Cancer Chemother Pharmacol (1987) 19: 118–122

Cancer Chemotherapy and Pharmacology

Studies on the mechanism of cytotoxicity of 3'-deoxyadenosine N¹-oxide in different strains of Ehrlich ascites tumor cells*

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Summary. The correlation between the metabolic processing of 3'-deoxyadenosine N^1-oxide (3'-dANO) in vitro and its effect on tumor growth in vivo has been investigated in seven different strains of Ehrlich ascites tumor cells.

The metabolism of 3'-dANO is initiated by reduction to 3'-deoxyadenosine (3'-dA). This process is the rate-limiting process. The 3'-dA does not accumulate, but is converted to 3'-deoxyadenosine triphosphate (3'-dATP) or 3'-deoxyinosine (3'-dI). The ratio between 3'-dATP and 3'-dI inosine corresponds to the ratio between the activities of adenosine kinase and adenosine deaminase in the cell.

Two of the cell lines were markedly inhibited by 3'-dANO in vivo. In these cells the accumulation of 3'-dATP was 1.4–2.2 nmol/h per mg cells, which accounts for the major part of the metabolized 3'-dANO. Five of the cell lines were not inhibited by 3'-dANO and the formation of 3'-dATP was 5–10 times less in these than in the sensitive strains. The low level of 3'-dATP is caused primarily by a low ratio between the activities of adenosine kinase and adenosine deaminase, which is 15 time less than in the sensitive cell lines. The rate of reduction of 3'-dANO seems to be of minor importance.

These results indicate a correlation between the inhibition of tumor growth by 3'-dANO and the ability of the cell to accumulate 3'-dATP from 3'-dANO and show that this conversion is determined solely by the rate of reduction of 3'-dANO (3'-dANO reductase activity) and the ratio between the activities of adenosine kinase and adenosine deaminase in the cell. Consequently, the estimation of these enzyme activities in cell lysate of a given tumor can be used to predict whether the tumor is susceptible to inhibition by 3'-dANO.

* This work was supported by The Danish Medical Research Council, Gerda and Åge Haensch Fond, Direktor Åge Henriksens Fond, P. Carl Petersens Fond and the Danish Cancer Society

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Abbreviation: 3'-dANO, 3'-deoxyadenosine N^1-oxide; 3'-dA, 3'-deoxyadenosine; 3'-dI, 3'-deoxyinosine; 3'-dATP; 3'-deoxyadenosine triphosphate

Introduction

3'-deoxyadenosine N^1-oxide (3'-dANO) is a synthetic N^1-oxidized derivative of the naturally occurring 3'-deoxyadenosine (3'-dA) [2, 11]. 3'-dANO has previously been shown to inhibit growth of Ehrlich ascites cells in vivo [8], and the metabolism of 3'-dANO and 3'-dA is well characterized (for review see [7]). In Ehrlich cells 3'-dANO is reduced to 3'-dA, which is then phosphorylated to the triphosphate. 3'-deoxyadenosine triphosphate (3'-dATP) inhibit nucleic acid synthesis [5] and nucleotide metabolism [16, 20]. 3'-deoxyinosine (3'-dI), which is formed by deamination of 3'-dA, has no effect on nucleic acid synthesis [13]. 3'-dATP is incorporated into the growing RNA chain in lieu of ATP and functions as a chain terminator [15, 23]. This causes inhibition of the synthesis of ribosomal RNA [24], LMW RNA [10], poly A [3, 19], and mRNA [1].

3'-dANO is not a substrate for adenosine deaminase (adenosine aminohydrolase; E. C. 3.5.4.4) [6] or adenosine kinase (ATP: adenosine 5'phosphotransferase; E. C. 2.7.1.20) [17]. These properties imply that 3'-dANO is metabolically inert until it enters a target cell, which is able to reduce 3'-dANO to 3'-dA. In this case 3'-dA may be phosphorylated by adenosine kinase, leading to the antimetabolic agent 3'-dATP, or deaminated by adenosine deaminase to the inactive 3'-dI.

These data suggest that for 3'-dANO to be able to inhibit the growth of a cell, two conditions need to be fulfilled: (1) the cell must be able to reduce 3'-dANO to 3'-dA, and (2) it must be able to accumulate 3'-dATP from 3'-dA, which will depend on a favorable ratio between the activities of adenosine kinase and adenosine deaminase in the cell.

To test this hypothesis, the correlation between the following parameters has been investigated: (1) The effect of 3'-dANO on cell growth in vivo; (2) the metabolic processing of 3'-dANO in cells in vitro; and (3) the ratio between the activities of adenosine kinase and adenosine deaminase in the cell lysates. The experiments were carried out on seven different cell lines of Ehrlich ascites tumors, which exhibited different levels of sensitivity to 3'-dANO.

Materials and methods

Preparation of 3'-deoxyadenosine N^1-oxide. 3'-dANO was prepared according to published procedures [5, 14] but modified for large-scale preparation as follows: 253 g 3'-deoxyadenosine (0.94 mol) was suspended in 27 l dis-

tilled water at 20 °C, and 200 ml concentrated ammonium hydroxide and 6.8 mol monoperphthalic acid [18] in 10.5 l ether solution were added during stirring. The pH value of the reaction mixture was kept at pH 7.0 by successive additions of concentrated ammonium hydroxide (total 1 l). Samples were taken from the reaction mixture after 3 h and 3.75 h of stirring. Titration of the monoperphthalic acid with 0.1 *N* sodium thiosulfate showed that the amounts of acid left were 13% and 8%, respectively. The reaction was stopped after 4 h. The product was purified by chromatography of 4 l batches of the reaction mixture on a column (15 × 150 cm) of Dowex-1-×8 formate, 100–200 mesh. The columns were eluted with 0.01 *M* ammonium formate, pH 9.0. The pooled fractions of 3'-dANO (total volume 190 l) were adjusted to pH 7.0 with ammonium hydroxide and evaporated at 50 °C in vacuo to about 10 l. The pH was monitored repeatedly during evaporation and adjusted to 7.0 with ammoniom hydroxide. 3'-dANO was allowed to crystallize from the solution at 4 °C and then removed by filtration and dried in a desiccator over potassium hydroxide. Coprecipitated ammonium formate was removed by sublimation at 40 °C under oil pump vacum and the remaining 3'-dANO was recrystallized from 70% dioxan. The overall yield was 45%.

The 3'-dANO was found to be more than 99.5% pure by the following criteria: (1) A characteristic N^1-oxide spectrum in which the ratio of molar extinction coefficients at 233 nm and 260 nm was 5 [22]; (2) a comparison of the infrared spectrum with those of a previously prepared batch of 3'-dANO [5], 3'-deoxyadensine [9], 2'-deoxyadenosine, adenosine, adenine, adenosine N^1-oxide, adenine N^1-oxide and 2'-deoxyadenosine N^1-oxide [14]; (3) chromatography on thin-layer plates with cellulose powder in the following solutions: water (R_f 0.66), *n*-butanol saturated with water (R_f 0.20), 2% boric acid (R_f 0.73), 5% boric acid (R_f 0.81), 0.1 *M* sodium borate (R_f 0.77) and 0.005 *M* HCl (R_f 0.77); (4) reverse phase chromatography on a HPLC column (Sperisorb 5S OD5) in gradients of methanol in glycine buffer.

Cell lines. Seven different Ehrlich ascites tumors characterized by their chromosome number were used in these investigations. Three cell lines (ELT, ELD and Karolinska) were kindly supplied by Prof. G. Klein, Karolinska Institutet, Stockholm; the Åarhus cell line, by Dr. E. B. Thorling, Institute of Cancer Research, Århus; the NCI cell line, by Dr. T. Skovsgaard, Finsen Institute, Copenhagen, and the Fibiger cell line, by Dr. J. Kieler, the Fibiger Laboratory, Copenhagen. The New Klein line was also originally supplied by Prof. G. Klein, Karolinska Institutet, Stockholm, in 1972, and it has since been maintained in our laboratory.

Mice bearing Ehrlich tumor ascites cell were treated with colchicine on the 4th or 5th after transplantation. Each mouse received 15 μg colchicine in 0.154 *M* NaCl by i.p. injection. The mice were killed 16 h later, and the cells harvested. The cells were subjected to a standard fixation procedure using KCl swelling and methanol-acetic acid, spread on slides, and stained with Giemsa, after which 50 or more metaphase cells with well-spread chromosomes were photographed and the chromosome number determined.

Determination of tumor growth. Female mice (Theiller's original non-inbred strain, Tucks and Son Ltd, Essex, England) weighing 22–24 g received transplants of 2×10^7 cells by i.p. injection. The number of mice in each group ranged from 8 to 16.

3'-dANO was dissolved in 0.154 *M* NaCl solution and sterilized by filtration. The solution was injected i.p. into tumor-bearing mice in a volume varying from 0.4 ml to 0.6 ml. Control mice received the same volume of sterile 0.154 *M* NaCl.

The mice were killed and the ascites cells removed quantitatively by washing with 0.154 *M* NaCl containing heparin (3 IU/ml). The cells were counted in methylviolet-acetic acid in a counting chamber [8].

Metabolism of 3'-deoxyadenosine N^1-oxide. Six days after transplantation the mice were killed and the cells were harvested in heparinized tubes and isolated by centrifugation at 800 *g* for 5 min. The cells were washed with 3 vol. Krebs-Ringer bicarbonate solution [4], pH 7.4, equilibrated with 5% CO_2 in atmospheric air, and finally suspended in the medium to give cell volume of 15% (vol./vol.).

The incubation of cells was carried out in 200-ml Erlenmeyer flasks equipped with a gas flow device and a side-arm near the bottom to permit sampling without interrupting the gas flow through the flask. The flasks were placed in a metabolic shaker at 50 oscillations per min and 37 °C. The cell suspensions were supplemented with glucose and sodium succinate at concentrations of 10 m*M* and 5 m*M*, respectively. The suspensions were then equilibrated with the gas phase for 10 min before the experiment was started by the addition of 3'-dANO. At different time intervals samples of the cell suspension were deproteinized with ice-cold perchloric acid. The acid-soluble fraction was neutralized with KOH, the $KClO_4$ precipitate (0 °C) was discarded and the supernatant was applied to PEI-cellulose thin-layer plates and chromatographed for 3 h in 0.15% LiCl-1% boric acid solvent.

The spot containing both 3'-dANO and 3'-dI (R_f value 0.90) and the spot corresponding to 3'-dA were eluted with 2.0 ml 0.02 *M* Tris-HCl, pH 7.4. The 3'-dI in the eluate was hydrolyzed and hypoxanthine determined enzymatically using xanthine oxidase (E. C. 1.2.3.2) according to the method of Kalckar [12]. The concentration of hypoxanthine was calculated assuming a differential molar extinction coefficient of 12300 M^{-1} cm^{-1} at 292.5 nm for the conversion of hypoxanthine to uric acid at pH 8.0.

3'-dANO in the eluate was determined from the absorbance at 233 nm, corrected for the absorbance exerted by 3'-dI in the eluate, using the following molar extinction coefficients at pH 7.4 and 233 nm: 3'-dANO, 41000 M^{-1} cm^{-1} and 3'-dI, 6100 M^{-1} cm^{-1}. The 3'-dATP was determined in the neutralized acid-soluble fraction from cells after chromatography on thin-layer cellulose plates in *n*-butanol-H_2O (86:18) for 4 h. (This treatment removes 3'-dANO and other nucleosides and bases from the application spot.) The plates were then further chromatographed for about 5 h in 0.32 *M* ammonium acetate, 0.22 *M* lithium borate pH 8.2 in 50% ethanol. In this solvent the ribonucleotides form complexes with borate and remain at the origin, whereas 3'-deoxyadenosine mono-, di-, and triphosphate migrate [21]. The UV-absorbing spots of 3'-deoxyadenosine phosphates were eluted with water and the concentration was calculates from the absorbance at 260 nm, assuming a molar extinction coefficient for the 3'-deoxyadenosine phosphates of 15000 M^{-1} cm^{-1}.

Table 1. Inhibitory effect of 3'-dANO on growth of different Ehrlich ascites tumor cell lines in vivo

Tumor cell line	Dose of 3'-dANO (mg kg^{-1} day^{-1})	Percentage inhibition
ELD	67	88
	100	95
	175	97
ELT	67	93
	100	93
	175	98
New Klein	150	0
Fibiger	200	0
NCI	150	0
Aarhus	400	0
Karolinska	100	0

Enzyme assays. Cells were washed with 0.15 *M* NaCl containing 1 m*M* EDTA pH 7.4 and suspended in 4 times their weight of 20 m*M* Tris-HCl buffer (pH 7.5) containing 1 m*M* EDTA and 10 m*M* dithiothreitol, and lysed by three freeze-thaw cycles in a −80 °C bath. The lysate was used for enzyme assays after dilution 5–50 times with 20 m*M* Tris-HCl buffer, pH 7.5, containing 0.5 mg bovine serum albumin per ml.

The adenosine deaminase assay (35 µl) contained 50 m*M* Tris-HCl buffer (pH 7.5), 1 m*M* EDTA, 3 m*M* dithiothreitol, 1.2 m*M* [U-^{14}C]adenosine (1.75 mCi/mmol) and cell lysate equivalent to 0.2–1 mg cells wet weight. The reaction mixture was incubated at 37 °C, and five aliquots were taken during the 30 min of incubation and chromatographed on PEI-cellulose thin-layer plates with a mixture of adenosine, inosine, and hypoxanthine as carriers. The thin-layer plate was developed for 3 h in 0.15% LiCl – 1% boric acid solvent. UV-absorbing spots were cut out and the radioactivity determined.

The adenosine kinase assay (35 µl) contained 90 m*M* Tris-HCl buffer (pH 7.5), 1.0 m*M* $MgCl_2$, 2 m*M* dithiothreitol, 5 m*M* ATP, 0.3 µ*M* Coformycin, 30 µ*M* [U-^{14}C] adenosine (44 mCi/mmol) and cell lysate equivalent to 0.02–0.1 mg cells. The reaction mixture was incubated at 37 °C, and four aliquots were taken during the 30 min of incubation and chromatographed on PEI-cellulose thin-layer plates with adenosine as carrier. The plate was developed for 3 h in 0.15% LiCl – 1% boric acid solvent. The adenosine spot and the adenine nucleotide spots were cut out and the radioactivity determined.

The kinase and deaminase reaction were linear during the time of incubation.

Results

Tumor characterization

Each of the tumor cell lines was characterized by its chromosome number. The cell lines are listed below with their chromosome numbers (means and SEM) and the percentages of subclones with different chromosome numbers. New Klein: 44.1 ± 0.22, 2% with 89.0 ± 3.65; Fibiger: 33% with 53.3 ± 1.62 and 66% with 75.4 ± 0.63; NCI: 45% with 42.9 ± 0.73, 45% with 80.2 ± 1.23, and 10% with 124 ± 7. Åarhus: 89.0 ± 0.56, fewer than 5% hyperdiploid and fewer than 5% triploid; Karolinska: 80.9 ± 0.39, fewer than 5% hyperdiploid; ELD: 47.7 ± 0.21, fewer than 1% hypotetraploid; ELT: 49.0 ± 0.26, fewer than 10% tri- to tetraploid. The mean chromosome numbers in ELD cells and ELT cells were 47.7 and 49.0, respectively. Statistical evaluation of 60 metaphase spreads (Student's *t*-test) showed that the chromosome numbers were different at the significance level $P = 0.01$.

Effect of 3'-dANO on tumor growth in vivo

The effect of 3'-dANO on tumor growth in vivo in the seven Ehrlich cell lines is shown in Table 1. The mice received 3'-dANO i.p. daily for 4 days, starting on the 3 day after transplantaton. The tumor cell content of the peritoneal cavity was determined on the 7th day after transplantation. Two cell lines (ELD and ELT) showed 88%–98% inhibition when treated with 67, 100, and 175 mg/kg. The cell counts were evaluated by Student's *t*-test, and inhibition was significant at $P = 0.01$. 3'-dANO in doses varying from 100 to 400 mg/kg had no effect on tumor growth in the following cell lines: Karolinska, New Klein, NCI, Fibiger, and Åarhus.

No toxic symptoms were observed in mice receiving up to 400 mg 3'-dANO per kg body weight for 4 days.

Table 2. Metabolism of 3'-dANO in different Ehrlich ascites tumor cell lines in vitro and activities of adenosine kinase and adenosine deaminase in homogenates of the cell lines[a]

Tumor cell line	Components formed or metabolized (nmol/h per mg cells)					Enzyme activity (nmol/h per mg cells)		
	3'-dATP formed	3'-dI formed	sum of 3'-dATP and 3'-dI	3'-dANO reduced	ratio 3'-dATP/3'-dI	adenosine kinase	adenosine deaminase	ratio kinase/deaminase
ELT	2.20	0.40	2.60	2.60	5.5	30	11	2.70
ELD	1.46	0.82	2.28	2.35	1.8	20	16	1.25
New Klein	0.28	1.20	1.48	1.50	0.23	11	60	0.18
Fibiger	0.28	0.85	1.13	1.15	0.30	13	78	0.17
NCI	0.27	1.31	1.58	1.67	0.21	13	78	0.17
Aarhus	0.25	1.80	2.05	2.00	0.14	13	83	0.16
Karolinska	0.25	1.20	1.45	1.50	0.21	12	68	0.18

[a] Ascites tumor cell suspensions were incubated at 37 °C with 2 m*M* 3'-dANO. Samples were taken over 3 h and analyzed for 3'-dANO, 3'-dATP, and 3'-dI. Linear increases in the concentrations of 3'-dATP and 3'-dI were observed during the first 2 h. Data shown in the table are values obtained after 1 h of incubation. Enzyme activities were determined in diluted cell homogenates at 37 °C. Values given are averages from three experiments

Metabolism of 3'-dANO in vitro

The ability of the different cell lines to reduce 3'-dANO to 3'-dA and to form 3'-dATP and 3'-dI was determined in cells incubated in the presence of 2 m*M* 3'-dANO, corresponding to 534 mg/l. These results are shown in Table 2.

The reduction of 3'-dANO to 3'-dA was observed in all cell lines, varying from 1.15 to 2.60 nmol/h per mg cells wet weight. The pools of 3'-dA and 3'-deoxyadenosine monophosphate during the incubation were so small that the actual concentrationsof these compounds could not be detected by the analytical methods used, and only an accumulation of 3'-dI and 3'-dATP was observed. In accordance with this observation the amount of 3'-dANO reduced in each cell line was identical, within experimental error, to the sum of 3'-dI and 3'-dATP formed, leaving no possibility for alternative major metabolic processing.

These data show that the reaction which limits the metabolic processing of 3'-dANO is the redution of 3'-dANO. The 3'-dA formed by this reaction is quickly consumed by the competitive action of adenosine kinase and adenosine deaminase, giving rise to the formation of 3'-dATP or 3'-dI. The activity of adenosine kinase and adenosine deaminase determined in cell lysates of the different cell lines is shown on the right of Table 2. It can be seen that the ratio between the amounts of 3'-dATP and 3'-dI formed in each cell line corresponds to the ratio between the activities of adenosine kinase and adenosine deaminase. From these results it can be concluded that the accumulation of 3'-dATP formed from 3'-dANO is determined by the rate of reduction of 3'-dANO to 3'-dA and the ratio between the adenosine kinase and adenosine deaminase in the cell.

The largest accumulation of 3'-dATP was seen in the ELT cell line, which also possessed the highest rate of 3'-dANO reduction and the highest kinase-to-deaminase ratio. It can be seen that 2.20 nmol 3'-dATP was formed per hour and milligram of cells, which corresponds to 85% of the amount of 3'-dANO reduced. The ELT cell line was strongly inhibited by 3'-dANO in vivo (Table 1). In the ELD cell line the accumulation of 3'-dATP was 35% less than in the ELT cell line. The decrease in the formation of 3'-dATP corresponds to the lower kinase-to-deaminase ratio in this cell line. The ELD cell line was also strongly inhibited by 3'-dANO in vivo.

In five cell lines the accumulation of 3'-dATP was 0.25–0.28 mmol/h per mg cells, corresponding to only 10%–12% of the 3'-dATP formed in the ELT cell line. The low values of 3'-dATP are caused primarily by a very low kinase-to-deaminase ratio in all the cell lines. To a lesser extent the rate of 3'-dANO reduction may also limit the accumulation of 3'-dATP; the rate of 3'-dANO reduction in several of these cell lines was significantly lower than in the ELT cell line. These five cell lines were not inhibited by 3'-dANO in vivo.

Discussion

The effect of 3'-dANO on the growth of seven different Ehrlich ascites tumor cell lines in vivo has been correlated with the metabolic processing of 3'-dANO in vitro.

The metabolism of 3'-dANO is initiated by reduction to 3'-dA. This process is the rate-limiting process. The 3'-dA does not accumulate, but is converted to 3'-dATP or 3'-dI by the competitive action of adenosine kinase and adenosine deaminase, in such a way that the ratio between 3'-dATP and 3'-dI corresponds to the ratio between the activities of adenosine kinase and adenosine deaminase (Table 2). Even though 3'-dA is present in extremely low concentrations, this compound is a key component in the processing of 3'-dANO. This fact can also be shown experimentally by addition of both 3'-dANO and the adenosine deaminase inhibitor EHNA to resistent cells, when 3'-dATP only accumulates and the cells become sensitive to 3'-dANO (results to be published).

From these patterns it can be seen that two factors determine the accumulation of 3'-dATP, namely the rate of reduction of 3'-dANO and the ratio between the activities of adenosine kinase and adenosine deaminase.

Two of the cell lines investigated were markedly inhibited by 3'-dANO in vivo (Table 1). In these cells the accumulation of 3'-dATP was 1.46–2.20 nmol/h per mg cells, which accounted for most of the metabolized 3'-dANO. Five of the cell lines were not inhibited by 3'-dANO, and the formation of 3'-dATP was 5–10 times less in these than in the sensitive strains. The low level of 3'-dATP is primarily caused by a low ratio between the activities of adenosine kinase and adenosine deaminase, which is 15 times less than in the sensitive cell lines. The low level of 3'-dATP is less influenced by the decreased rate in the reduction of 3'-dANO.

It has been shown [8] that several-fold doses of 3'-dA are needed compared with 3'-dANO to produce the same degree of growth inhibition in an Ehrlich ascites tumor cell line. This is because 3'-dANO is not deaminated until it reaches a target cell where it is reduced at a slow rate. This makes 3'-dANO a more selective and effective cytostatic agent than 3'-dA.

From the present results it is concluded that following treatment with 3'-dANO a given tumor will accumulate 3'-dATP in dependence on the activity of 3'-dANO reductase and the ratio between the activities of adenosine kinase and adeosine deaminase. It should therefore be possible to predict whether a tumor will be sensitive to 3'-dANO from an estimation of the 3'-dANO reductase, adenosine kinase, and adenosine deaminase in a homogenate of the tumor.

Such screening experiments are in progress in different human tumors transplanted into nude mice.

Acknowledgements. 3'-Deoxyadenosine was a generous gift from Novo Pharmaceutical Products, Copenhagen, Denmark. We also thank Novo Pharmaceutical Products for placing facilities for large scale chromatography at our disposal.

We thank Kirsten Samuelsen, Charlotte Goos Iversen, Jette Simonsen, Rasmus Bredahl and Rita Jensen for technical assistance.

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Received June 10, 1986/Accepted October 23, 1986