REFERENCES

1. M. M. Appleman, W. J. Thompson and T. R. Russell, *Adv. Cyclic Nucleotide Res.* **3**, 65 (1973).
2. J. N. Wells and J. G. Hardman, *Adv. Cyclic Nucleotide Res.* **8**, 119 (1977).
3. J. N. Wells, Y. J. Wu, C. E. Baird and J. G. Hardman, *Molec. Pharmac.* **11**, 775 (1975).
4. G. L. Kramer, J. E. Garst, S. S. Mitchel and J. N. Wells, *Biochemistry* **16**, 3316 (1977).
5. H. Brana and F. Chytil, *Folia microbiol., Prague* **11**, 43 (1966).
6. Y. Y. Chang, *Science* **161**, 57 (1968).
7. P. F. Gulyassy, *J. clin. Invest.* **47**, 2458 (1968).
8. G. Schultz, E. Böhme and J. G. Hardman, *Meth. Enzym.* **38**, 9 (1974).
9. W. Y. Cheung, *Biochim. biophys. Acta* **191**, 303 (1969).
10. J. N. Wells, C. E. Baird, Y. J. Wu and J. G. Hardman, *Biochim. biophys. Acta* **383**, 430 (1975).
11. Y. M. Lin, Y. P. Liu and W. J. Cheung, *J. biol. Chem.* **249**, 4943 (1974).

Biochemical Pharmacology, Vol. 30, No. 16, pp. 2374–2376, 1981.
Printed in Great Britain.

0006-2952/81/162374-03 $02.00/0

Actions of 3-deazaguanine and 3-deazaguanosine on variant lines of Chinese hamster ovary cells

(*Received* 24 *November* 1980; *accepted* 24 *February* 1981)

3-Deazaguanine and its 9-ribosyl derivative, 3-deazaguanosine, were synthesized recently by Cook, *et al.* [1] as potential antitumor and antiviral agents. 3-Deazaguanine has exhibited significant activity against a variety of rodent neoplasms including several mammary adenocarcinoma lines [2–4]. The several cell types that are inhibited *in vitro* by 3-deazaguanine include L1210 [2], HeLa [2], and Ehrlich ascites tumor cells [5]. These compounds also exhibit activity against bacteria and a variety of DNA and RNA viruses [6–8].

In a study of the effects of 3-deazaguanine and its derivatives on purine nucleotide biosynthesis in Ehrlich ascites cells, Streeter and Koyama [5] describe the utilization of hypoxanthine-[^{14}C] in the presence of these agents at a concentration of 1 mM. Of the enzymes considered, they found IMP dehydrogenase (EC 1.2.1.14) to be the most sensitive to inhibition by all compounds tested—3-deazaguanine, 3-deazaguanosine and 3-deazaguanylic acid. HGPRT* was partially inhibited by 3-deazaguanosine and 3-deazaguanylic acid but only slightly by the free base, 3-deazaguanine. Thus, at 1 mM, 3-deazaguanine partially interfered with the biosynthesis of guanine nucleotides in these cells. These investigators also reported that 3-deazaguanosine was cleaved to 3-deazaguanine by extracts of Ehrlich ascites cells during an 18-hr incubation. Schwartz *et al.* [4] have reported the metabolism of 3-deazaguanine to 3-deazaguanosine-5′-triphosphate as well as its incorporation into nucleic acids in L1210 cells.

More recently Cook *et al.* [1] have compared the antiviral and antibacterial activities of 3-deazaguanine, 3-deazaguanosine, 3-deazaguanylic acid, and their imidazolecarboxamide precursors. Although the latter were more active, the antiviral spectra were similar for all. These investigators also reported partial inhibition of IMP dehydrogenase in Ehrlich ascites cells by 3-deazaguanine and its derivatives.

We have developed procedures to study the biochemical transformations of 3-deazaguanine and its derivatives using CHO cell mutants that are deficient in specific enzymes involved in purine nucleotide synthesis and interconversion. This type of approach, which has been employed effectively by Bennett *et al.* [9] and others, can be very useful in clarifying biochemical routes, particularly in comparing agents of similar structure.

CHO cells were carried in monolayer culture using McCoy's 5A growth medium supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY) as described previously [10]. Dialyzed fetal calf serum was used in all experiments relating to drug inhibition and utilization to avoid competition by natural serum purines.

Conventional methods were employed for mutagenesis and subsequent isolation of drug resistant cell lines [11]. CHO cells were dispensed into 250-ml plastic flasks (5×10^6 cells/flask) and treated with ethyl methanesulfonate (0.1 to 0.5 mg/ml) for 18 hr. The medium was removed, the cells were washed, and fresh medium was added to the flask. After 5 days, single-cell suspensions of the cells from those lines exhibiting 10–20 per cent survival were obtained by trypsinization and were dispensed into 250-ml flasks (2×10^5 cells/flask) in medium containing dialyzed fetal calf serum and an inhibitory concentration of the desired selective agent. After incubation for 10 days those flasks containing resistant clones were treated with trypsin, and single-cell suspensions were obtained by repeated pipetting (mechanical pipet aids were used throughout these procedures). A portion of each cell suspension was diluted and dispensed into 25-cm^2 flasks such that there was approximately 1 cell per flask. After incubation for 10 days those flasks containing single clones were kept, and the cells were allowed to propagate as pure cell lines.

For the determination of a minimum inhibitory drug concentration, logarithmically growing CHO cells were trypsinized and appropriately diluted with medium containing dialyzed fetal calf serum. They were dispensed into

* Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8); APRT, adenine phosphoribosyltransferase (EC 2.4.2.7); AK, adenosine kinase (EC 2.7.1.20); dCK, deoxycytidine kinase (EC 2.7.1.74); CHO cells, Chinese hamster ovary cells; 8-azaGua, 8-azaguanine; 8-azaAde, 8-azaadenine; TCN, tricyclic nucleoside (3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentazaacenaphthylene); ara-C, arabinosylcytosine; EHNA, *erthro*-9-(2-hydroxy-3-nonyl)adenine; DTT, dithiothreitol; and PRPP, phosphoribosyl pyrophosphate.

a series of 35-mm plastic petri dishes (approximately 200 cells/dish) and were allowed to form clones in a broad range of drug concentrations: 0.5, 1, 5, 10, 50, 100, 200, 500, 1000, 2000 and 3000 μM. After incubation for 7 days in humidified CO_2 at 37.5° the media were poured off, and the clones were fixed with 10% formaldehyde for 10 min and stained with 0.1% crystal violet. The minimum inhibitory drug concentration was the lowest tested concentration of drug that resulted in the formation of clones containing fewer than 50 cells.

Large quantities of cells for preparation of crude extracts were grown in glass roller bottles and harvested by scraping into phosphate-buffered saline. The cells were gently pelleted by centrifugation and stored at −90° until use. To prepare extracts, cells were suspended in 2 vol. of buffer containing 0.05 M Tris–HCl (pH 7.8), 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, and 6 mM $MgCl_2$. Cell debris was removed by centrifugation in an SS 34 rotor at 12,000 rpm for 20 min in a Sorvall RC2-B refrigerated centrifuge. Protein was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard.

HGPRT and APRT were assayed according to Fenwick and Caskey [12]. Reaction mixtures contained 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1.5 mM PRPP, 0.08 mM hypoxanthine-[8-^{14}C] (56 mCi/mmole) or adenine-[8-^{14}C] (62 mCi/mmole), and crude extract in a final volume of 50 μl. Incubation was at 37°. Aliquots of 10 μl were removed at desired intervals and applied onto Whatman DE 81 filter paper circles previously spotted with 25 μl of 0.1 M EDTA. The circles were then washed repeatedly with 1 mM ammonium formate, twice with deionized H_2O, dried, and counted in Aquasol (New England Nuclear Corp., Boston, MA). The amount of product formed in this and the other assays was calculated using appropriate corrections for quenching and counting efficiency.

Reaction mixtures for adenosine kinase contained 0.1 M Tris–maleate buffer (pH 5.5), 4 mM ATP, 1.5 mM $MgCl_2$, 5 μM EHNA (adenosine deaminase inhibitor), 50 μM adenosine[8^{14}C] (59 μCi/μmole), and extract in a final volume of 50 μl [13]. Incubation was at 37°. At appropriate intervals 10 μl samples were removed and applied to Whatman DE 81 paper circles, which were then washed four times in 1 mM ammonium formate, twice with deonized H_2O, dried, and counted in Aquasol.

Incubation mixtures for deoxycytidine kinase contained 5 mM ATP (neturalized), 50 μM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, and 25 μM deoxycytidine[8-^{3}H] (4 Ci/mmole) in a final volume of 100 μl. At 10-min intervals 10 μl samples were removed and applied to DE 81 circles that were washed as described above and placed in vials containing 1 ml of 1 N HCl. After 10 min, 10 ml of Aquasol was added and mixed, and the solution was counted.

Table 1 describes some of the variant cell lines that were derived from CHO cells for this and similar studies. All the cell lines were stable and were maintained in the absence of drugs. Tests for mycoplasma contamination were negative. Enzyme specific activities are expressed as nmoles product formed per minute per mg protein.

The parent CHO line was quite sensitive to 3-deazaguanine (Table 2); however, it was even more sensitive to the nucleoside derivative. The HGPRT deficient cell line AG^R-1 was used to determine if this activity reflected degradation of 3-deazaguanosine to the free base. AG^R-1 cells were extremely resistant to 3-deazaguanine, but they retained their sensitivity to 3-deazaguanosine. This observation suggests the existence of a mechanism for activation or inhibition apart from one involving degradation to the free base by purine nucleoside phosphorylase.

Since the adenosine kinase from H.E.P. #2 cells has been shown to utilize certain inosine analogs, but not inosine itself [14], a mutant CHO clone deficient in both HGPRT and AK was isolated to determine its sensitivity to 3-deazaguanosine. Strain AG^R-1/T^R-5 was derived from AG^R-1 and selected for resistance to tricyclic nucleoside (TCN). TCN has been shown to require "activation" by AK [15] and resistance to it generally reflects a deficiency in AK activity. Strain AG^R-1/T^R-5, which was deficient in AK activity and completely resistant to tubercidin as well as TCN, had not acquired resistance to 3-deazaguanosine other than that conferred by the HGPRT deficiency. This suggests that 3-deazaguanosine is not a substrate for AK, and, if phosphorylation is involved, another kinase must be implicated. Similarly, strain aC^R-7, which was deficient in dCK, had also retained sensitivity to 3-deazaguanosine, thus eliminating that enzyme as a possible mechanism of phosphorylation.

Another of the CHO lines, strain AA^R-6, which was deficient in APRT demonstrated partial resistance to 3-deazaguanine, 500 μM being necessary for inhibition (Table 2). This suggests that APRT may accept 3-deazaguanine as a substrate to a limited extent in these cells. We have not observed this phenomenon with other guanine analogs. The cell line that was deficient in both HGPRT and APRT, AA^R-6/AG^R-7, was resistant to 3-deazaguanine at concentrations greater than 2 mM, which was higher than either AA^R-6 or AG^R-1 cells would tolerate.

In summary, 3-deazaguanine appeared to require phosphoribosylation by HGPRT to be active. 3-Deazaguanosine, which was active in the absence of HGPRT, was not phosphorylated by adenosine kinase or deoxycytidine kinase. Although this suggests the possibility of a guanosine phosphorylating activity in these cells, further investigation will be necessary to determine if this is the case or if this agent is capable of inhibitory activity without being phosphorylated.

Table 1. Enzyme deficiencies of variant CHO cell lines

Cell line	Selective agent(s)	Enzyme specific activities*			
		HGPRT	APRT	AK	dCK
CHO		3.27	1.92	1.12	0.023
AG^R-1	8-azaGua	0.19	1.15	0.74	0.021
AA^R-6	8-azaAde	3.18	0.05	1.02	0.030
AG^R-1/T^R-5	8-azaGua, TCN	0.01	1.21	< 0.01	0.023
AA^R-6/AG^R-7	8-azaAde, 8-azaGua	< 0.01	< 0.01	0.81	0.027
aC^R-7	Ara-C	3.50	1.84	1.29	0.005

* Specific activities are expressed as nanomoles product formed per min per mg protein.

Table 2. Growth inhibition of CHO and mutant cell lines by 3-deazaguanine and 3-deazaguanosine*

Cell line	Enzyme deficiencies	Minimum inhibitory concentration (μM)	
		3-Deazaguanine	3-Deazaguanosine
CHO	None	10	1
AG^R-1	HGPRT	2000	5-10
AA^R-6	APRT	500	ND†
AG^R-1/T^R-5	HGPRT, AK	2000	10
AA^R-6/AG^R-7	HGPRT, APRT	> 2000	20
aC^R-7	dCK	10	1

* Procedures were as described in the text.
† Not determined.

In view of the growth inhibition properties of 3-deazaguanosine, its apparently unique action, and its relatively high solubility in water, it may be worthwhile to consider the clinical potential of this agent as well as that of 3-deazaguanine.

Acknowledgements—This work was supported by American Cancer Society Grant ACS CD-60 and, in part, by USPHS Grants CA 11520, CA 14528 and RR-5511-15.

Department of Developmental Therapeutics
The University of Texas System Cancer Center
M.D. Anderson Hospital and Tumor Institute
Houston, TX 77030, U.S.A.

PRISCILLA P. SAUNDERS*
LIAN-YU CHAO
TI LI LOO

Department of Chemistry
Brigham Young University
Provo, UT 84601, *U.S.A.*

ROLAND K. ROBINS

REFERENCES

1. P. D. Cook, R. J. Rousseau, A. M. Mian, P. Dea, R. B. Meyer, Jr. and R. K. Robins, *J. Am. chem. Soc.* **98**, 1942 (1976).
2. T. A. Khwaja, L. Kigwana, R. B. Meyer and R. K. Robins, *Proc. Am. Ass. Cancer Res.* **16**, 162 (1975).
3. T. A. Khwaja and J. Varven, *Proc. Am. Ass. Cancer Res.* **17**, 200 (1976).
4. P. Schwartz, D. Hammon and T. A. Khwaja, *Proc. Am. Ass. Cancer Res.* **18**, 153 (1977).
5. D. G. Streeter and H. H. P. Koyama, *Biochem. Pharmac.* **25**, 2413 (1976).
6. L. B. Allen, J. H. Huffman, R. B. Meyer, Jr., P. D. Cook, J. T. Witkowski, L. N. Simon, R. K. Robins and R. W. Sidwell, *Fifteenth Interscience Conference on Antimicrobial Agents and Chemotherapy*, Abstr. 245, Washington, DC, September 1975.
7. P. P. Saunders, L. Y. Chao, R. K. Robins and T. L. Loo, *Molec. Pharmac.* **15**, 691 (1979).
8. T. R. Matthews, D. W. Yotter, P. D. Cook, R. W. Sidwell, R. K. Robins and P. F. Dougherty, *Sixteenth Interscience Conference on Antimicrobial Agents and Chemotherapy*, Abstr. 425, Chicago, October 1976.
9. L. L. Bennett, Jr., M. H. Vail, P. W. Allan and S. C. Shaddix, *Biochem. Pharmac.* **22**, 1221 (1973).
10. P. P. Saunders and L. Y. Chao, *Cancer Res.* **34**, 2464 (1974).
11. F. T. Kao and T. T. Puck, in *Methods in Cell Physiology* (Ed. D. Prescott), Vol. VIII, p. 23. Academic Press, New York (1974).
12. R. G. Fenwick, Jr. and C. T. Caskey, *Cell* **5**, 114 (1975).
13. M. S. Hershfield, F. F. Snyder and J. E. Seegmiller, *Science* **197**, 1284 (1977).
14. L. L. Bennett, Jr., D. L. Hill and P. W. Allan, *Biochem. Pharmac.* **27**, 83 (1978).
15. P. G. W. Plagemann, *J. natn. Cancer Inst.* **57**, 1283 (1976).