inhibitors of S-180/KR than of S-180 cells, are listed in Table 2. The increases in sensitivity of S-180/KR varied from 1.8- to 2.2-fold and were consistent. Bennett et al. (2) also observed increased sensitivity to 6-mercaptopurine and 5-fluorodeoxyuridine in H. Ep. cells resistant to adenosine analogs. Even a 2-fold increase in sensitivity to an antineoplastic agent, if occurring in vivo, would be significant. No explanation for this increase in sensitivity can be offered at this time.

Stability of Drug Resistance. The resistance of S-180/KR cells to growth inhibition by adenosine analogs was stable when the cells were maintained in the absence of KR for prolonged periods of time. Thus, S-180/KR cells after being grown for 5 months in the absence of the drug were fully resistant to KR and IPAR, and the degree of resistance to toyocamycin was unaltered.

Adenosine Kinase. Initial comparison of the 2 cell extracts for adenosine kinase activity was performed with MMPR-35S as the substrate (Table 3). This compound is known to resist the attack of adenosine deaminase and nucleoside phosphorylase, enzymes abundant in crude cell extracts. While MMPR was phosphorylated by S-180 extracts at a rate of 4.3 nmoles/min/mg protein, no phosphorylation by S-180/KR extracts was observed even when the protein content was raised 14-fold and the incubation was continued for 3 hr. Extracts of S-180/KR cells and of cells maintained for 4 months in the absence of KR also failed to catalyze the phosphorylation of IPAR-8-14C (Table 3).

Since resistance of S-180/KR to the more potent adenosine analogs (Table 1) was not absolute but only relative, complete absence of adenosine kinase in S-180/KR cells was unlikely. To allow for a more sensitive and accurate assay of the possible presence of adenosine kinase in the resistant cells, adenosine deaminase was removed by fractionation on DEAE-cellulose column as was described for S-180 cells (7). Fractions 59 to 64, which were free of adenosine deaminase and now could be assayed with adenosine-8-14C (47 μCi/μmole) as the substrate, contained low but distinct
Resistance to Adenosine Analogs

Table 3

<table>
<thead>
<tr>
<th></th>
<th>S-180</th>
<th>S-180/KR (4M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-180 Crude cell</td>
<td>nmoles/min/mg protein</td>
<td>K_m (μM)</td>
</tr>
<tr>
<td>extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMPR-35-1^S, 1.0 mM</td>
<td>4.3^b</td>
<td>&lt;0.036^b</td>
</tr>
<tr>
<td>IPAR-8-^14C, 0.1 mM</td>
<td>2.1^c</td>
<td>&lt;0.004^c</td>
</tr>
<tr>
<td>Fractions from</td>
<td>4.65^d</td>
<td>0.5</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
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<tr>
<td>adenosine-8-^14C, 0.0044 mM</td>
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</table>

a These cells had been maintained in the absence of KR for 4 months.

b In the case of S-180 or S-180/KR, MMPR-35-1^S was incubated for 20 min with 0.5 mg or for 3 hr with 7 mg of cell extract protein, respectively, as described in "Materials and Methods."

c In the case of S-180, S-180/KR, or S-180/KR (4M), IPAR-8-^14C was incubated for 10 min with 0.23 mg, 60 min with 2 mg, or 60 min with 2.7 mg of cell extract protein, respectively, as described in "Materials and Methods."

d Activity expressed per mg equivalents of cell extract protein which had been applied to the DEAE-cellulose column. In the case of S-180 or S-180/KR, the incubations were carried for 2 min with 20 µl or 2 hr with 240 µl of the fractions, respectively.

Chart 1. Adenosine kinase of S-180/KR cells. A, fractionation of the extract (equal to 37.7 mg of protein) of S-180/KR cells on DEAE-cellulose column as described elsewhere (7). Linear NaCl gradient was applied at the point indicated by the arrow. Adenosine kinase activity was assayed by incubating 4.4 mM adenosine-8-14C with 240 µl of each fraction for 2 hr as described in "Materials and Methods." Inset, fractions which in the case of S-180 cells contained the adenosine kinase activity (7). B, Lineweaver-Burk plot (29) for adenosine kinase in pooled and concentrated fractions (59 to 64) from DEAE-cellulose column; the reactions were carried in the presence of 2.5 mM ATP and 0.25 mM MgCl₂. K_m 0.5 μM, V_max 1.5 pmol/min/mg protein.

Adenosine kinase activity (Chart 1A). The elution pattern of these fractions was identical with that of adenosine kinase of S-180 cells. It is seen from Table 3 that the extracts of the parent cells had 22,000 times higher adenosine kinase activity than the extracts of the resistant cells, assuming that no loss of adenosine kinase activity occurred during purification of either cell extract.

For further comparison of the 2 adenosine kinases with each other, the fractions containing adenosine kinase derived from S-180/KR cells were pooled and concentrated 10-fold by evaporation in a dialysis bag at 4° in the presence of 500 µg bovine serum albumin per ml as a stabilizer (7). Although this procedure caused some inactivation of the enzyme, the K_m value for adenosine could be estimated (Chart 1B) and was found to be 0.5 µM, identical to that for enzyme in S-180 cells (7). This was determined in the presence of 2.5 mM ATP, 12.5 times the K_m for ATP.

Nucleoside Phosphorylase. It can be seen from Chart 2, that the nucleoside phosphorylase activity in crude extracts of sensitive and resistant cells was almost equal when tested with either inosine-8-14C or IPAR-8-14C as the substrate. In the experimental conditions used inosine was cleaved 7 to 9 times faster than IPAR. By using the extract of the parent cells and by running the reactions in Tris buffer, we found that the cleavage of both substrates required phosphate (Chart 2). The traces of activity, observed in Tris buffer, most likely reflect the presence of some endogenous phosphate in these cell extracts. At equimolar and at 10-fold concentration, inosine inhibited the cleavage of IPAR by 15 and 40%, respectively.

Adenosine Deaminase. The average specific activity in 3
Divekar, Fleysher, Slocum, Kenny, and Hakala

different crude extracts of S-180/KR cells was 45% higher (144 ± 15 nmoles/min/mg protein) than the corresponding value for S-180 cells (103 ± 4.8 nmoles/min/mg protein). The $K_m$ value for adenosine (29 $\mu$M) was the same for both enzymes. After S-180/KR cells were maintained for 4 months in the absence of KR, the activity of adenosine deaminase still remained 45% higher (149 ± 1.5 nmoles/min/mg protein) than in S-180 cells indicating that the increase was as stable as the resistance to adenosine analogs.

**N°-Substituted Adenosines as Substrates of Adenosine Deaminase.** With the use of a spectrophotometric assay (27) KR and IPAR at 50 $\mu$M appeared not to be substrates of adenosine deaminase in the crude extract of either cell. No decrease of absorbance at 265 nm was observed during incubation for 60 min at 37° in 0.05 M potassium phosphate, pH 7.0, in the presence of 1 mg of protein per 3 ml. This indicated that the cleavage of KR and IPAR to inosine by both cell extracts, if any, must have been less than 0.03 nmole/mg protein/min.

In a more sensitive assay 100 $\mu$M IPAR-8-14C (3.1 $\mu$Ci/µmole) was incubated with the extracts (4 mg protein) of sensitive and resistant cells for 60 min at 30° in 0.05 M Tris-chloride buffer, pH 7.0, in a total volume of 0.4 ml. Tris buffer was used to reduce the cleavage of IPAR to the free base by the nucleoside phosphorylase. No formation of inosine-8-14C was observed while 10% of IPAR had been cleaved to the free base.

Although KR and IPAR did not appear to be substrates of adenosine deaminase these compounds inhibited the deamination of adenosine in a competitive manner. However, their affinity to the enzyme was poor as revealed by the $K_i$ values of 1.3 and 2.3 mM for IPAR and KR, respectively.

**Cellular Uptake of Adenosine-8-14C.** The uptake per mg of cells, when tested at 1 to 10 $\mu$M concentration of adenosine, was linear with time for at least 20 min. On this basis a 15-min period was chosen for the kinetic studies.

![Chart 2. Nucleoside phosphorylase activity in crude extracts of S-180 (circles) and S-180/KR cells (squares). Solid symbols, activity for inosine-8-14C as the substrate, open symbols, IPAR-8-14C activity. All activity determinations were performed in the presence of 100 $\mu$M substrate in 0.05 M phosphate buffer, pH 7.0, except those marked with Tris which indicate 0.05 M Tris-HCl buffer, pH 7.0.](chart2)

![Chart 3. Velocity of uptake of adenosine-8-14C in S-180 and S-180/KR cells at varied concentrations of adenosine. The uptake was measured with the use of monolayer cultures of cells grown in Roux bottles as described in "Materials and Methods."](chart3)

![Chart 4. Double reciprocal plots for adenosine-8-14C (AR) uptake by S-180 and S-180/KR cells. The data were obtained as described in legend for Chart 3 by incubating the monolayers of cells for 15 min. For S-180: $K_t$, 47 $\mu$M; $V_{max}$, 18.2 nmoles/hr/mg cells. For S-180/KR: $K_t$, 20 $\mu$M; $V_{max}$, 5.1 nmoles/hr/mg cells.](chart4)

The relationships between the extracellular adenosine concentrations and the velocity of uptake differed in S-180 and S-180/KR cells. In S-180 cells, maximal velocity was reached at about 100 $\mu$M adenosine. In contrast, in S-180/KR cells the uptake velocity continued to increase even at 3 mM adenosine. The double reciprocal plots for adenosine uptake in the 2 cell lines are shown in Chart 4. It is seen that the maximal velocity of adenosine uptake was almost 4 times higher in S-180 than in S-180/KR cells; also the cell:medium ratio at 10 $\mu$M adenosine was 2 to 3 times higher in sensitive [125] than in resistant cells [47] after 15 min incubation.
Resistance to Adenosine Analogs

None or only a small fraction of the intracellular $^{14}$C was unaltered adenosine in any of the conditions used; the majority was in the form of ATP (22).

**Cellular Uptake of IPAR-$^{14}$C.** The velocity of uptake of IPAR by S-180 cells was much slower than that of adenosine, and in resistant cells the uptake of IPAR was drastically reduced, being only one-fortieth that in sensitive cells (Chart 5). Kinetic studies revealed $K_t$ values of 24 $\mu$M for S-180 and 14 $\mu$M for S-180/KR. The corresponding $V_{max}$ values were 3.3 and 0.08 nmoles/hr/mg of cells for S-180 and S-180/KR, respectively. In 15 min at 10 $\mu$M IPAR the cell: medium ratio for S-180 cells was 31 while for S-180/KR it was only 1.0.

**Cellular Metabolism of IPAR-$^{14}$C.** The results of these studies are presented in Table 4. In sensitive cells incubated at 8.5 $\mu$M IPAR, the amount of IPAMP was 55 times higher than in S-180/KR cells incubated at 9.5 $\mu$M IPAR and comprised 75% of the total intracellular $^{14}$C. In these conditions the cell water in S-180 cells contained 400 $\mu$M concentration of IPAMP. Only small amounts of the unaltered nucleoside were present in both cells. The identity of IPAMP was confirmed by digestion with snake venom 5'-nucleotidase as described previously (7) followed by paper chromatography with Solvent A described in “Materials and Methods,” as well as the Solvent A described by Hacker (16). Traces of $^{14}$C were found to be incorporated into NaOH-soluble material of both cells. This was most likely due to the traces of adenine present in IPAR-$^{14}$C preparation (see “Materials and Methods”). The small amount of adenosine in the preparation could hardly have entered the cells, since IPAR is a potent inhibitor of adenosine uptake (21, 22). The stability of IPAR-$^{14}$C in the incubation medium throughout these studies was shown by the fact that after 2 hr incubation at 36° no other $^{14}$C-labeled compound was detected by paper chromatography in Solvent B.

**Utilization of Adenosine for Growth.** When the de novo synthesis of purine ribonucleotides, TMP, and glycine is inhibited by amethopterin, the growth of cells becomes dependent on preformed glycine, thymidine, and a source of purines (17, 23). In such conditions, when the source of purines was adenosine (Chart 6), it was observed that S-180/KR cells did not grow quite as well as S-180 cells. The growth responses differed also in that S-180 cells grew better than controls at 300 $\mu$M adenosine and 1 mM adenosine inhibited growth completely while S-180/KR cells never achieved the control growth and were only slightly inhibited at 1 mM adenosine. These adenosine utilization curves have an interesting similarity with the curves for adenosine uptake in the 2 cell lines (Chart 3).

**DISCUSSION**

Previous studies on resistance to adenosine analogs include studies on H. Ep. cells (human carcinoma of larynx) grown in vitro and Ehrlich ascites cells of mouse (2, 5, 30). Both of these were cases of drug-induced resistance developed by using MMPR as the selecting agent where the ability to convert the analog to its 5'-monophosphate was lost. The resistant H. Ep./MMPR cells were shown to be cross-resistant to a number of other adenosine analogs and the resistance was stable “for many generations.” Although the selecting agent for resistance in the present case was an $N^\alpha$-substituted adenosine, the characteristics of the resulting S-180/KR cell line are in some respects similar to those observed for H. Ep./MMPR. The similarities include resistance to other adenosine analogs,
increased sensitivity to certain nucleosides, and reduction of adenosine kinase activity. However, our study differs from the others by demonstrating traces of adenosine kinase, increase in adenosine deaminase, and unaltered nucleoside phosphorylase in the resistant cells. The study of the uptake of adenosine and IPAR in both cells and demonstration of intracellular accumulation of IPAMP in the sensitive cells is also included.

The demonstration of traces of adenosine kinase in resistant cells was possible only through partial purification of the enzyme to permit use of the natural substrate for assay. The presence of adenosine kinase in S-180/KR explains why the growth of these cells, although 50 to 90 times more resistant to toyoacamycin, sangivamycin, and tubercidin than the parent cells, still is inhibited 50% at 2.5, 4.4, and 17 µM concentrations of these compounds, respectively. These nucleoside analogs are not deaminated (14) or cleaved by nucleoside phosphorylase (3), and consequently their cytotoxic action appears to require direct phosphorylation by adenosine kinase. The adenosine kinase in the resistant cells was similar to the enzyme in sensitive cells with respect to K_m and elution pattern on DEAE-cellulose under comparable conditions. This suggests that it is rather the quantity and not the quality of the enzyme which is altered. The stability of the alteration (no change in 5 months in absence of KR) indicates a permanent mutant selected during the development of S-180/KR subline. To prove this assumption much more work would be required.

IPAR was not cleaved to inosine in crude extracts of S-180 cells but was a poor competitive inhibitor of adenosine deaminase (K_i, 1.3 mM). Another N^6-substituted adenosine analog, N^6-methyladenosine, has been shown to be a competitive inhibitor of adenosine deaminase of ox heart, rat heart, and rat red cells with a K_i of 6 (35), 12, and 13 µM (1), respectively, and a substrate of adenosine deaminase of calf intestinal mucosa with a K_m of 11 µM and V_max of 0.16% of that for adenosine (6). With an increase in the size of the N^6-substituent affinity for the enzyme was lost (6). However, IPAR was shown to be a poor substrate of that enzyme (K_m 100 µM, being cleaved at a rate which was 0.03% of that for adenosine (24). The adenosine deaminase of calf serum, on the other hand, seems to differ from other mammalian deaminases in having a K_m for adenosine and K_i for IPAR in the order of 1 µM (39). No substrate activity for IPAR in the calf serum system was found to be identical.

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Resistance to Adenosine Analogs


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