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Interrelationship of 3-deazaguanine-induced growth inhibitory actions in L1210 cells*

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3-Deazaguanine (c^3Gua)† is a guanine analog whose cancer chemotherapeutic properties are currently being investigated clinically [1]. Studies from this laboratory using L1210 leukemic cells *in vitro* have shown that c^3Gua-induced inhibition of DNA synthesis and protein synthesis is closely correlated with the cytotoxic action of the compound [2]. The actions of c^3Gua on DNA and protein synthesis have been associated previously with incorporation of c^3Gua into DNA [2], and with inhibition of initiation of translation [3], respectively. In addition, in L1210 cells, GTP pools decline after c^3G exposure, whereas c^3Gua is anabolized to c^3GTP, which accumulates and is incorporated into RNA [2].

The objective of this study was to assess the roles of various drug effects in cytotoxicity by examining cell recovery following drug removal from the incubation medium. In addition, the possible impairment of translation into protein of mRNA containing c^3Gua was studied.

Materials and methods

[*Methyl*-^{3}H]Thymidine (Thd) (20 Ci/mmol) and [4,5-^{3}H]L-leucine (58 Ci/mmol) were supplied by the New England Nuclear Corp. and ICN Biochemicals, Inc. respectively. [2-^{14}C]c^3Gua mesylate (9.47 mCi/mmol) and c^3Gua mesylate were provided by Dr. R. Jackson, Warner-Lambert Co., Ann Arbor, MI. Additional c^3Gua has been donated previously by ICN Biochemicals, Inc. c^3GTP was made available by Dr. Priscilla Saunders, M. D. Anderson Hospital and Tumor Institute, Houston, TX.

Tissue culture. The L1210 cell line was maintained in RPMI 1640 medium supplemented with 10% dialyzed horse serum (Gibco), 60 μg/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells were grown under 5% CO_2/air at 37° and were diluted regularly to maintain logarithmic growth.

Assessment of recovery of cells following c^3Gua-induced exposure. Cells (100 ml, 5×10^4 cells/ml) were incubated with 4, 10, or 30 μM c^3Gua for 12 or 24 hr, were washed to remove the drug, resuspended in the original volume of drug-free medium, and reincubated at 37° to permit cell recovery. Two aliquots of 2 ml were taken from each culture at 0, 6, 12, 18, and 24 hr after drug removal and were assayed for [^{3}H]Thd and [^{3}H]leucine incorporation as previously described [2, 4]. In other aliquots, GTP and c^3GTP levels were analyzed by high pressure liquid chromatography [2]. Amounts of c^3GTP in extracts were quantified by comparing area under the curve for the c^3GTP peak to a standard curve derived from cells treated with [^{14}C]c^3Gua. Area under the curve for the UV-absorbing eluate was consistent with the radioactivity data ($r^2 = 0.84$). GTP was quantified similarly from AUC standards.

Assessment of the effect of c^3Gua on viability. L1210 cells were incubated with 0, 4, 10, or 30 μM c^3Gua for 24 hr and then allowed 0, 12, 18, or 24 hr recovery in drug-free medium before plating. Colony formation assays were then performed [5].

RNA isolation and analysis. To study the activity of c^3Gua-containing mRNA in *in vitro* translation, L1210 cultures (5×10^5 cells/ml, 200 ml) were incubated with 0, 4, 10 or 30 μM c^3Gua for 24 hr, RNA was isolated by the method of Chirgwin *et al.* [6] and was then enriched for poly A$^+$ RNA by oligo-deoxythymidylate cellulose chromatography. The amount of poly A$^+$ RNA isolated from 10^8 L1210 cells incubated with 0–30 μM c^3Gua for 24 hr ranged from 16.8 to 21 μg, with A_{260}/A_{280} ratios of 2.00 ± 0.03.

To assess incorporation of ^{14}C-labeled c^3Gua into mRNA, L1210 cells (2×10^5 cells/ml) were incubated with 20 μM [2-^{14}C]c^3Gua (4.41 mCi/mmol) for 24 hr, and RNA was isolated by cesium sulfate density gradient centrifugation [2, 7]. mRNA from these cells prepared as above was collected on pre-wetted Whatman GF/C filters to which 50 μg calf thymus DNA had been added as a carrier. The filters were washed twice with 5 ml of cold 5% tri-

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† Abbreviations: dT, deoxythymidylate; c^3Gua, 3-deazaguanine; c^3GTP, 3-deazaguanosine triphosphate; and Thd, thymidine.

chloroacetic acid and once with 8 ml of cold 100% ethanol. The filters were dried, added to 10 ml of toluene : Omnifluor (4 g/L) mixture, and counted in a scintillation counter.

In vitro *translation*. A rabbit reticulocyte *in vitro* translation system (Bethesda Research Laboratories, Gaithersburg, MD) was used to assess the ability of mRNA isolated from control and c^3Gua-treated L1210 cells to be translated into protein [8]. mRNA (0.1 to 0.4 μg) isolated from L1210 cells previously incubated with 0, 4, 10, or 30 μM c^3Gua, for 24 hr, was added to each reaction tube according to the protocol of the manufacturer. After incubation in the presence of [^{3}H]leucine, triplicate aliquots were assayed for radioactivity.

Results

Varying recoveries from c^3Gua-induced drug effects: Inhibition of DNA and protein synthesis, GTP depletion and c^3GTP formation. Cultures incubated with 4 μM c^3Gua regained control rates of DNA and protein synthesis within 24 hr after removal from drug-containing medium (Fig. 1, A and B). Cultures incubated with 30 μM c^3Gua, however, had levels of DNA and protein synthesis significantly lower than those of control cultures (35 and 70% of control values respectively) even 24 hr after drug removal. Levels of DNA synthesis were also depressed significantly in cells incubated with 10 μM c^3Gua for 24 hr and then allowed 24 hr to recover (Fig. 1A).

A 24-hr exposure to c^3Gua depleted intracellular GTP (expressed as percent of control) by up to 70% (Fig. 1C). Within 24 hr after removal from drug-containing medium, however, L1210 cells previously incubated with up to 30 μM c^3Gua contained amounts of GTP not significantly different from those in control cells.

A recovery period of 24 hr in drug-free medium allowed for loss of c^3GTP accumulation from c^3Gua-treated cells. In cells previously incubated with 4 μM c^3Gua for 24 hr, no c^3GTP could be detected. In cells incubated with 10 to 30 μM c^3Gua, about 75% of the accumulated c^3GTP was lost (Fig. 1D).

Lack of recovery of L1210 cells from c^3Gua-induced cytotoxicity. Incubation with c^3Gua decreased L1210 cell viability to 80.5, 3.8 ± 0.45, and 0.77 ± 0.03% of control in cells incubated for 24 hr with 4, 10, or 30 μM c^3Gua respectively. Allowing the cultures to recover in drug-free medium for 24 hr before plating resulted in small but significant increases in viability (89, 6.0 ± 0.5 and 1.7 ± 0.1% of untreated cultures respectively). All values are mean ± SE and are derived from three determinations performed in triplicate. Values without SE were derived from two determinations done in triplicate.

Incorporation of c^3Gua into poly A$^+$ RNA. RNA isolated by cesium sulfate density gradient centrifugation from cells incubated for 24 hr with 20 μM [^{14}C]c^3Gua contained 6.85 pmol c^3Gua/μg RNA. Enrichment of the RNA by oligo-dT cellulose chromatography produced a poly A$^+$ RNA fraction with an A_{260}/A_{280} ratio of 2.05 and which contained 21.1 pmol c^3Gua/μg. These results suggest that c^3Gua was incorporated preferentially into L1210 mRNA.

Effect of c^3Gua incorporation on poly A$^+$ mRNA function. The results of *in vitro* translation of L1210 cell mRNAs are shown in Fig. 2. mRNA from control cells stimulated *in vitro* incorporation of [^{3}H]leucine into protein in a dose-dependent manner over a range of 0.1 to 0.4 μg mRNA per reaction. Maximal ^{3}H incorporation was nine times greater than background levels. mRNA from cells incubated for 24 hr with 30 μM c^3Gua had only 64% of the activity of control mRNA in stimulating protein synthesis. Significant diminution in activity also could be demonstrated for several of the samples of mRNA from cells incubated for 24 hr with 10 μM c^3Gua.

Discussion

The actions of c^3Gua on DNA synthesis and protein

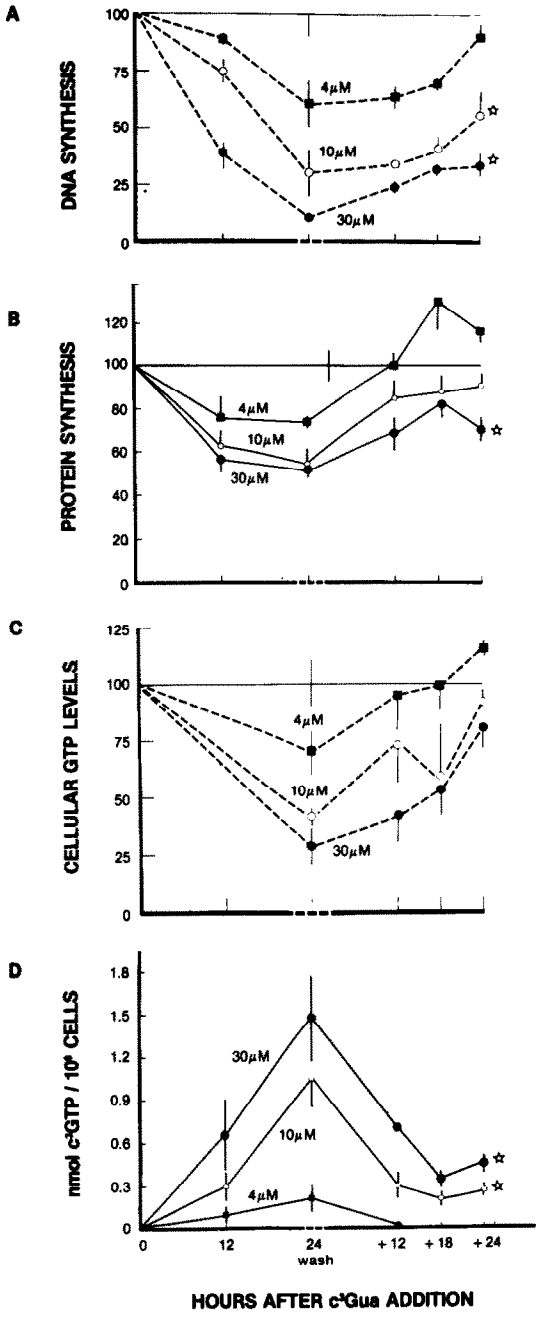

Fig. 1. Recoveries of L1210 cells from c^3Gua-induced inhibition of DNA synthesis and protein synthesis, GTP depletion and c^3GTP accumulation. Levels of DNA (A) and protein (B) synthesis, intracellular GTP (C), and intracellular c^3GTP (D) (expressed as percent of control except for c^3GTP levels in nmol/10^6 cells) were determined in cultures incubated with 0, 4 (■), 10 (○), or 30 (●) μM c^3Gua during 24 hr of incubation with the drug, and 12, 18 and 24 hr after removal of the drug from the medium. Each point represents the mean ± SE of three to four experiments performed in duplicate. Asterisks at 24-hr points indicate values significantly different ($P < 0.05$, Student's t-test) from control values. Typical control values were 812 and 34 dpm/10^3 cells for DNA and protein synthesis respectively; levels of GTP in control cells were 0.56 ± 0.05 nmol/10^6 cells.

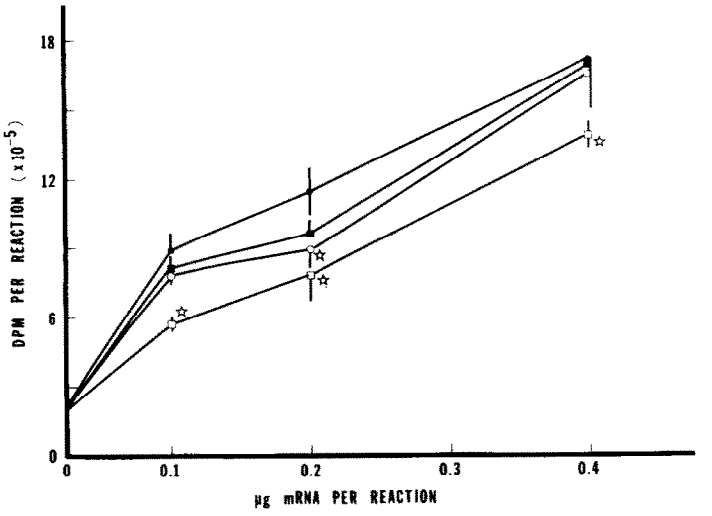

Fig. 2. *In vitro* translational activity of mRNA from c^{3}Gua-treated cells. L1210 cultures were incubated with 0 (●), 4 (■), 10 (○), or 30 (□) μM c^{3}Gua for 24 hr. Poly A$^+$ RNA was then isolated from each culture as described. mRNA (0.1 to 0.4 μg) from each group was added to an *in vitro* translation system containing [^{3}H]leucine in a final reaction volume of 30 μl. Acid-precipitable ^{3}H-dpm were assessed and used as a measure of mRNA activity. Each point is the mean ± SE (bars) of three to four experiments performed in triplicate. Asterisks indicate values significantly different ($P < 0.05$, Student's *t*-test) from control values.

synthesis in L1210 cells, and their association to c^{3}Gua-induced cytotoxicity have been well established [2, 3]. Although the effects of c^{3}Gua on DNA and protein synthesis have been linked to incorporation of c^{3}Gua into DNA [2], and inhibition of translation [3], respectively, other actions of c^{3}Gua such as accumulation of c^{3}GTP, depletion of GTP pools (probably via inhibition of IMP dehydrogenase [9]) and incorporation of c^{3}Gua into RNA may also be involved. The present study was an attempt to assess the possible interrelationships between c^{3}Gua-induced effects on GTP and c^{3}GTP pool sizes, inhibition of DNA and protein synthesis, and cytotoxicity.

Although it was impossible to dissociate the various drug effects previously reported for cells growing in the presence of inhibitory concentrations of c^{3}Gua, differing rates of recoveries of these effects following drug removal permitted several such dissections.

A 24-hr exposure to 30 μM c^{3}Gua inhibited DNA synthesis, and to a lesser extent, protein synthesis even 24 hr after removal of the drug from the medium. These persistent effects of c^{3}Gua stand in contrast to the rapid repletion of intracellular GTP pools and the elimination of c^{3}GTP accumulation following c^{3}Gua removal. Of note also is that L1210 cells incubated with c^{3}Gua for 24 hr and then allowed to recover for up to 24 hr in drug-free medium showed only a very small but significant increase in viability relative to cells allowed no recovery time.

The incomplete recovery of c^{3}Gua-treated cells from inhibition of DNA synthesis, as well as from cytotoxicity within 24 hr of drug removal, suggests that these two effects may be closely linked. Although persistent inhibition of protein synthesis can also be demonstrated in cells allowed 24 hr to recover from 30 μ c^{3}Gua treatment, at lower drug concentrations recovery of protein synthesis was virtually complete, like that of GTP levels and the loss of c^{3}GTP. This suggests that the altered nucleoside phosphate levels may play an important role in the effect of the drug on protein synthesis.

The diminished ability of c^{3}Gua-containing mRNA to stimulate protein synthesis *in vitro* suggests that incorporation of c^{3}Gua into mRNA may be an additional mechanism by which c^{3}Gua inhibits protein synthesis. A decrease in the ability of analog-containing mRNA to be translated *in vitro* has been associated with decreased protein synthesis and cytotoxicity with at least two other purine analogs [8, 10]. Decreased translation *in vitro* could be explained by a generalized slowing of translation of c^{3}Gua-containing mRNA, perhaps due to a conformational change in the mRNA, as has been suggested for 8-azaguanine [10]. Another possibility, however, is that c^{3}Gua-containing mRNA may be translated less efficiently *in vitro* because it binds poorly to ribosomal initiation complexes, perhaps due to an altered 5′-c^{3}Gua-containing cap structure [11].

This study has permitted some analysis of the multiple biochemical effects resulting from the actions of c^{3}Gua on L1210 cells. Drug removal following growth inhibition led to virtually complete recovery of GTP levels and the loss of c^{3}GTP, together with an essentially normal rate of protein synthesis in cells previously exposed to the lower c^{3}Gua concentration. In contrast, recoveries of DNA synthesis and cytotoxicity were extremely limited, implying a close linkage between these two drug effects. c^{3}Gua was preferably incorporated into mRNA leading to impaired translation into protein which may also contribute to the analog's inhibition of protein synthesis.

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