Differential sensitivity of RSV^{ts} (temperature-sensitive Rous-sarcoma virus)-infected rat kidney cells to nucleoside antibiotics at permissive and non-permissive temperatures

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Among a variety of anti-tumour agents tested, oxanosine and 5-azacytidine were found to be significantly more effective in inhibiting growth of rat kidney cells infected with a temperature-sensitive mutant of Rous sarcoma virus at a permissive temperature (33 °C) than at a non-permissive temperature (39 °C). These two nucleoside antibiotics were antagonistic to each other in cytotoxicity. They seem to share the same carrier-mediated membrane-transport system, because dipyrindamole, a potent inhibitor of nucleoside transport, protected cells from the cytotoxicity of both drugs. Thymidine transport, which is twice as fast in cells at 33 °C as at 39 °C, was competitively inhibited by both drugs. Thus the differential toxicity of oxanosine and 5-azacytidine at the two temperatures is thought to be due to their increased transport via the thymidine-transport system, which is somehow under the influence of the active *src*-gene product.

INTRODUCTION

Evidence has been accumulating that malfunctioning of cellular oncogenes is the major cause of human cancer and that most cellular oncogenes have their viral relatives (for reviews, see Bishop, 1983). From the viewpoint of cancer chemotherapy, it is important to find drugs that are preferentially active against tumour cells where oncogenes are abnormal or abnormally expressed, but not active against non-tumorous counterparts. For this purpose, we chose the cells of a normal rat kidney (NRK) line that had been infected with a mutant of Rous-sarcoma virus (RSV) Prague strain whose oncogene *src* is temperature-sensitive (*ts*/NRK), because the cells shift between the tumorous and non-tumorous state simply on change of culture temperature (Chen *et al.*, 1977). We previously found an increase in the rate of thymidine transport in cells at 33 °C compared with those at 39 °C that was the result of the qualitative change in the *src*-gene product (Uehara *et al.*, 1984). Here we report that oxanosine and 5-azacytidine are preferentially active against the cells in which the *src* gene is expressed and that this selective toxicity results from the increased transport of these two nucleoside antibiotics via the thymidine-transport system, which is somehow altered by the active *src*-gene product.

MATERIALS AND METHODS

Materials

Normal rat kidney (NRK) cells infected with *ts*25, a T-class mutant of RSV Prague strain, were provided by Dr. M. Yoshida, Cancer Institute, Tokyo, Japan (Chen *et al.*, 1977). These cells will be referred to as '*ts*/NRK'.

Oxanosine was obtained from Nippon Kayaku Co., Tokyo, Japan. NBMPR was obtained from Professor A. R. P. Paterson, Cancer Research Group, University of Alberta, Edmonton, Alberta, Canada. 5-Azacytidine, dipyrindamole and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.), Calbiochem-Behring (San Diego, CA, U.S.A.) and Wako Pure Chemical Industries (Osaka, Japan). Radioactive compounds were purchased from Amersham International (Amersham, Bucks., U.K.). Cell-culture materials were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.).

Cell culture

The *ts*/NRK cells were grown in DME supplemented with 10% heat-inactivated calf serum in humidified air with 5% CO₂. Cells were maintained by culture at 33 °C and, unless otherwise indicated, 3 × 10⁴ cells were seeded in each 35 mm plastic dish (day 0) and cultured overnight at 33 °C or 39 °C. Drugs were then added to the cultures (day 1) and the incubation was continued at either temperature. The cells were twice washed with 2 ml of Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954)/dish on day 3 and incubated further in 2 ml of the fresh medium/dish. On day 5 the cells were trypsin-treated [0.5 ml of 0.05% trypsin/0.02% EDTA in Hanks (1948) solution lacking Ca²⁺ and Mg²⁺/dish] and the cell number was counted in a model ZM Coulter counter. Runs were duplicated. Mouse leukaemic L1210 cells were also cultured in the same medium, harvested at exponential-growth phase and submitted to tests of growth inhibition by drugs in Falcon tissue-culture plates (24 wells) by seeding 1 × 10⁵ cells/ml per 2 cm² well. Drugs were added at the time of seeding and, after 48 h of incubation at 33 °C or 39 °C, cells were counted.

Abbreviations used: RSV^{ts}, temperature-sensitive Rous-sarcoma virus; NBMPR, nitrobenzylmercaptapurine riboside; DME, Dulbecco's modified Eagle medium.

Table 1. Effect of the concentration of serum on the growth of *ts*/NRK cells at permissive and non-permissive temperatures

The inoculum size was 0.45×10^5 /60 mm dish; the incubation time was 120 h. Each value represents the mean $\pm$ S.E.M. of triplicate runs within a single experiment.

[Calf serum] (%, v/v)	Temperature... 33 °C		39 °C	
	$10^{-5} \times$ No. of cells/dish	Doubling time (h)	$10^{-5} \times$ No. of cells/dish	Doubling time (h)
10	7.5 ± 0.41	30	4.5 ± 0.30	36
5	6.0 ± 0.43	32	2.2 ± 0.16	52
0.5	2.7 ± 0.08	46	0.7 ± 0.03	188

Table 2. Growth inhibition by various anti-tumour agents

IC₅₀ represents the drug concentration that gave 50% inhibition of growth at each temperature compared with the growth of controls. The IC₅₀ values were read from nearly linear segments of the curves (duplicate plots) of dose (log scale) versus percentage growth inhibition (regular scale).

Anti-tumour agent	Temperature...	IC ₅₀ (μg/ml)		Ratio 39 °C/33 °C
		33 °C	39 °C	
Aclacinomycin		0.009	0.012	1.3
Actinomycin D		0.009	0.009	1.0
Adriamycin		0.045	0.040	0.9
Auromomycin		0.55	0.60	1.1
Bactobolin		0.1	0.3	3.0
Clazamycin		1.8	2.8	1.6
Diketocorilin B		1.9	1.9	1.0
Hydroxyurea		5.0	6.0	1.2
Methotrexate		0.045	0.045	1.0
Mitomycin C		0.03	0.03	1.0
Neothramycin		0.06	0.06	1.0
Peplomycin		1.3	1.4	1.1
Rifamycin SV		6.0	6.0	1.0
Streptovaricin		55	65	1.2
Tunicamycin		0.09	0.10	1.1

Determination of cell membrane transport

Uptake of [³H]thymidine or [³H]adenosine by replicate monolayer cultures was measured at 20 °C by using the cluster-tray method (Gazzola *et al.*, 1981). Briefly, cells were grown at 33 °C or 39 °C in 24-well Falcon cluster trays to form cell layers of 10^5 cells/2 cm² well. The cell layers were twice washed with Dulbecco's phosphate-buffered saline containing 0.1% glucose, exposed for 30 s to 0.2 ml of phosphate-buffered saline/glucose solution containing each labelled nucleoside with or without an inhibitor, and rapidly washed with 3×1.5 ml of ice-cold phosphate-buffered saline containing 5 μM-dipyridamole, a potent nucleoside-transport inhibitor (Paterson *et al.*, 1981). After solubilization of the cell layers in 0.5 M-KOH, the radioactivity was determined as described previously (Uehara *et al.*, 1984).

RESULTS AND DISCUSSION

The *ts*/NRK cells were grown at 33 °C (permissive temperature) and 39 °C (non-permissive temperature) with the transformed phenotype and normal phenotype

respectively (Chen *et al.*, 1977). We tried to find drugs having selective toxicity to cells grown at 33 °C. For this purpose, we thought that cell-growth inhibition by various drugs should be determined under conditions where the doubling times of both phenotypes were similar. To find such conditions, the serum-dependency for cell growth at the two temperatures was determined (Table 1). At 33 °C, the doubling time did not change greatly, even at low serum concentrations. In contrast, at 39 °C, the doubling time became significantly longer when the concentration of the serum was lowered to 0.5%. With 10% (v/v) calf serum, the cells of both phenotypes grew at a similar speed, and the following experiments were therefore conducted under these conditions (10% calf serum). A variety of anti-tumour agents, including DNA-binding and/or DNA-reacting drugs (Table 2) and purine and pyrimidine analogues (Table 3), were tested. The concentrations required for 50% growth inhibition (IC₅₀) of each drug to cells at 33 °C and 39 °C were almost the same, although the values varied greatly from one drug to another. Oxanosine and 5-azacytidine (Fig. 1), both nucleoside antibiotics, were

Table 3. Growth inhibition by purine and pyrimidine analogues

Results are expressed as in Table 2.

Analogue	Temperature...	IC ₅₀ (μg/ml)		Ratio 39 °C/33 °C
		33 °C	39 °C	
9-β-D-Arabinofuranosyladenine (Ara A)		9	14	1.6
1-β-D-Arabinofuranosylcytosine (Ara C)		0.025	0.035	1.4
8-Azaguanine		3.0	5.5	1.8
6-Azauridine		1.0	4.8	4.8
5-Bromo-2'-deoxycytidine		20	22	1.1
5-Bromo-2'-deoxyuridine		4.0	1.0	0.25
3-Deazauridine		4.2	3.5	0.8
Decoyinine		400	350	0.9
2,6-Diaminopurine		0.8	1.3	1.6
5-Fluorouracil		0.07	0.18	2.6
5-Iodo-2'-deoxycytidine		9	20	2.2
5-Iodo-2'-deoxyuridine		1.5	2.0	1.3
6-Mercaptopurine		0.3	0.4	1.3
6-Mercaptopurine riboside		0.5	0.5	1.0
Pyrazofurine		0.065	0.13	2.0
Showdomycin		9	20	2.2
6-Thioguanine		0.027	0.05	1.9

Fig. 1. Structures of oxanosine and 5-azacytidine

exceptional in that they inhibited growth of cells at 33 °C more strongly than at 39 °C; the IC₅₀ of each of the two drugs for cells at 33 °C was less than one-tenth that for cells at 39 °C (Table 4). The differential effect became more pronounced when dialysed serum was used. With mouse leukaemic L1210 cells, however, no such differences in the toxicity of either drug between the two temperatures were observed (Table 4). This indicates that the observed differential toxicity of the two drugs was not simply due to their possible degradation at high temperatures, although 5-azacytidine has been reported to be chemically unstable (Notari & DeYoung, 1975).

We suspected that there was a common reason why the two drugs showed selective toxicity to cells at 33 °C in which the *src* gene was expressed. To test this possibility,

Table 4. Inhibition of cell growth by oxanosine and 5-azacytidine

Treatment was for 48 h. The doubling times for L1210 cells were 19 h at 33 °C and 13 h at 39 °C. The values represent the means ± S.E.M. of duplicate assays, with the numbers of separate experiments in parentheses. Statistical significance of the difference between IC₅₀ values of each pair (33 °C and 39 °C) was **P* < 0.05; ***P* < 0.01; NS, not significant.

Nucleoside antibiotic	Cells and conditions	Temperature...	IC ₅₀ (μg/ml)		Ratio 39 °C/33 °C
			33 °C	39 °C	
Oxanosine	<i>ts</i> /NRK cells				
	Normal serum		1.8 ± 0.3 (4)	22 ± 7.4 (4)**	12.2
	Dialysed serum		1.2	50	42
	L1210 cells				
5-Azacytidine	Normal serum		0.5 ± 0.1 (2)	0.8 ± 0.2 (2) ^{NS}	1.6
	<i>ts</i> /NRK cells				
	Normal serum		0.17 ± 0.05 (4)	2.5 ± 1.4 (3)*	14.7
	Dialysed serum		0.08	5.5	69
L1210 cells	Normal serum		0.3	0.3	1.0

Fig. 2. Antagonism between oxanosine and 5-azacytidine at (a) 33 °C and (b) 39 °C

Cells were plated at the cell density of $3 \times 10^4/2$ ml per 35 mm dish and incubated overnight at 33 °C or 39 °C. Then oxanosine and 5-azacytidine were added together and incubation was continued for 2 days at each temperature. The medium was replaced by fresh drug-free medium, incubation was resumed and the cell numbers were counted 2 days later. O, Control (no 5-azacytidine added); Δ , 5-azacytidine added at the concentrations ($\mu\text{g/ml}$) indicated; $-\square-$, theoretical values for drug combination at either temperature on the assumption that both drugs act in an additive fashion. The values shown represent the means for duplicate dishes, with a maximum range of 10%.

Table 5. Protection of *ts*/NRK cells by dipyrindamole against cell-growth inhibition by oxanosine and 5-azacytidine

Dipyridamole (10 μM for 48 h) inhibited cell growth by 8% and 29% of control at 33 °C and 39 °C respectively. For each determination, two separate duplicate runs were made at 33 °C, or one duplicate run at 39 °C. Each value represents the mean $\pm$ S.E.M. Statistical significance of difference between the values of each pair (with and without dipyridamole) was $**P < 0.01$ and $***P < 0.001$.

Drug	Concn. ($\mu\text{g/ml}$)	Temperature... Dipyridamole...	Growth (% of control)			
			33 °C		39 °C	
			—	+	—	+
Oxanosine	2		42 $\pm$ 2.5	87 $\pm$ 1.9***	—	—
	10		12.5 $\pm$ 5.0	73 $\pm$ 4.4***	65 $\pm$ 1.5	99 $\pm$ 1.5**
	50		—	—	24 $\pm$ 1.0	91 $\pm$ 6.5
5-Azacytidine	0.2		46.5 $\pm$ 2.9	76 $\pm$ 5.4**	—	—
	1		15.7 $\pm$ 7.8	52 $\pm$ 5.9**	44 $\pm$ 2.5	74 $\pm$ 0.5**
	5		—	—	19 $\pm$ 0.5	48 $\pm$ 1.0

we determined the toxicity of the two drugs in combination. As Fig. 2 shows, the effect of the two drugs was antagonistic rather than additive; the combined effect of the two drugs over a wide range of combination ratios was above the broken line (Fig. 2) drawn on the assumption that they would act in an additive manner (Berenbaum, 1977). We studied how the two drugs antagonize each other in inhibiting cell growth. It is known that 5-azacytidine is incorporated into DNA and RNA and inhibits protein synthesis (Cihak & Vesely, 1972; Plagemann *et al.*, 1978; Vesely & Cihak, 1978), whereas oxanosine inhibits guanine nucleotide synthesis, thereby inhibiting DNA and RNA syntheses (Uehara

et al., 1985). It seemed difficult to explain the antagonism between the two compounds on the basis of their modes of action. Since both are nucleosides in structure, we thought that they might compete for a common membrane-transport system, resulting in antagonism in their activities. This possibility was tested. Dipyridamole, a potent inhibitor of nucleoside transport (Paterson *et al.*, 1981), protected cells at both 33 °C and 39 °C from the toxicity of oxanosine and 5-azacytidine, as shown in Table 5. The results suggest that: (1) these two antibiotics enter into cells via the same nucleoside transporter, which is sensitive to dipyridamole, and (2) efficient transport through this system is important for the cytotoxicity of

Fig. 3. Inhibition of thymidine transport by NBMPR and dipyridamole

Cells grown at 33 °C (●, ▲) or 39 °C (○, △) were incubated with the indicated concentrations of NBMPR (●, ○) or dipyridamole (▲, △) for 20 min at 20 °C. [³H]Thymidine (1 μM, 1 μCi/ml) uptake was determined for 30 s at 20 °C as described in the Materials and methods section. Control values of the uptake of thymidine at 33 °C and 39 °C cells were 12 and 7 pmol/min per 10⁶ cells respectively. The values shown represent the means for duplicate wells, with a maximum range of 5%.

both drugs. The selective toxicity of oxanosine and 5-azacytidine to cells at 33 °C compared with that to cells at 39 °C could be due to an elevated activity of the transport system in cells at 33 °C which is common to both antibiotics. Because of our previous observation that the thymidine-transport system, among others tested, was more active in cells at 33 °C than at 39 °C, the transport system was considered a possible candidate. We examined a possible correlation between the alteration of thymidine transport in cells at 33 °C and the selective toxicity of the two drugs to these cells. To characterize the thymidine-transport system in more detail, we determined the effect of various nucleoside-transport inhibitors on thymidine transport. As shown in Fig. 3, the inhibition of thymidine transport by NBMPR was very weak and about 10 times less effective than that by

dipyridamole on a molar basis. In agreement with the weaker inhibition by NBMPR (Fig. 3, IC₅₀ 10 μM) than that reported with other cell systems (IC₅₀ 1–10 nM) (Cass & Paterson, 1977; Paterson *et al.*, 1981), we found no specific binding site(s) for this analogue in our cell systems (results not shown). Other investigators also failed to find any NBMPR-binding sites on cultured cells of Walker 256 rat carcinoma (Paterson *et al.*, 1983). The NBMPR-insensitive character of nucleoside transport in Walker 256 cells (Paterson *et al.*, 1983; Belt, 1983), in rat Novikoff hepatoma cells (Wohlhueter *et al.*, 1978, Paterson *et al.*, 1983) and in rat hepatoma 3924A cells (Zhen *et al.*, 1983; A. R. P. Paterson, personal communication) was also reported. Therefore the transport system in NRK cells, and probably in other cells of rat origin, should be intrinsically insensitive to NBMPR. The nucleoside-transport system(s) of mammalian cells must be heterogeneous, as has been reported by Belt (1983). NBMPR did not protect cells at either 33 °C or 39 °C from the toxicity of oxanosine and 5-azacytidine (Table 6), consistent with its weak inhibition of thymidine transport. These effects were in marked contrast with that of dipyridamole. In order to confirm that oxanosine and 5-azacytidine share a membrane transport system with thymidine, we examined whether these drugs inhibit the cellular uptake of thymidine. From a kinetic analysis of the inhibition by oxanosine and 5-azacytidine of thymidine transport, competition was observed between these drugs and thymidine in the higher-affinity system (Uehara *et al.*, 1984) of thymidine transport (Fig. 4). The apparent *K_i* values for oxanosine and 5-azacytidine with cells at 33 °C were about 0.1 and 0.4 mM respectively, whereas those with cells at 39 °C were 0.2 and 0.3 mM (Table 6). Moreover, the inhibition by these drugs of the transport of other nucleosides, such as adenosine, occurred in their low-affinity systems, and the *K_i* values were much higher than those for thymidine transport (Table 7), supporting the notion that they possess especially higher affinity to the thymidine-transport systems of these cells. All these results indicate that the selective toxicity of the two drugs to 33 °C cells is based on their faster uptake into 33 °C cells than into 39 °C cells via the activated thymidine-transport system in response to expression of the *src* gene.

In mammalian cells, most nucleoside drugs are reported to be taken up by carrier-mediated transport

Table 6. Lack of protection by NBMPR against *ts*/NRK cell-growth inhibition by oxanosine and 5-azacytidine

The concentration of NBMPR, when added, was 11 μM. Cell growth rates at 33 °C and 39 °C were 95% and 89% of the corresponding controls respectively. The difference between a pair of mean values (with or without NBMPR) was not statistically significant, except * *P* < 0.05.

Drug	Concn. (μg/ml)	Temperature... NBMPR...	Growth (% of control)			
			33 °C		39 °C	
			—	+	—	+
Oxanosine	1		54 ± 2.5	66 ± 2.0	—	—
	10		15 ± 1.0	22 ± 0*	—	—
5-Azacytidine	0.2		75 ± 2.0	70 ± 4.0	85 ± 2.5	93 ± 4.5
	1		23 ± 0.5	26 ± 1.0	42 ± 3.0	47 ± 2.0

Fig. 4. Competitive inhibition of thymidine transport in *ts*/NRK cells by oxanosine and 5-azacytidine

Replicate cultures grown at (a) 33 °C or (b) 39 °C in a 24-well Multitray were kept at 20 °C for 30 s with graded concentrations (40 μM or less) of [³H]thymidine in Dulbecco's phosphate-buffered saline containing glucose with no inhibitor (control, ○) or with oxanosine (100 μg/ml) (●) or 5-azacytidine (100 μg/ml) (▲). At the end of the incubation, the cells were washed rapidly with cold phosphate-buffered saline containing 5 μM-dipyridamole, and the radioactivity taken up into the cells was determined as described in the Materials and methods section. The values shown represent the means for duplicate wells, with a maximum range of 10%.

Table 7. Inhibition of thymidine and adenosine uptake by oxanosine and 5-azacytidine

Carrier-mediated uptake of thymidine or adenosine over 30 s was measured in the presence or absence of the competing compound (100 μg/ml). Determinations of K_i for the high- and low-affinity components were made by double-reciprocal plots of velocity versus concentration in the presence or absence of inhibitor. Where indicated, results are means $\pm$ S.E.M.

Nucleoside	Temperature (° C)	K_m (μM)	K_i (μM)	
			Oxanosine	5-Azacytidine
Thymidine	33	30 $\pm$ 6	110 $\pm$ 15	430
		170	*	*
	39	60 $\pm$ 10	200 $\pm$ 20	330
		250	*	*
Adenosine	33	13 $\pm$ 2	*	*
		50 $\pm$ 16	460	930

* No competition was observed.

system(s) and metabolized further to active forms (for reviews, see Paterson, 1981; Wohlhueter & Plagemann, 1980), but little is known about the multiplicity and specificity of the transport system(s). Although some experiments have demonstrated the presence of only a single type of transport with two efficiencies (Plagemann & Wohlhueter, 1980), there are some exceptions. All the following observations indicate the heterogeneity of nucleoside transport in mammalian cells: high- and low-affinity transport systems for thymidine in rat hepatocytes (Ungemach & Hegner, 1978) and for adenosine in mouse lymphocytes (Strauss *et al.*, 1976), L1210 cells (Chello *et al.*, 1983) and human erythrocytes (Kolassa *et al.*, 1978), a thymidine-transport-deficient mutant isolated from 2-bromodeoxyuridine-resistant mutants of HeLa cells (Lynch *et al.*, 1977), and the NBMPR-sensitive and -insensitive nucleoside-transport mechanisms in a variety of cultured neoplastic cells (Belt, 1983). If there are any general differences in nucleoside transport between the normal and the tumorous state, as

we observed with cells at 33 °C and 39 °C, it would be possible to prepare appropriate drugs that will distinguish the differences. Our present study might be an example. Another approach would be to improve the selectivity of chemotherapeutic action of nucleoside analogues by modifying the transport system, as has been reported by Rabinovitz *et al.* (1979).

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