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Synergistic effect of 3'-deoxyadenosine N^1-oxide and adenosine deaminase inhibitors on growth of Ehrlich ascites tumor cells in vivo*

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Summary. The simultaneous administration of 3'-deoxyadenosine N^1-oxide (3'-dANO) and the adenosine deaminase inhibitors erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) or 2'-deoxycoformycin (2'-dCF) to mice bearing Ehrlich ascites tumor cells resistant to 3'-dANO resulted in 80%–90% inhibition of tumor growth in vivo. 3'-dANO and 2'-dCF increased the survival time of tumor-bearing mice by a factor of 2. In vitro studies showed that the 3'-dANO resistant Ehrlich cells initiate the metabolism of 3'-dANO by a reduction to 3'-deoxyadenosine, which is converted primarily to 3'-deoxyinosine by adenosine deaminase and, to a small extent, phosphorylated to the cell toxic agent 3'-dATP. By the addition of EHNA or 2'-dCF it was possible to block the formation of 3'-deoxyinosine, resulting in a profound stimulation in the accumulation of 3'-dATP. The development of resistance to 3'-dANO was studied in cell cultures and found to be accompanied by changes in the enzyme activities of the reductase, the adenosine kinase, and the adenosine deaminase.

Introduction

In a previous work we have demonstrated that the growth of two lines of Ehrlich ascites tumor cells (ELT and ELD) is inhibited about 95% by 3'-deoxyadenosine N^1-oxide (3'-dANO) when tumor-bearing mice are treated for 4 days with 100 mg/kg per day [19]. Other lines of Ehrlich ascites cells were not inhibited by doses of 400 mg/kg per day. The inhibitory effect of 3'-dANO was found to be correlated to the ratio between the activities of adenosine kinase and adenosine deaminase [19]. In sensitive Ehrlich cells the activity ratio of kinase/deaminase was 1.25–2.70, and in resistant cells the ratio was 0.16–0.18.

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Abbreviations: 3'-dANO, 3'-deoxyadenosine N^1-oxide; 3'-dA, 3'-deoxyadenosine; 3'-dI, 3'-deoxyinosine; 3'-dATP, 3'-deoxyadenosine triphosphate; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; 2'-dCF, 2'-deoxycoformycin

3'-dANO is not a substrate for adenosine kinase [11] or adenosine deaminase [5] but is slowly reduced to 3'-deoxyadenosine [4], which is either deaminated to the inactive 3'-deoxyinosine (3'-dI) or phosphorylated to 3'-deoxyadenosine triphosphate (3'-dATP), which is the active inhibitor. This compound is incorporated into RNA and functions as a chain terminator [6, 19].

In resistant cell lines, the 3'-dANO is preferentially metabolized to 3'-dI due to a low kinase/deaminase ratio. This ratio may be increased in the cells by the addition of a specific adenosine deaminase inhibitor, such as EHNA [erythro-9-(2-hydroxy-3-nonyl) adenine] [15] or 2'-dCF (2'-deoxycoformycin) [20], which may lead to an increased accumulation of 3'-dATP in the cells, thereby making the insensitive cells line sensitive to 3'-dANO. The adenosine deaminase inhibitors EHNA and 2'-dCF have previously been used to enhance the biological activity and therapeutic effectiveness of arabinosyl adenine and 3'-deoxyadenosine [9, 14].

The present paper describes the inhibitory effect of 3'-dANO when administered together with either EHNA or 2'-dCF to a 3'-dANO-resistant line of Ehrlich ascites cells. The metabolism of 3'-dANO in the presence of the two deaminase inhibitors was studied in the cells in vitro, and the development of resistance was studied in cell cultures.

Materials and methods

3'-Deoxyadenosine N^1-oxide (3'-dANO) was prepared as previously described [19]. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was obtained from Burroughs Wellcome Co., and 2'-deoxycoformycin (2'-dCF) was obtained from the Natural Products Branch, Division of Cancer Treatment, NCI (Bethesda, Md, USA). Female mice of Theiller's original non-inbred strain were obtained from Tucks and Son Limited (Essex, England). Mice weighing 22–24 g were used in the experiments. Ehrlich ascites tumor cells were kindly supplied by Dr. E. B. Thorling, Institute of Cancer Research, Århus, Denmark (Århus line) and by Dr. G. Klein, Institute of Cancer Research, Karolinska Institute, Stockholm, Sweden (ELT line).

Tumor growth was determined by counting the cells in the peritoneal cavity of the mice [7]. Each mouse was transplanted with 2×10^7 cells by intraperitoneal injection. 3'-dANO, EHNA, and 2'-dCF were injected i.p. in sterile, filtered 0.154 *M* NaCl, and control mice received the same

volume of 0.154 *M* NaCl. After treatment, the mice were killed and the ascites fluid removed quantitatively by washing with 0.154 *M* NaCl containing heparin (3 IU/ml).

Ehrlich ascites cells were harvested 6 days after transplantation and incubated in Krebs-Ringer's bicarbonate solution with shaking. Aliquots were taken at the times indicated, and the amounts of 3'-dANO, 3'-dI, and 3'-dATP were determined by thin-layer chromatography as earlier described [19].

Ehrlich cells were made resistant to 3'-dANO in cell cultures. The sensitive ELT cell line [19] transplanted into Theiller mice was established in cell culture in NCTC 135 medium [3] with 10% fetal calf serum. The cells were found to grow faster in RPMI 1640 medium [13] with 10% fetal calf serum, which was therefore used in the present experiments. Twenty-four separate cultures were grown in medium containing 3'-dANO (0.8 μmol/ml). This treatment gave rise to giant cell formation and multinuclear cells, and heavy cell death was seen. In this period the doubling time increased from 22 h to 82 h (range 61–118 h), and every second or third passage was made in medium without 3'-dANO in order to allow the cells to recover. After about 2 months the cell morphology became normal. The cells then grew permanently in medium containing 3'-dANO (0.8 μmol/ml), and after 5 months the doubling time was 24 h (range 21–29 h). The 3'-dANO, 3'-dA, and 3'-dI in these cultures were measured by HPLC chromatography on a reverse-phase column using gradients of methanol and glycine buffers. Adenosine kinase and adenosine deaminase were measured as described previously [19].

Results

In a previous investigation we found that the growth in vivo of two lines of Ehrlich ascites tumor cells are highly sensitive to 3'-dANO (100 mg/kg per day), whereas other cell lines are not inhibited by doses up to 400 mg/kg per day [19]. Erhlich cell lines resistant to 3'-dANO had a much higher adenosine deaminase activity than sensitive cell lines. In the present paper we investigated the effect of 3'-dANO in combination with EHNA or 2'-dCF on the growth of the 3'-dANO-resistant "Århus" cell line.

Groups of 10 mice were treated daily for 4 days, beginning on the 3rd day after transplantation. On the 7th day the animals were killed and tumor growth was determined. I.p. injection of either 3'-dANO (400 mg/kg per day) or EHNA (25 mg/kg per day) had no effect on tumor growth (Table 1). Injection of 3'-dANO (100 mg/kg) plus EHNA (10 or 25 mg/kg) inhibited growth by 71%–72%, and injection of 3'-dANO (200 mg/kg) plus EHNA (10 or 25 mg/kg) inhibited growth by 68% and 80%, respectively (Table 1). Injection of 3'-dANO in doses of 400 mg/kg together with EHNA (10 or 25 mg/kg) resulted in the death of about 70% of the animals. No toxic symptoms were observed in mice injected with the doses shown in Table 1.

The results show that the 3'-dANO-resistant tumor became sensitive to 3'-dANO by treatment with 3'-dANO in combination with EHNA. In order to investigate whether this treatment in fact gave rise to an increased accumulation of 3'-dATP , cells of the resistant Arhus line were incubated with 2 m*M* 3'-dANO, in combination with different concentrations of EHNA comparable with those used in the in vivo experiment. The results are shown in Fig. 1.

Table 1. Inhibitory effect of 3'-dANO and EHNA on the growth of a 3'-dANO-resistant Ehrlich ascites tumor-cell line

Exp. no.	3'-dANO (mg/kg)	EHNA (mg/kg)	Inhibition (%)
1	400	0	0
2	0	25	0
3	100	10	72
4	100	25	71
5	200	10	68
6	200	25	80

Groups of 10 mice were treated daily for 4 days, starting the 3rd day after tumor transplantation with the doses indicated. The number of tumor cells was determined on the 7th day after transplantation. The cell counts were evaluated by the Student's *t*-test, and the inhibition was found to be significant at $P = 0.01$. The inhibition in experiment 6 is significantly higher than those obtained in experiments 3, 4, and 5 ($P = 0.01$)

Figure 1A shows the rate of reduction of 3'-dANO at 4 EHNA concentrations ranging from 1.2×10^{-4} to 2.5×10^{-2} mg/ml (0.43–90 μ*M*). During the first 2 h of incubation, the rate of reduction was not affected by any of the EHNA concentrations used. After this time, a small inhibition is observed at the highest concentrations of EHNA.

Figure 1B shows the rate of accumulation of 3'-dATP at the different EHNA concentrations. The rate of 3'-dATP accumulation increased with increasing EHNA concentrations, reaching a maximal rate at the two highest concentrations of EHNA. Under these conditions, the adenosine deaminase is completely blocked since no accumulation of 3'-dI takes place, as shown in Fig. 1C, and all the 3'-dANO which is reduced to 3'-dA is converted quantitatively to 3'-dATP. The accumulation of 3'-dATP is increased by a factor of about 10 under these conditions. At lower concentrations of EHNA (1.2×10^{-4} and 6×10^{-4} mg/ml), both 3'-dI and 3'-dATP accumulate, and the sum of these corresponds in each case to the amount of 3'-dANO reduced.

In a similar experiment using 2'-dCF in place of EHNA, the effect of 2'-dCF on the metabolism of 3'-dANO was found to follow the same pattern as seen with EHNA. Four concentrations of 2'-dCF were used, ranging from 0.4×10^{-3} μg/ml (1.5 n*M*) to 0.4 μg/ml (1.5 μ*M*). None of these concentrations of 2'-dCF had any effect on the reduction of 3'-dANO to 3'-dA (results not shown). Figure 2 shows that formation of 3'-dI decreased with increasing concentrations of 2'-dCF. At a 2'-dCF concentration of 0.4 μg/ml, no formation of 3'-dI is observed; this means that the adenosine deaminase is completely blocked at this concentration, which is comparable with that used in the in vivo studies.

The effect of 3'-dANO and 2'-dCF on tumor growth is shown in Table 2. The K_i value of 2'-dCF is about 10^3 lower than that of EHNA. The schedule of treatment with 2'-dCF was therefore varied in order to obtain maximal inhibition and avoid toxic side effects.

Daily i.p. injection for 3 days with 3'-dANO (100 mg/kg) and 2'-dCF (1.0 mg/kg) inhibited tumor growth by 75%. Treatment was initiated the 3rd day after transplantation, and tumor growth was determined one day after the last treatment. This treatment gave rise to neurotoxic symptoms not seen when mice were treated with EHNA

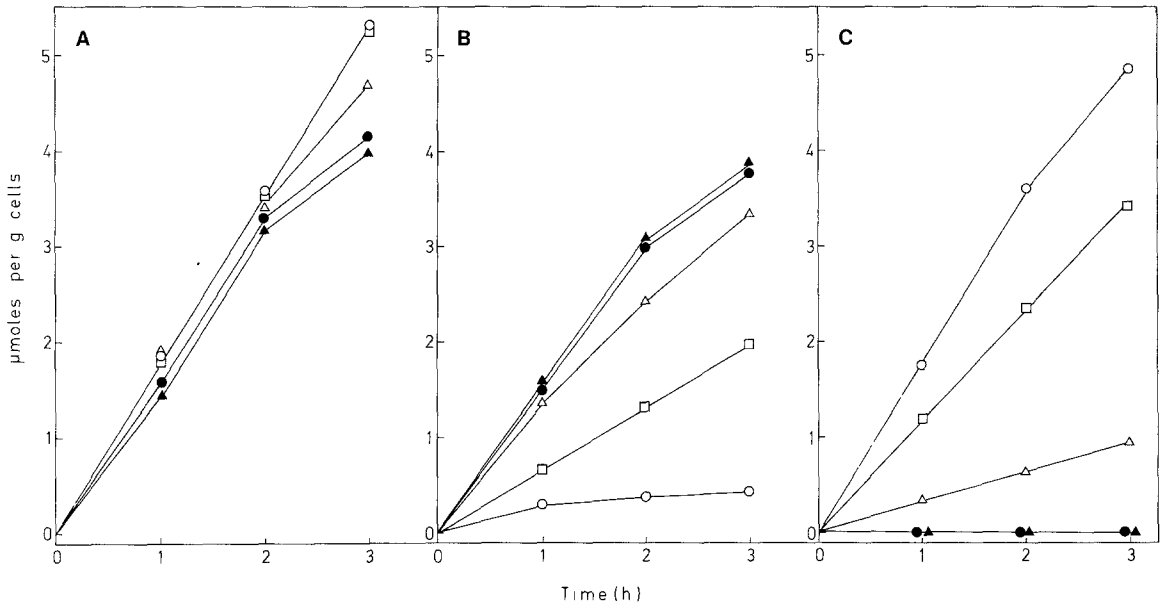

Fig. 1 A–C. The effect of EHNA on the metabolism of 3'-dANO in Ehrlich ascites tumor cells. Ehrlich cells (line Århus) suspended in Krebs-Ringer's bicarbonate medium, pH 7.4 (containing 10 m*M* glucose and 5 m*M* sodium succinate; cell density 12.0% wet weight) were incubated at 37° C with 2.3 m*M* 3'-dANO in the presence of the following concentrations of EHNA: none (○); 1.2×10^{-4} mg/ml (0.43 μ*M*) (□); 6×10^{-4} mg/ml (2.2 μ*M*) (△); 1.0×10^{-2} mg/ml (36 μ*M*) (●); and 2.5×10^{-2}mg/ml (90 μ*M*) (▲). Aliquots were taken at the times indicated and analyzed for 3'-dANO, 3'-dI, and 3'-dATP. **A** Reduction of 3'-dANO; **B** Formation of 3'-dATP; **C** Formation of 3'-dI

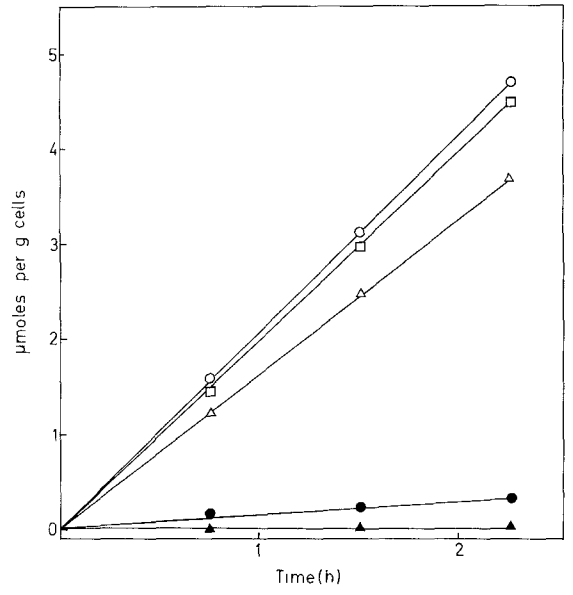

Fig. 2. The effect of 2'-dCF on the accumulation of 3'-dI in Ehrlich cells incubated with 3'-dANO. Ehrlich ascites cells (Århus line) were incubated with 2.3 m*M* 3'-dANO as described in Fig. 1 in the presence of the following concentrations of 2'-dCF: none (○); 4×10^{-7} mg/ml (1.5 n*M*) (□); 4×10^{-6} mg/ml (15 n*M*) (△); 4×10^{-5} mg/ml (0.15 μ*M*) (●); 4×10^{-4} mg/ml (1,5 μ*M*) (▲)

and 3'-dANO in higher concentrations (200 mg/kg), or with 3'-dANO (400 mg/kg) alone. The neurotoxic symptoms involved continuous up-and-down movements of the head and very frequent turns in circles when the mice were running around in the cage. When the dose of 3'-dANO injected together with 2'-dCF was lowered to 50 mg/kg, no toxic symptoms were seen. These doses of 3'-dANO and 2'-dCF also prolonged the survival time of tumor-bearing mice by a factor of 2 (Table 2).

The combined effect of 3'-dANO (100 mg/kg) and 2'-dCF (1.0 mg/kg) caused a 75% inhibition of tumor growth (Table 2), and it was of interest to know whether the remaining cells had become resistant to the treatment. Cells from these mice were therefore transplanted into fresh mice and treated again with 3'-dANO and 2'-dCF. To make certain that the transplanted tumor cells had started to grow, the treatment was first initiated on the 4th day after transplantation. The growth of these tumor cells was inhibited by 94%, showing that no resistance had developed at this stage of treatment. When treatment (50 mg/kg, 3'-dANO; 0.5 mg/kg, 2'-dCF) was initiated 1 day after transplantation, tumor growth was inhibited by 82% and the survival time increased by a factor of 2.

In the experiments with EHNA and 2'-dCF, a line of Ehrlich ascites cells (Århus) resistant to 3'-dANO was used. The reason for this resistance was found to be a high adenosine deaminase activity relative to the adenosine kinase activity [19]. The development of resistance to 3'-dANO was studied in vitro. An Ehrlich cell line (ELT) highly sensitive to 3'-dANO was grown in vitro in the presence of 3'-dANO. Twenty-four separate cultures were grown in the presence of 3'-dANO (0.8 μmol/ml), and the effect on cell growth, the change in metabolism of 3'-dANO, and the enzyme activities were studied over a 5-month period. During the first weeks, 3'-dANO caused severe cell damage and cell death. Giant cells and multinuclear cells appeared, and the doubling time increased from 22 h to 82 h. After about 2 months the cell morphology was normal, and after 5 months the doubling time was 24 h.

In a previous investigation [19], it was shown that three enzyme activities in the tumor cells are important for the inhibitory effect of 3'-dANO, these being the reductase, adenosine kinase, and adenosine deaminase. These enzyme activities were therefore determined in the ELT cells that were made resistant. The cell cultures were grown for

Table 2. Inhibitory effect of 3'-dANO and 2'-dCF on the growth of a 3'-dANO-resistant Ehrlich ascites cell line

Exp. no.	3'-dANO (mg/kg)	2'-dCF (mg/kg)	Inhibition (%)	Survival time (days)	Treatment initiated, days after transpl.	Treatment schedule
1	0	1.0	0	–	3	3 days
	100	0	0	–	3	3 –
	100	1.0	75	–	3	3 –
2[a]	100	0.5	94	–	4	3 days
3	20	0.5	51	–	3	3 days
4	20	0.5	60	–	3	9 days
5	100	0.5	92	–	2	2 days, pause 2 days
	100	0.5	–	10 days (60%)[b] 28 – (40%)	2	2 days
6	50	0.5	82	–	1	3 days, pause 2 days
	50	0.5	–	33	1	2 days
7	0	0	–	16	–	no treatment

Groups of 10 mice were used and tumor growth was determined 1 day after the last treatment

[a] Cells from mice treated with 3'-dANO (100 mg/kg) and 2'-dCF (1.0 mg/kg) (experiment 1, 75% inhibition) were transplanted to fresh mice

[b] 6 mice died immediately after end of treatment

Table 3. Correlation of 3'-dANO metabolism to the pattern of adenosine kinase and adenosine deminase activity in Ehrlich cell (ELT) cultures made resistant to 3'-dANO

Cultures no.	3'-dANO metabolized to 3'-dI (nmol/ml)	Kinase activity (nmol/h per mg cells)	Deaminase activity (nmol/h per mg cells)
8	11.2± 2.4	14.1±0.9	166±15
4	24.0± 1.6	8.0±1.3	143±21
3	26.4± 1.6	13.0±1.0	202±13
4	53.6± 4.0	8.0±0.8	197±20
5	95.2±20.8	17.6±1.8	254±28
Sensitive ELT cells [19]		30	11

Twenty-four separate cultures of the ELT line of Ehrlich ascites cells were made resistant to 3'-dANO after growth for 5 months in RPMI medium containing this drug. ELT cells (5×10^4 cells/ml) were inoculated in medium containing 3'-dANO (0.8 μmol/ml). After 3 days, the amounts of 3'-dANO and 3'-dI were measured by HPLC chromatography. Larger amounts of the cell cultures were grown in medium without 3'-dANO, and the enzyme activities were measured as previously described [19]

3 days in medium with 3'-dANO, and the amount of 3'-dANO, 3'-dA, and 3'-dI was determined by HPLC chromatography. No 3'-dA was found, and the amount of 3'-dI formed was taken as a measure of reductase activity. This assumption is permissible because the reductase activity is low compared with that of the deaminase, and because the amount of 3'-dATP formed is insignificant compared to the total amount of 3'-dANO and 3'-dI. As shown in Table 3, the different cell cultures had developed a very different capacity to reduce 3'-dANO to 3'-dA. For 3 days during growth, the cultures could reduce from about 1% to about 12% of the added 3'-dANO. In the resistant cell cultures, the adenosine kinase activity was 2- to 4-fold lower than in the sensitive cells, and the adenosine deaminase activity was 13- to 23-fold higher than in the sensitive cells (Table 3).

Discussion

It has previously been demonstrated that the in vivo growth of some Ehrlich ascites tumor-cell lines are strongly inhibited by 3'-dANO (67 mg/kg), whereas other cell lines are not inhibited even at doses of 400 mg/kg [19]. This variation in sensitivity to 3'-dANO was found to be related primarily to the ratio between the activities of adenosine kinase and adenosine deaminase in the different cell lines. The variation in the rate of reduction of 3'-dANO to 3'-dA in the different cell lines was much less pronounced and thus could not explain the difference in sensitivity to 3'-dANO. This conclusion is supported by the present study of the development of resistance to 3'-dANO in 24 separate Ehrlich cell cultures. In cultures which had acquired a very different capacity to reduce 3'-dANO, resistance was achieved by development of low kinase and high deaminase activity in the cells.

Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) and 2'-deoxycoformycin (2'-dCF) are two well-studied inhibitors of adenosine deaminase [8]. The K_i value of EHNA is on the order of $2-4 \times 10^{-9}$ *M*, and that of 2'-dCF is in the range of $2.5-15 \times 10^{-12}$ *M* [1, 2]. Reactivation of adenosine deaminase inhibited with 2'-dCF is exceedingly slow, i.e., $T_{1/2}$ = 8–29 h, and in intact cells, such as erythrocytes, reactivation of inhibited adenosine deaminase has not been demonstrated [2]. When given to patients with solid tumors, 2'-dCF can cause lymphopenia [17] as well as complete lysis of leukemic cells [12]. In erythrocytes, 2'-dCF causes an accumulation of 2'-dATP and a profound reduction in the ATP level, which may account in part for the toxicity of 2'-dCF [16]. 2'-dCF has been shown to increase the inhibitory effect of 3'-dA in 3 mammalian

tumor-cell lines in culture, and, when given together with 3'-dA to mice bearing P388 ascites cells, the survival time is prolonged significantly [9].

In mice, a single oral dose of EHNA (50 mg/kg) totally inhibits adenosine deaminase for 4 h and causes a large increase in the conversion of ^{3}H-adenosine to ATP. Lower doses of EHNA (3 mg/kg) have decreased the deamination of adenosine by 50% for 2–6 h [10]. EHNA can likewise inhibit the deamination of arabinosyl adenine in mice and increase the apparent plasma half-life by a factor of 2–3 [18]. In mice bearing Ehrlich ascites tumor cells, arabinosyl adenine (50 mg/kg) has had no significant effect on survival time, but this effect could be doubled by simultaneous addition of EHNA (3.1 mg/kg) [14]. The inhibitory effect of arabinosyl adenine and 3'-deoxyadenosine on the growth of L-cells in culture is greatly potentiated by the simultaneous addition of EHNA [14].

In the present study, EHNA in concentrations of 10–25 mg/kg injected i.p. together with moderate doses of 3'-dANO (100 and 200 mg/kg) caused a 70%–80% inhibition of tumor growth.

The combined effect of 2'-dCF (0.5 mg/kg) and 3'-dANO (50 and 100 mg/kg) caused a 75%–94% inhibition of tumor growth. 3'-dANO (100 mg/kg) injected together with 2'-dCF resulted in neurotoxic symptoms (total dose 300 mg/kg) and killed 70% of the mice in survival experiments. Lowering the amount of 3'-dANO to 50 mg/kg increased the survival time of tumor-bearing mice by a factor of 2 without causing toxic symptoms.

The neurotoxic symptoms seen when large amounts of 3'-dANO are injected i.p. together with either EHNA or 2'-dCF are most likely due to the accumulation of 3'-dATP. Neither EHNA, 2'-dCF, nor 3'-dANO alone is toxic in the concentrations used. The LD_{10} for 3'-dANO is 675 mg/kg when injected i.p. (unpublished data). By variation in the schedule of treatment, it may be possible to inhibit tumor growth completely. When 3'-dANO-treated tumor cells (75% inhibition, Table 2, experiment 1) were transplanted into fresh mice, which were again treated with 3'-dANO and 2'-dCF, the inhibitory effect on tumor growth was 94%, showing that resistant cells were not accumulated in this period.

The effect of EHNA and 2'-dCF supports previous results [4, 19] showing that 3'-dANO is reduced to 3'-dA, which is then phosphorylated to 3'-dATP. During the conversion of 3'-dANO to 3'-dATP in the cells, the concentration of 3'-dA is so small that it cannot be detected by the present analytical method. However, the effect of the deaminase inhibitors show that 3'-dA is the key intermediate in this process. Under conditions where adenosine deaminase is completely blocked by EHNA or 2'-dCF, the amount of 3'-dANO which is reduced is converted quantitatively to the cell-toxic 3'-dATP.

In a previous paper [19] it was suggested that the ratio between the activities of adenosine kinase and adenosine deaminase determines the sensitivity of a cell line to 3'-dANO. The present results, obtained with adenosine deaminase inhibitors, support this assumption. The results also demonstrate that the deaminase inhibitors can make a 3'-dANO-resistant tumor sensitive to 3'-dANO, which is important from a therapeutic point of view.

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