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Studies on the mechanism of cytotoxicity of 3-deazaguanosine in human cancer cells

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Summary. *The mechanism of toxicity of 3-deazaguanosine was studied in a number of human tumor cell lines by determination of the effects of various purine compounds on the growth of the cells in the presence of the drug and by studies of the effects of 3-deazaguanosine on the metabolism of radiolabeled precursors in these cells. The drug was found to be toxic to all of the cell lines tested. The toxicity was reversible with removal of the drug. None of the purine bases tested could restore normal growth after 48 h exposure to 3-deazaguanosine; the bases were more effective in preventing cytotoxicity when added simultaneously with the drug. Metabolic studies indicated decreased synthesis of DNA, variable inhibition of de novo purine synthesis, and complete inhibition of the enzyme guanosine monophosphate reductase by 3-deazaguanosine.*

Introduction

The purine analog 3-deazaguanine [10] has been shown to possess an interesting variety of antiviral [1, 4, 21], antibacterial [17, 26], and antitumor activities [9–11, 13]. Its effectiveness against the murine R3230AC mammary adenocarcinoma, L1210 leukemia, and colon adenocarcinoma 38 indicated its potential use as a chemotherapeutic agent [9]. More recently the 9-*B*-D-ribofuranosyl derivative, 3-deazaguanosine, has been synthesized and shown to have even greater antitumor activity than the parent compound [13, 16, 18]. However, the precise mode of action of these drugs is not thoroughly understood.

The metabolism of 3-deazaguanine and 3-deazaguanosine has been studied by several groups. It is likely that both compounds act via a common intermediate. Streeter and Koyama [25], using the method of Snyder [23] to measure the activity of the enzymes of purine metabolism in intact Ehrlich ascites cells, found that 3-deazaguanine, its nucleoside, and 5′-nucleotide showed a similar pattern of inhibition: hypoxanthine-guanine phosphoribosyl transferase (HPRT) and inosine monophosphate (IMP) dehydrogenase activities were reduced by all three compounds. Saunders et al. [18], using variant lines of Chinese hamster ovary cells, showed that cells lacking HPRT were resistant to 3-deazaguanine but not to 3-deazaguanosine. This implies that formation of the nucleotide is necessary for toxicity, and that deazaguanosine may be activated without prior degradation to the base. Khwaja et al. [11] showed that 3-deazaguanine is converted to its corresponding nucleotides and incorporated into nucleic acids.

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The basis of the toxic effect of 3-deazaguanosine and 3-deazaguanine appears most likely to be the suppression of DNA synthesis [16, 19]. For example, Schwartz et al. [19] reported that a concentration of 10^{-4} *M* 3-deazaguanine caused 70% suppression of DNA syntehsis within 12 h in L1210 cells. Whether this is a direct consequence of the incorporation of the drug into DNA or whether toxicity is mediated by inhibition of the enzymes involved in the synthesis or regulation of nucleotides is not clear. Based on incorporation studies with radiolabeled uridine, thymidine, and leucine, Saunders et al. reported that RNA and protein synthesis were not affected in Chinese hamster ovary cells by 10 μ*M* 3-deazaguanine [16], whereas Rivest et al. have reported that both DNA and protein synthesis were inhibited in L1210 cells by the drug at a concentration of 50 μ*M* [15].

This study was designed to assess the effect of 3-deazaguanosine on human tumor cell lines. The mechanism of the toxic effect was investigated by determinating the effects of various purine compounds on the growth of cells in the presence of 3-deazaguanosine and by studying the metabolism of radiolabeled purine precursors by these cells in the presence of the drug. Growth inhibiton was seen in all of the cell lines tested. Metabolic studies showed inhibition of guanosine monophosphate reductase and de novo purine synthesis, but gave no evidence of IMP dehydrogenase or HPRT inhibition.

Materials and methods

The drugs 3-deazaguanosine and 3-deazaguanosine-5′-monophosphate were prepared as previously reported [3]. The ^{14}C-labeled compounds hypoxanthine (53 mCi/mmol), formate (52.6 mCi/mmol), guanine (54.6 mCi/mmol), thymidine (63 mCi/mmol), and adenine (49.1 mCi/mmol) were obtained from New England Nuclear, Boston, Mass, [^{14}C]guanosine monophosphate (GMP; 562 mCi/mmol) was obtained from Amersham, Arlington Heights, Ill. Fetal bovine serum was obtained from Reheis, Kankakee Ill.

The human lung adenocarcinoma line SkLu-1 was provided by Dr Jorgen Fogh, Sloane-Kettering, Rye, NY. The human cell lines Sk-Co (colon adenocarcinoma), MCF7L (breast adenocarcinoma), J82 (bladder carcinoma), HT-1080 (fibrosarcoma), T24 (bladder carcinoma), A172 (glioblastoma), and A549 (lung carcinoma) were provided by Dr Robert Hoffman, University of California, San Diego.

Cultures of cells were grown in monolayers and maintained at 37° C in a 5% CO_2/95% air atmosphere in a modified

Eagle's MEM [8] containing 10% fetal bovine serum (FBS). For growth studies, 3-deazaguanosine and stock solutions containing the various purine compounds were prepared in culture medium and stored frozen at −20° C. Cell were plated and exposed to the drug (200 μ*M*) subsequent to attachment. For the rescue experiments, the test compound was added to a concentration of 100 μ*M* either simultaneously with the drug or after 2 days' exposure to the drug. Zero time points were densities measured at the time of addition of the drug. All time points were the average of counts from triplicate plates.

For the determination of DNA synthesis, 3-deazaguanosine (700 μ*M*) was added 1 day after the cells were plated. Cells were grown as above except that the FBS supplement was 15%.

When the cells reached 80% −90% confluency, radiolabeled thymidine was added to the medium at a concentration of 2 μCi/ml. After 4 h the medium was removed, the plate was washed twice with phosphate-buffered saline (PBS), and the cells were harvested by trypsinization. The cell pellet was extracted with 0.8 *M* perchloric acid, and the acid-insoluble material was washed twice with PBS and counted in a liquid scintillation counter.

Purine metabolism was analyzed in logarithmic cultures after 2 days of exposure to 3-deazaguanosine (500 μ*M*). Cells were detached by trypsinization, washed twice with PBS, and suspended at 10^6 cells/ml in PBS supplemented with 4 mg/ml human serum albumin and 0.1 mg/ml glucose. When cells had been cultured in 3-deazaguanosine, the drug was added to the suspension at a concentration of 500 μ*M*. The cells were then pulse-labeled with adenine, guanine, hypoxanthine, or formate, as described earlier [2]. Results were normalized by purine content as described elsewhere [14].

Guanosine monophosphate reductase (E.C. 1.6.6.8) was assayed in human erythrocyte lysates, which provided a more abundant source of the enzyme. The cells were suspended at a concentration of 5 : 1 in a buffer containing 33 m*M* HEPES, 1 m*M* EDTA, and 12 m*M* cysteine at pH 6.95 and lysed by osmotic shock. Debris were removed by centrifugation and the lysates were dialyzed overnight in this buffer. For the assay of GMP reductase activity, 100 μl of this lysate was incubated with 10 μ*M* NADPH and 1 μ*M* [^{14}C]GMP. After 20 min incubation at 37° C the protein was precipitated with perchloric acid and the substrate and product separated by thin-layer chromatography [8]. The spots were cut from the sheet and counted in a liquid scintillation counter. This assay was shown to be linear with time and protein under these conditions.

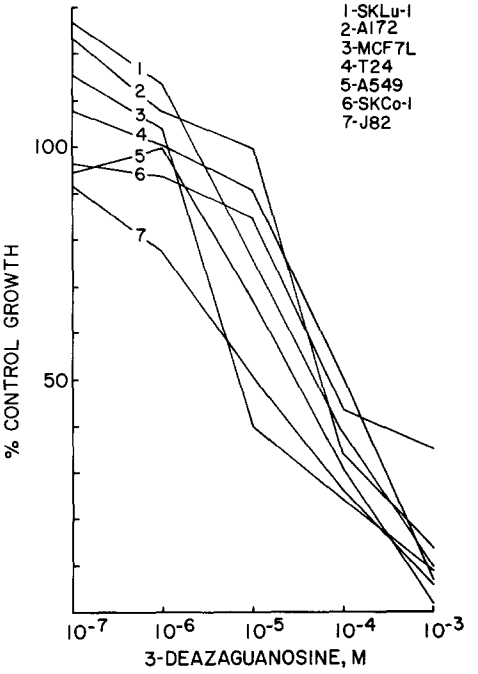

Fig. 1. Growth of human cancer cell lines as a function of 3-deazaguanosine concentration

Table 1. Effects of purine bases and nucleosides on the antiproliferative effect of 3-deazaguanosine

Tumor cell line	Base	SkLu-1	SkCo-1	J-82	A-549
3-Deazaguanosine					
+	–	4.2 (20%)	0.9 (6%)	3.2 (22%)	2.3 (23%)
–	–	21.4	14.2	14.4	10.1
+	Ade	11.1 (49%)	2.2 (21%)	3.8 (28%)	7.6 (69%)
–	Ade	22.7	10.2	14.0	11.0
+	Gua	9.6 (57%)	2.6 (19%)	3.0 (50%)	5.8 (100%)
–	Gua	16.8	13.7	6.0	5.8
+	Hyp	10.4 (44%)	3.2 (22%)	5.6 (33%)	7.9 (69%)
–	Hyp	23.9	15.0	4.2	11.4
+	–	4.5 (27%)	0.4 (4%)	2.6 (22%)	1.1 (10%)
–	–	16.8	8.7	12.2	10.7
+	Ade	7.1 (30%)	0.6 (9%)	2.8 (27%)	5.4 (46%)
–	Ade	23.8	6.4	10.6	11.7
+	Gua	5.5 (46%)	0.4 (5%)	1.6 (26%)	5.5 (76%)
–	Gua	11.8	8.4	5.4	7.2
+	Hyp	6.1 (22%)	0.6 (7%)	3.2 (26%)	6.4 (58%)
–	Hyp	28.1	8.3	12.2	11.1

All bases were at a concentration of 100 μM. Additions were made either together with the drug (*top panel*), or 48 h subsequent to 3-deazaguanosine exposure (*bottom panel*). The concentration of 3-deazaguanosine was 200 μM. The initial plating density was 20,000 cells per dish. The reported final densities are the average of total cells in duplicate plates × 10^{-5}. The number in parentheses is the percent of growth with purine base alone obtained with drug and purine base added. *Abbreviations:* Hyp, hypoxanthine; Ade, adenine; Gua, guanine

Results

The antiproliferative effect of 3-deazaguanosine on several human cancer cell lines is shown in Fig. 1. The growth of tumor cells was inversely proportional to the concentration of 3-deazaguanosine in the medium. The drug produced inhibition of growth in every line, although the degree of this inhibition was variable. Growth inhibition was reversible. When the drug was removed from the medium following 2–8 days of exposure, growth rates similar to those of controls resumed within 24 h.

The effects of various purine compounds on the growth of cells treated with 3-deazaguanosine is shown in Table 1. None of the compounds tested was able to restore normal growth when added after 48 h exposure to the drug under the experimental conditions, though in some cases addition of a purine compound increased growth in the presence of the drug. The purine bases seem to be more effective at protecting the cells from cytotoxicity when added simultaneously with the drug (Table 1). The purine bases were included in the controls, since these bases alone have various effects on cell growth.

DNA synthesis was also decreased by 3-deazaguanosine. At a concentration of 700 μM the drug caused an 85% decrease in the incorporation of labeled thymidine into acid-insoluble nucleic acids in SkLu-1 cells.

Results of the incorporation of radiolabeled precursors into purine compounds is shown in Table 2. The incorporation of guanine into adenine nucleotides was completely prevented by 3-deazaguanosine; this inhibition was seen in each of the cell lines assayed (Table 3). In addition, the drug increased the ratio of guanine/adenine incorporation with every precursor. The incorporation of formate and hypoxanthine into adenine nucleotides was also diminished. The incorporation of adenine into purine compounds was not affected.

In the presence of 3-deazaguanosine the incorporation of formate into purine compounds was affected to varying degrees in the different cell lines (Table 4). Inhibition varied from approximately half in SkLu to complete in SkCo, T24, and A172.

In dialyzed hemolysates, the control GMP reductase activity of 2.21 pmol/min/mg protein was reduced to 1.93 (12.5% inhibition) by 100 μM 3-deazaguanosine. Complete inhibiton was seen with 100 μM 3-deazaguanosine monophosphate.

Table 2. Incorporation of radiolabeled purine precursors into purine compounds in SkLu cells

Precursor	Drug	Incorporation into adenine compounds	Incorporation into guanine compounds
Adenine	–	33,274	< 1
Adenine	+	36,414	91
Formate	–	2,423	136
Formate	+	1,396	116
Guanine	–	1,224	28,950
Guanine	+	< 1	27,547
Hypoxanthine	–	30,106	2,771
Hypoxanthine	+	20,609	3,717

Incorporation is in units of picomoles incorporation/100 nanomoles cellular purines [14]. Each number is the average of the analyses of two separate cell harvests

Table 3. Effect of 3-deazaguanosine on the incorporation of guanine into adenine nucleotides in human cancer cell lines

Line	Without 3-deazaguanosine	With 3-deazaguanosine
SkLu	1,224	< 1
SkCo	1,037	< 1
J82	1,546	< 1
A549	107	< 1
A172	222	< 1
T24	972	< 1

Incorporation is expressed in units of picomoles of adenine incorporation/100 nmoles cellular purines [14]

Table 4. Inhibition of the incorporation of formate into purine compounds by 3-deazaguanosine in human cancer cell lines

Line	Without 3-deazaguanosine	With 3-deazaguanosine
SkLu	2,857	1,513
SkCo	1,708	< 1
J82	3,156	52
MCF7L	1,855	340
A549	385	< 1
A172	708	126
T24	973	< 1

Incorporation is in units of picomoles of incorporation/100 nmoles of cellular purines [14]

Discussion

The inhibition of the growth of human cancer cell lines by 3-deazaguanosine appears to be comparable to that observed with the various animal lines studied [9, 10, 13, 18, 19]. The drug was toxic to all lines tested. As in the case of the animal cell lines, toxicity seems to involve the suppresssion of DNA synthesis.

Several theories have been advanced to explain the inhibition of DNA synthesis by 3-deazaguanosine. One possibility is that reduced DNA synthesis results from incorporation of the drug into the cellular nucleic acids [11]. However, the speed with which growth inhibition occurs after exposure to the drug and the fact that cells resume normal logarithmic growth with 24 h after its removal are not characteristic of drugs that are incorporated into nucleic acids.

Several investigators have reported inhibition of IMP dehydrogenase by 3-deazaguanosine in animal cell lines [4, 18]. In our studies with human cell lines, we see no decrease in the incorporation of hypoxanthine into guanine nucleotides in the presence of the drug. In growth studies, guanine is not able to improve growth in the presence of the drug in all of the lines tested; in the presence of an IMP dehydrogenase inhibitor such as tiazofuran [6, 22] growth is consistently improved by guanine. Inhibition of HPRT has been noted with Chinese hamster ovary cells [15]. Although we see a decrease in the incorporation of hypoxanthine into adenine nucleotides, there is no parallel decrease in incorporation into guanine nucleotides, and no decrease in guanine incorporation.

The inhibition of incorporation of formate into purine compounds varied widely among the different cell lines (Table 4), from approximately half in SkLu to complete in SkCo, A549, and T24. Again, the fact that purine bases are not helpful in restoring growth in the presence of 3-deazaguano-

sine in each of the cell lines indicates that inhibition of de novo synthesis is not the only significant toxic effect of the drug.

The most striking finding in these studies was the inhibition of guanosine monophosphate reductase. This was evidenced as the complete inhibiton of the incorporation of guanine into adenine nucleotides in intact cells in the presence of 3-deazaguanosine, and the complete inhibition of the conversion of GMP to IMP in cell extracts in the presence of 3-deazaguanosine monophosphate. Other investigators have shown that 3-deazaguanine is converted to its nucleotides [10] and that this conversion is necessary for the expression of toxicity [18].

The importance of GMP reductase in the regulation of guanine nucleotide metabolism has not been studied extensively, so it is difficult to assess the significance of the loss of this activity to purine metabolism. In human erythrocytes reductive deamination of GMP is the major catabolic route [7]. Inhibition of GMP reductase could therefore conceivably increase guanine nucleotide pools. Such an increase might have several effects. Sidi and Mitchell have shown that an accumulation of GTP in lymphoblasts treated with the purine nucleoside phosphorylase inhibitor 8-aminoguanosine is associated with an arrest in the S phase of the cell cycle which results in growth inhibition [20]. The mechanism of this inhibition is not clear. Alternatively, an increase in ribonucleotides might cause an increase in deoxyribonucleotides. DeoxyGTP (dGTP), in particular, is known to have cytotoxic properties and is believed to be the cytotoxic agent responsible for T-cell deficiency in purine nucleoside phosphorylase deficiency [12]; dGTP may exert this effect by inhibition of ribonucleotide reductase [27] or by direct inhibition of DNA polymerase [24].

Clearly 3-deazaguanosine exerts several toxic effects on human cancer cells. The relative importance of these effects will be the subject of future cell culture and metabolic studies.

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