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SYNERGISTIC INHIBITION OF HUMAN LEUKEMIA CELL GROWTH BY DEOXYGUANOSINE AND 1-β-D-ARABINOFURANOSYLCYTOSINE*

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Abstract—We studied the ability of 2′-deoxyguanosine (dGuo) to influence 1-β-D-arabinofuranosylcytosine (ara-C) inhibition of soft agar cloning of the cultured human leukemia cell line K562. Ara-C alone inhibited cloning in concentrations of >10 nM, with a steep drop in colony formation observed between 10 and 100 nM. dGuo and ara-C synergistically inhibited cloning; the combination of ineffective concentrations of dGuo (10–50 μM) and ara-C (≤10 nM) inhibited cloning by 40–70%. In K562 cells, dGuo is metabolized by both nucleoside kinase and purine nucleoside phosphorylase (PNP), resulting in augmentation of both the GTP pool (to more than 200% of control after a 3 hr incubation with 500 μM dGuo) and the dGTP pool (to more than 2700% of control after 3 hr with 500 μM dGuo). dGuo (50–500 μM) caused a decrease in the dCTP and dTTP pools and an increase in the dATP pool. Synergistic concentrations of dGuo plus 10 nM ara-C augmented the ara-CTP pool up to 800% of control after 3 hr to levels equivalent to those observed after incubation with 500 nM ara-C alone. Incorporation of 10 nM ara-CTP into DNA also increased in the presence of dGuo (up to a maximum of 300% of control), but only to a level that approximated the value observed with 50 nM ara-C alone. The disparity between enlargement of the ara-CTP pool and augmentation of ara-C incorporation into DNA is consistent with the observation of Steinberg *et al.* [*Cancer Res.* **39**, 4330 (1979)] that high concentrations of dGTP may inhibit DNA polymerase activity. Thus, synergy between dGuo and ara-C is multifactorial, possibly involving inhibition of DNA polymerase by elevated dGTP and ara-CTP pools and augmented incorporation of ara-C into DNA.

Compounds which affect pyrimidine biosynthesis are being recognized as possible potentiators of antimetabolite antitumor agents. Enhancement of antimetabolite ara-C‡ activity by agents which affect the *de novo* synthesis of dCTP [1–8], or which inhibit the metabolic degradation of the cytosine base [9–11], is currently the subject of intensive investigation. Thymidine, through conversion to dTTP, inhibits the *de novo* synthesis of dCTP, principally by allosteric inhibition of ribonucleotide reductase, the enzyme responsible for converting CDP to dCDP [12–16]. Since dCTP is a potent feed-back inhibitor of dCyd kinase (EC 2.7.1.74) [17–20], depletion of the dCTP pool by dThd enhances phosphorylation of dCyd and ara-C, increases ara-CTP pool size, and augments incorporation of ara-C into nucleic acid, both *in vitro* [6–8, 21] and *in vivo* [2]. Since antitumor activity of ara-C correlates positively with the ara-CTP pool size [20, 22–28] and negatively with the dCTP pool size [17, 29–31], dThd-induced depletion of the dCTP pool causes enhanced cytotoxicity of ara-C *in vitro* [1, 3–5, 8] and *in vivo* [2, 21].

In a previous report, we compared the effects of the naturally occurring deoxynucleosides on the dCTP pool, in the human leukemia cell lines HL-60 and K562, and found that 750 μM dAdo or 750 μM dGuo reduces the dCTP pool to a significantly greater degree than does dThd, with dGuo having the greatest effect [32]. These data led us to the present studies, in which we investigated the effect of dGuo on ara-C activity against the human cultured leukemia K562, employing inhibition of soft agar cloning as a measure of cell growth inhibition.

MATERIALS AND METHODS

Cell culture. K562 is a human cell line derived from pleural fluid cells of a patient with chronic myelogenous leukemia in blast crisis [33]. Culture conditions have been described previously [32]. K562 cells clone spontaneously in soft agar, without exogenous stimulator. Drug and nucleoside combinations were tested against K562 cloning by incu-

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‡ Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, the 5′-Triphosphate derivative of ara-C; dThd, thymidine; dCyd, 2′-deoxycytidine; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; PNP, purine nucleoside phosphorylase (EC 2.4.2.1); HPLC, high pressure liquid chromatography; dNTP, deoxynucleoside triphosphate; ara-U, 1-β-D-arabinofuranosyluracil; and IC_{50}, the concentration causing 50% inhibition of cell growth in soft agar culture.

bating 500 K562 cells/ml in medium plus 0.3% agar and appropriate concentrations of ara-C and dGuo for two weeks in 35 mm Petri dishes (Falcon Plastic, Oxnard, CA), at 37°, 7.5% CO_2. After 2 weeks colonies of 50 cells or greater were counted with a 40× dissecting microscope. Cloning efficiency of control cultures was 14–30%.

Drugs. All nucleosides, all nucleotides, phosphoenolpyruvate, and pyruvate kinase were obtained from the Sigma Chemical Co., St. Louis, MO. [5-^{3}H]Ara-C (sp. act. 15.5 Ci/mmole) was purchased from Amersham/Searle (Arlington Heights, IL) and was diluted to the appropriate concentration in Roswell Park Memorial Institute medium 1640 without fetal calf serum prior to use.

Ara-C metabolic studies. One to two million logarithmically growing K562 cells were incubated with 10 nM [5-^{3}H]ara-C plus dGuo, or with various concentrations of [5-^{3}H]ara-C alone at 37° for 3 hr. This time period was chosen because we observed that, after 3 hr, the formation of ara-C nucleotides from 10 nM ara-C alone reached and maintained maximal equilibrium values (data not shown); thus we ensured that the observed effects of dGuo on ara-C metabolism were sustained and not transient kinetic ones. After incubation, the cells were cooled on ice for 5 min, sedimented by centrifugation at 4°, 400 *g* for 5 min, and then washed twice in ice-cold 154 mM NaCl. After washing, determinations were made in triplicate, on separate samples, of acid precipitable radioactivity, acid soluble radioactivity, and methanol-soluble radioactivity by previously published methods [32, 34].

Ara-CTP pool sizes were determined by applying 25-μl aliquots of the methanol-extracted supernatant fraction, along with appropriate standards, to a Spectra-Physics model 3500 B high pressure liquid chromatograph (Spectra-Physics, Santa Clara, CA), equipped with a 4.6 mm × 25 cm Whatman Partisil SAX/10 anion exchange column (Whatman Inc., Clifton, NJ) and a 2.1 mm × 7 cm guard column filled with anion exchange packing. Samples were eluted at 60° with an 8–89% buffer gradient of distilled water: 1 M KH_2PO_4, pH 3.3. over 25 min, at a flow rate of 1.5 ml/min. Absorption peaks were detected at 254 nm by a model 770 Chromatronix Spectrophotometric Detector (Schoeffel Instrument Co., Westwood, NJ). Eluates were collected in a fraction collector in 30-sec fractions for 15 min following injection of sample, and radioactivity of each fraction was determined. This method permits effective separation of ara-CTP from ara-UTP.

dGuo and ara-C degradation. The metabolic fates of dGuo and ara-C in liquid cultures of one million K562 cells/ml were determined. After cells were removed by centrifugation at 4°, 400 *g* for 10 min, they were extracted with methanol. The amounts of dGuo or ara-C and their metabolites remaining in the extracted supernatant fractions were determined by applying aliquots to a Spectra-Physics Spherisorb 10 uODS (3 mm × 25 cm) reversed phase HPLC column and determining absorption at 254 (dGuo) or 280 (ara-C) nm.

Enzyme assays. For all enzyme assays, crude K562 cellular cytosol supernatant fractions were prepared by harvesting one to ten million log phase growth cells by centrifugation at 4°, 400 *g*. Cell pellets were washed twice in 10 vol. of ice-cold phosphate-buffered normal saline prior to lysis. Lysis was accomplished by four freeze-thaw cycles in the buffer appropriate for each assay. Fifteen homogenization strokes in a Dounce homogenizer followed freeze–thaw lysis in the purine nucleoside phosphorylase (EC 2.4.2.1) assay. Lysates were clarified by centrifugation prior to assay. PNP activity was assayed by the method of Stoeckler *et al.* [35]. One unit of activity corresponds to 1 μmole of product produced/min. Assay for ara-C phosphorylation was performed by the method described by Coleman *et al.* [36]. Protein content of the supernatant fraction was determined by the method of Lowry *et al.* [37].

Ribonucleotide and deoxyribonucleotide triphosphate pool size. Ribonucleotide and deoxyribonucleotide triphosphate pool sizes were determined after incubating 50 to 100 million K562 cells with various concentrations of dGuo for 3 hr. Cells were harvested by centrifugation at 400 *g*, 4° for 10 min. Pellets were not washed; residual supernatant fractions were wiped away with tissue paper. Methanol extractable material was collected as outlined above. Ribonucleotide triphosphate pools were determined by applying aliquots to the HPLC as described above for ara-CTP pools, using dual wavelength detection at 254 and 280 nm. Peak heights were determined by manual measurement and normalized with an internal standard. Deoxyribonucleotide triphosphate pools were determined on the same system after ribonucleotide triphosphates had been removed by the method Garrett and Santi [38].

RESULTS

Ara-C and dGuo synergy for growth inhibition. Ara-C had no effect on K562 cloning in concentrations <10 nM. Above 10 nM, there was a steep dose response to ara-C; 50 nM ara-C caused 50% inhibition of cloning; 100 nM ara-C caused 100% inhibition of cloning. dGuo also had no effect on K562 cloning at concentrations below 50 μM; higher concentrations inhibited K562 cloning with an IC_{50} of

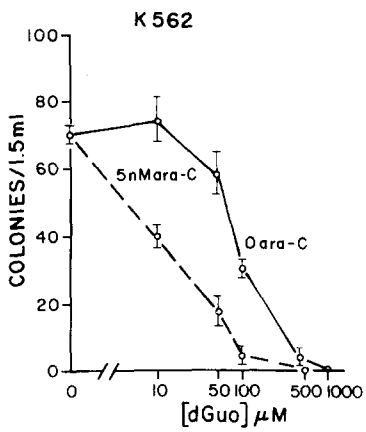

Fig. 1. Effect of dGuo and ara-C on cloning efficiency of K562 cells. Each plot represents a different concentration of ara-C, either 0 or 5 nM. Each point represents the mean ±S.D. of three samples.

Table 1. Effect of a 3-hr incubation with dGuo on dNTP and GTP pool sizes*

dGuo (μM)	dNTP or GTP pool size (pmoles/10^7 cells) dCTP	dTTP	dATP	dGTP	GTP
0	61	886	357	105	34,100
	(100)	(100)	(100)	(100)	(100)
50	11†	517†	273	109	48,300
	(18)	(58)	(76)	(104)	(142)
100	19†	523†	355	239†	68,600†
	(31)	(59)	(100)	(228)	(201)
500	24†	769	782†	2,841†	74,200†
	(39)	(87)	(219)	(2,705)	(218)

* Pool sizes were determined as described in Materials and Methods. Reported are mean values of a representative experiment. Values in parentheses equal percent control.
† Significantly different than controls ($P < 0.05$).

100 μM. Synergy between dGuo and ara-C was demonstrated against K562 (Fig. 1). The combination of 5 nM ara-C and 10 μM dGuo produced 40% cloning inhibition, whereas neither compound alone affected K562 at these concentrations. A 5 nM concentration of ara-C plus 50 μM dGuo inhibited cloning 70%.

Metabolism of dGuo and ara-C. To investigate the mechanism of the synergistic interaction between dGuo and ara-C, we determined the metabolic fate of dGuo incubated with K562 cells in liquid culture. A 100 μM concentration of dGuo disappeared rapidly from the cultures, with 50% metabolized by 2 hr. By 6 hr, only 10% remained. The major metabolite was guanine, indicating that removal of deoxyribose from dGuo by purine nucleoside phosphorylase (PNP) was the major metabolic pathway. We confirmed this by measuring PNP activity directly in cell lysates. PNP activity in K562 was 54.1 mU/mg protein, indicating considerable activity as compared to published activities in mammalian liver [35]. Incubation with dGuo caused increases in both the cellular GTP pool and the dGTP pool. GTP increased by more than 200% after a 3-hr exposure to up to 500 μM dGuo (Table 1). The intracellular dGTP pool increased by more than 2700% after 3 hr of exposure to dGuo (Table 1). This remarkable increase in the dGTP pool reflected not only increased salvage of guanine but also phosphorylation of dGuo directly to nucleotides by nucleoside kinase(s), as will be discussed below.

Incubation with dGuo was also associated with changes in other dNTP pool sizes. The dCTP pool was diminished by up to 80% after a 3-hr incubation with dGuo (Table 1). The dTTP pool was also lowered; the dATP pool was increased by high concentrations of dGuo.

We determined the metabolic fate of ara-C in K562 cultures. Ara-C at a 10 nM concentration disappeared from the culture system quite rapidly, with >90% reduction in ara-C concentration by 1 hr. The major metabolite co-chromatographed with ara-U, indicating that deamination is the major pathway of metabolism in K562.

Ara-C is activated by dCyd kinase catalyzed phosphorylation [17–20]. We observed ara-C phosphorylating activity in K562 cells to be 0.4 units. dGuo interfered with ara-C phosphorylation in a concentration-dependent fashion. Addition of 500 μM dGuo reduced ara-C phosphorylating activity to 0.3 units; addition of 1000 μM dGuo reduced it to 0.2 units. GTP and ATP at concentrations of 6 mM were equally efficacious as phosphate donors when used in the ara-C kinase assay of cell-free cells (data not shown).

Both ara-CTP pools and ara-C incorporation into K562 DNA increased with increasing concentration of ara-C (Table 2). At low concentrations of ara-C, much of the available ara-CTP was incorporated into DNA; however, as concentrations of ara-C increased, progressively smaller fractions of available ara-C were incorporated into DNA. Using cesium chloride density gradient purification of DNA and subsequent enzymatic degradation of the DNA, we confirmed the observation of Kufe *et al.* [39] that acid precipitable radioactivity represents ara-C incorporated into DNA (data not shown).

We measured the effect of dGuo on the metabolism of 10 nM ara-C, since this was the highest concentration of ara-C which by itself did not inhibit K562 cloning. dGuo increased the ara-CTP to a maximum of almost 800% of control at 60 μM dGuo, with the ara-CTP pool falling off at higher concentrations of dGuo (Fig. 2A). The largest ara-CTP pool produced by the combination of 10 nM ara-C and dGuo approximated those produced by 500 nM ara-C alone. Concentrations of dGuo synergistic with

Table 2. Concentration effects on ara-C incorporation into K562 DNA and ara-CTP pools*

Ara-C (nM)	Ara-C in DNA†‡	Ara-CTP†
10	0.03	0.06
50	0.15	0.12
100	0.25	0.15
500	0.68	0.75
1,000	1.15	3.10
5,000	1.48	23.38
10,000	1.48	43.73

* One to two million cells were incubated for 3 hr with [5-^{3}H]ara-C, adjusted to the concentrations shown with unlabeled ara-C, in all experiments. Determinations were performed as described in Materials and Methods.
† Expressed in pmoles per 10^6 cells per 3 hr.
‡ Mean of three samples. S.E.M. < 15% for all samples.

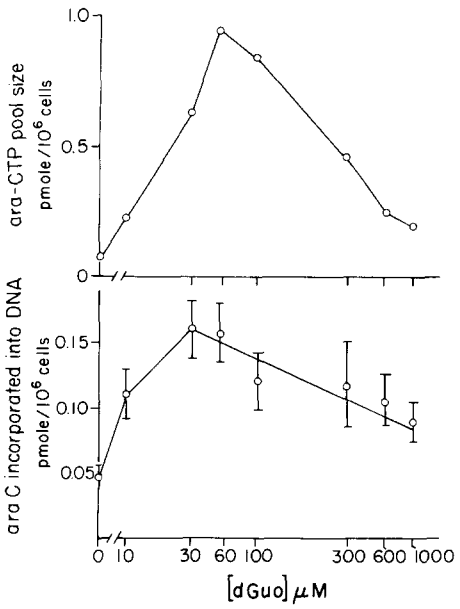

Fig. 2. (A) Effect of dGuo on ara-CTP pool size in K562 cells incubated with 10 nM [5-^{3}H] ara-C. Shown are the data from a single experiment which is representative of several identical experiments. (B) Effect of dGuo on the incorporation of 10 nM ara-C into K562 DNA. Each data point represents the mean ±S.E.M. of the combined results of four separate experiments.

10 nM ara-C in the cloning assay increased incorporation of ara-C into DNA to a maximum of 300% of control, to levels which approximated those observed with 50 nM alone (Fig. 2B).

Since dGuo is primarily converted to guanine by K562 cells, the effect of guanine on ara-C metabolism by K562 was also assessed. The poor solubility of guanine required that it be dissolved in medium containing 5 nM NaOH; therefore, controls were run with and without NaOH. NaOH caused a 10% decrease in ara-C metabolic variables (ara-CTP pool size and incorporation into DNA). Incubation with 10–500 μM guanine had only a marginal (100–120% of control) effect on ara-CTP pool size or incorporation of ara-C into DNA.

DISCUSSION

In our previous studies we found that, of the naturally occurring deoxynucleosides, dGuo was the most potent inhibitor of cultured human leukemic cell growth [32]. We have extended these studies, examining the metabolism of dGuo by K562 cells and the effect of incubation of dGuo with the potent antileukemia agent ara-C.

dGuo is converted to nucleotides by two pathways: (1) deoxyribose can be removed by PNP, producing guanine which can subsequently be converted to nucleotides by the phosphoribosyltransferase pathway; alternatively (2) phosphate groups can be added directly by nucleoside kinases. In some cell lines, dGuo is a high K_m substrate for dCyd kinase [19, 20, 40, 41]. In others, dGuo is phosphorylated by separate and distinct kinases [42]. We have shown that, at high concentrations, dGuo inhibited phosphorylation of ara-C by K562 dCyd kinase. This suggests that dGuo and ara-C are phosphorylated in this cell line by the same kinase. We have also noted considerable activity of PNP in K562. Thus, it appears that both pathways of dGuo metabolism are active in K562. PNP activity raises available guanine pools, resulting in augmentation of GTP and dGTP pools. Ara-C kinase directly converted dGuo to nucleotide, resulting in a remarkably inflated (>2700% of control) dGTP pool.

The regulation of dNTP pools in K562 by dGuo appears to be similar to what has been reported elsewhere [14, 40, 43]. dGuo elevated the dATP pool, while dCTP and dTTP pools were diminished. These pool size changes are consistent with the reported effects of dGTP on ribonucleotide reductase: stimulation of ADP reduction and inhibition of CDP reduction [14]. Therefore, dGuo may regulate clonal growth in part via the "ribonucleotide reductase hypothesis" [44]; i.e. high concentrations of the metabolites dGTP and dATP regulate the activity of ribonucleotide reductase such that pools of available precursors of DNA synthesis (dCTP and dTTP) are diminished. Lowered dCTP pools in K562 may be critical, since the dCTP pool of control cells appears to be the smallest of all dNTP pools. However, the reduction of pyrimidine nucleotide pools may not be the sole mechanism of growth inhibition by dGuo. The very elevated intracellular dGTP concentrations may inhibit DNA polymerase directly, as has been demonstrated in calf thymus by Steinberg *et al.* [45].

dGuo and ara-C synergistically inhibited cloning employing the definition of synergy of Harkrader *et al.* [3], who also demonstrated synergy between dGuo and ara-C in rat hepatoma cells. However, disturbances in nucleotide metabolism caused by dGuo appear to be different in rat hepatoma and human leukemia. Harkrader *et al.* found that 100 μM dGuo diminished the hepatoma dCTP pool but did not alter the ara-CTP pool. They concluded that the increased growth inhibition of ara-C in the presence of dGuo was mediated through the diminished dCTP pool. In K562, dGuo both augmented ara-CTP pools and diminished dCTP pools, enhancing incorporation of ara-C into DNA. Enhancement of incorporation of ara-C into DNA may be the mechanism of synergy, since Kufe *et al.* [39] have demonstrated correlation between cloning inhibition and amounts of ara-C incorporated into DNA. Guanine has no effect on ara-C metabolism in K562; therefore, the effect of dGuo on ara-C metabolism was inactivated by degradation of dGuo to guanine. This suggests that the increased dGTP pool, not the increased GTP pool, was responsible for augmentation of the ara-CTP pool. Even though GTP is an excellent phosphate donor, for the ara-C kinase mediated reaction the availability of phosphate donor is not rate-limiting for ara-C phosphorylation. Rather, ara-C phosphorylation is augmented by the diminution of the dCTP pool caused by the inflated dGTP pool. It has been well documented that diminished dCTP pools cause increased activity of dCyd kinase, the enzyme responsible for phosphorylation of ara-C [12–20].

High concentrations (greater than 100 μM) of dGuo caused a fall-off in ara-CTP pools. This fall-off appears to have been due to inhibition of ara-C phosphorylation by a competitive substrate, dGuo.

These data suggest that dGuo may be an important modifier of ara-C activity. We are currently pursuing this possibility, using animal studies.

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