Mechanism of Action of Granaticin: Inhibition of Ribosomal RNA Maturation and Cell Cycle Specificity

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Abstract □ Granaticin, an antibiotic produced by *Streptomyces* species was found to be cytotoxic (ED_{50} 3.2 μg/ml) against human oral epidermoid carcinoma (KB) cells. At ED_{50} concentrations RNA synthesis was inhibited to the greatest extent. Prelabeling of RNA in KB cells, followed by addition of granaticin (2.13 μg/ml) showed that ribosomal RNA maturation was inhibited. The inhibition of the formation of functional ribosomal RNA was determined by sucrose gradient centrifugation and showed that the accumulation of 45S preribosomal RNA was dependent on granaticin concentration and on the time granaticin was in contact with the KB cells. The effect of granaticin (6.3 μg/ml) on KB cells in the different cell cycle phases showed preferential inhibition (93%) of cell survival in the G_2 phase. However, RNA synthesis was only 20% inhibited by granaticin in KB cells in the G_2 phase. From these results, it was concluded that ribosomal RNA maturation was not the only site of action of granaticin toxicity.

Keyphrases □ Granaticin—cytotoxicity, mechanism of action, in human oral epidermoid carcinoma cells □ Cytotoxicity—granaticin, mechanism of action, in human epidermoid carcinoma cells □ Anticancer agents—granaticin, mechanism of action

Granaticin, an antibiotic produced by the *Streptomyces* species was first reported (1) as a fermentation product of *Streptomyces olivaceus*. Its structure was clarified by Keller-Schierlein *et al.* (2). Subsequently, granaticin or its L-rhodinoside, granaticin B, was isolated from *S. violaceoruber* (3), *S. litmogenes* (4), and *S. thermoviolaceus* (5). Granaticin was active against Gram-positive bacteria (6) and showed anticancer activity against P-388 lymphocytic leukemia in mice and cytotoxicity against human oral epidermoid carcinoma (KB) cells (7).

BACKGROUND

Granaticin was reported to inhibit RNA synthesis in bacteria (8). This inhibition was originally attributed to the interaction of granaticin with the DNA template. More recently, the mechanism of action of granaticin in bacteria has been attributed to the inhibition of aminoacylation of leucyl-tRNA by interaction with an aminoacyl-tRNA synthetase (9). It was not determined whether or not this inhibition was due to binding of granaticin to tRNA. Another report (10) showed that granaticin inhibited viral RNA-dependent DNA polymerase isolated from RNA tumor viruses, *i.e.*, Rauscher murine leukemia virus, simian sarcoma virus type 1, and avian myeloblastosis virus. From the sequential addition of granaticin to the reaction, it was proposed that granaticin interacts with the template (RNA) and not the reverse transcriptase. However, direct binding of granaticin to templates was not shown.

Since these results indicated an inhibitory action of granaticin on RNA metabolism, the mechanism of action of granaticin in mammalian cells was studied. In the present study, the effect of granaticin on the formation of functional ribosomal RNA in KB cells, on the uptake of uridine by KB cells and its subsequent phosphorylation, and on the cell cycle traverse of KB cells was investigated.

MATERIAL AND METHODS

Stock solutions of granaticin A[1] were prepared by dissolving 3 mg of granaticin in 0.03 ml of dimethyl sulfoxide and diluting to 10 ml with basal salt solution (11), pH 7.2.

Human oral epidermoid carcinoma cells[2] (KB cells) were grown in monolayer cultures with basal medium supplemented with 10% heat denatured (57°) fetal calf serum containing 100 U of penicillin and 100 g of streptomycin/ml (12, 13). Cells were maintained at 37° in an atmosphere of 5% CO_2 in air and subcultured every 2–3 days. For experiments, cells were detached from the glass support with trypsin–ethylenediaminetetraacetic acid[3], collected by centrifugation, washed and suspended in fresh medium, plated at a density of 5×10^4–1×10^5 cells/ml, and incubated for 3 hr at 37° to allow attachment to the support before an experiment was initiated.

Macromolecular synthesis was measured by the addition of [^{3}H]thymidine[4] (1 μCi/ml cells; 26.1 μCi/μmole) for DNA synthesis, [^{3}H]uridine[4] (1 μCi/ml cells; 22 μCi/μmole) for RNA synthesis, and [^{3}H]lysine[4] (1 μCi/ml cells; 50.4 μCi/μmole) for protein synthesis. Cells were disrupted and the macromolecules were precipitated with an equal volume of ice cold 10% trichloroacetic acid in 0.1 *M* pyrophosphate. The precipitates were collected on glass fiber filters[5], washed three times with trichloroacetic acid in 0.01 *M* pyrophosphate and two times with 0.1 *N* hydrochloric acid, and their radioactivity was determined.

Cell populations synchronized in the S-phase were obtained by a double-thymidine blockade (14–18). KB cells in the different phases were then treated with granaticin and RNA synthesis was measured through the incorporation of [^{3}H]uridine into acid-insoluble fractions. Cell survival after treatment with granaticin (6.2 μg/ml) for 1 hr in different phases of the cell cycle was measured by suspending washed cells in fresh medium. The cell suspension was diluted 1:1000, plated into plastic dishes, and incubated at 37° for 7 days. The number of colonies was determined under a microscope after staining with Giemsa.

The labeling, extraction, and isolation of nucleolar and cytoplasmic ribonucleic acid was described previously (19). KB cells at a concentration of 3.3×10^6 cells/ml were incubated with 1 μCi/ml [^{3}H]uridine (22 μCi/μmole) and 30 ml of medium. Identical incubations contained 2–5 μg of granaticin/ml of medium. Cells were harvested after 1–4 hr at 37°.

The uptake of [^{3}H]uridine and its subsequent conversion to uridine monophosphate, uridine diphosphate, and uridine triphosphate was measured by incubating 1.6–2.4×10^5 KB cells/ml with 1.1 μCi/ml

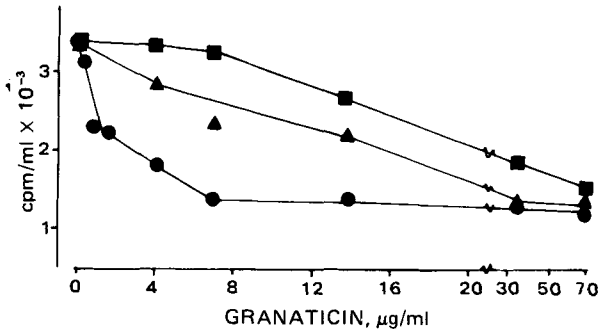

Figure 1—*Effect of granaticin on macromolecular synthesis. KB cells at a concentration of 1×10^5 cells/ml were plated 3 hrs before granaticin was added. DNA synthesis (■) was measured by the incorporation of [^{3}H]thymidine, RNA synthesis (●) was measured by the incorporation of [^{3}H]uridine, and protein synthesis (▲) was measured by the incorporation of [^{3}H]lysine. Granaticin was added 10 min before the radioactive precursor was added. The amount of acid-precipitated radioactivity was measured after the cells were in contact with granaticin for 1 hr.*

[1] Dr. H. G. Floss, Department of Medicinal Chemistry and Pharmacognosy, Purdue University.

[2] American Type Culture Collection (ATCC CL17).
[3] Grand Island Biological Co., Grand Island, N.Y.
[4] Amersham/Searle Corp., Arlington Heights, Ill.
[5] Grade GF/A, Whatman Inc.

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Table I—Effect of Granaticin on KB Cell Growth

Granaticin[a], μg/ml	Cells/ml × 10^{-5} [b] 24 hr	48 hr	72 hr
0	3.3	4.6	9.3
1.4	3.2	4.0	7.5
6.9	3.0	3.6	5.7
13.8	2.8	2.6	2.4
34.4	0.6	0.7	0.8

[a] Granaticin at the indicated concentrations remained in contact with the cells for 2 hr. The cells were then washed and incubated with fresh medium for 24, 48, and 72 hr. The number of cells remaining were determined by counting an aliquot in an eosinophil counter. Values are the average of three experiments.
[b] Cells were seeded at a concentration of 5 × 10^4 cells/ml 3 hr before granaticin was added.

[^{3}H]uridine (22 μCi/μmole) and with and without 1–2.75 μg of granaticin/ml. Aliquots were removed immediately after the addition of [^{3}H]-uridine and after 2 and 4 hr. The reaction was stopped with an equal volume of ice cold 10% trichloroacetic acid in 0.1 *M* pyrophosphate. The acid soluble fractions were analyzed by TLC followed by scintillation

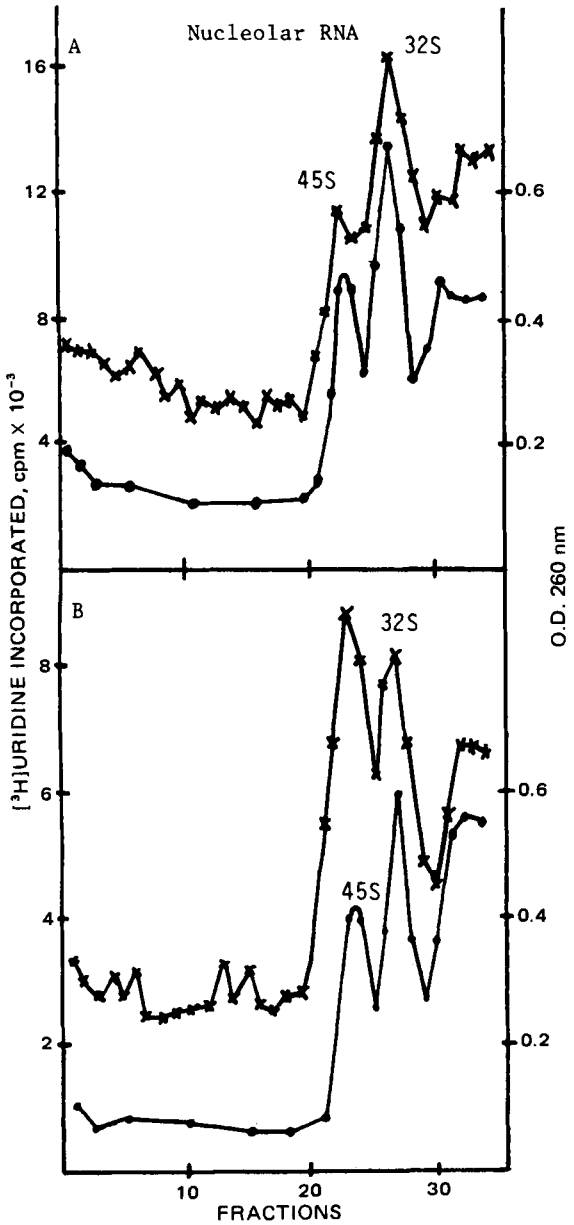

Figure 2—*Sucrose gradient analysis of nuceolar RNA from KB cells. RNA was labeled by the exposure of a total of 1 × 10^8 cells (3 × 10^6 cells/ml) for 1 hr to [^{3}H]uridine in the absence (A) or presence (B) of granaticin (2.13 μg/ml). Fractions were collected through a needle from 15–30% gradients and centrifuged for 12 hr at 20 × 10^3 rpm in an SW 40 rotor.*

counting (20) after the addition of cold uridine mono-, di-, and triphosphate[6] or by the method of Cheung and Suhadolnik (21).

Radioactivity was measured in a liquid scintillation spectrometer[7] in 10 ml of toluene scintillation cocktail or commercial scintillation solution[8] for water-soluble samples.

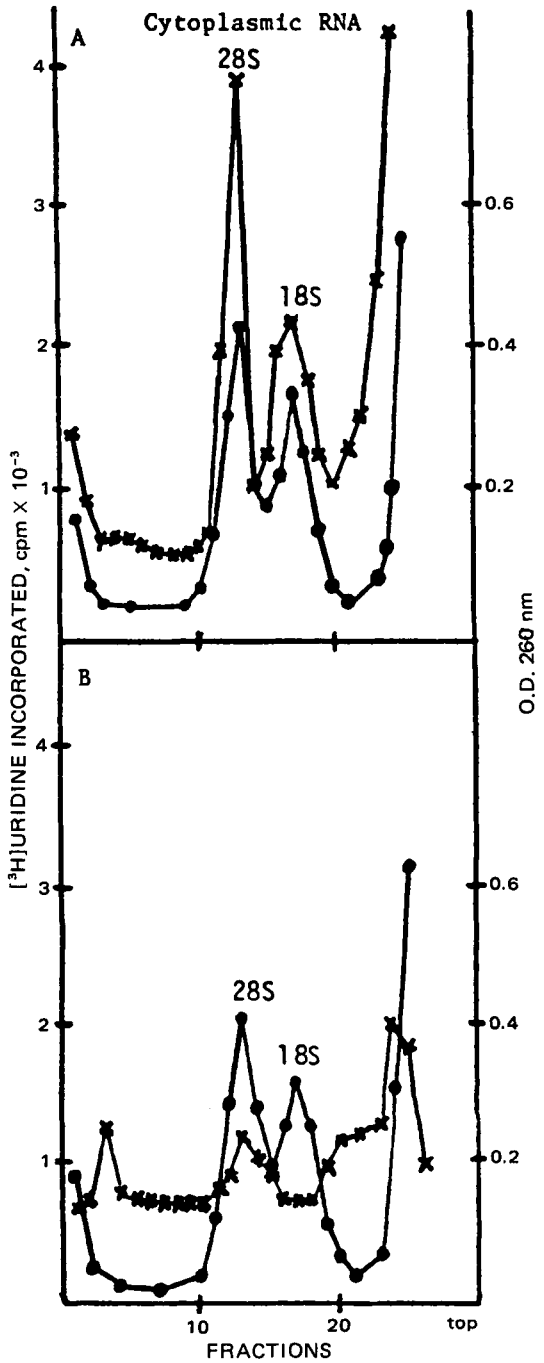

Figure 3—*Sucrose gradient analysis of cytoplasmic RNA from KB cells. Experimental condition was identical to the conditions in Fig. 2, except that the 15–30% sucrose gradients were centrifuged for 16 hr at 22 × 10^3 rpm. (A, control incubation; and B, 2.13 μg of granaticin/ml).*

RESULTS

To determine a concentration of granaticin that would allow study of the inhibition of cellular processes without excessive cell death, the effect of granaticin on cell survival was measured (Table I). Granaticin at a concentration of 1.4 mg/ml for 2 hr caused a 3% reduction in cell numbers

[6] Sigma Chemical Co., St. Louis, Mo.
[7] Beckman Instrument Co. model LS-250.
[8] Aquasol, New England Nuclear, Boston, Mass.

Table II—Effect of Granaticin Concentration and Contact Time on the Maturation of Ribosomal RNA in KB Cells [a]

Granaticin, μg/ml of medium	Incubation, hr	RNA Percent of control [b] 45S	32S	28S	18S
2.16	1	94	48	59	5
2.16	2	86	32	9	0
3.20	1	79	32	42	0
3.20	2	75	11	6	0
5.00	1	53	0	0	0

[a] Cells were seeded at a concentration of 3×10^6 cells/ml and treated with different concentrations of granaticin for 1–2 hr. [b] The radioactivity associated with the 45S, 32S, 28S, and 18S fractions was determined and is expressed as percentage of the radioactivity in control incubations without granaticin, analyzed after 1 and 2 hr.

after 24 hr compared to incubations without granaticin. In addition, the damage to the cells appeared to be permanent, since the reduction in cell number was 14 and 19% after 48 and 72 hr, respectively, at the same granaticin concentration. The cytotoxic effect of granaticin was more pronounced at higher concentrations of the inhibitor (Table I). From these results, concentration of 2 μg of granaticin/ml of cells (in some experiments up to 5 μg/ml) were used for subsequent experiments. In addition, the exposure time of the cells to granaticin was 1–2 hr to minimize cell death.

Synthesis of macromolecules was monitored by measuring the incorporation of radioactively labeled thymidine, uridine, and lysine into acid-insoluble fractions. Addition of granaticin at various concentrations, 10 min before the radioactive precursor was added to the cell cultures, showed (Fig. 1) that the formation of ribonucleic acid was inhibited 50% at a granaticin concentration of ~2 μg/ml. At this same granaticin concentration, DNA and protein synthesis were not appreciably affected.

The ribonucleic acids synthesized in the presence and absence of granaticin were analyzed by differential separation using sucrose gradient centrifugation of the cytoplasmic and nucleolar ribonucleic acids as described previously (19). The results (Figs. 2 and 3) indicated an accumulation of 45S RNA in the nucleoli of granaticin-treated cells (Fig. 3B) compared to untreated control cells (Fig. 2A). Similarly, virtually no 18S and very little 28S RNA apeared in the cytoplasm of granaticin-treated cells (Fig. 3B) compared with untreated cells (Fig. 3A). These results were obtained after 1-hr exposure of the cells to [^{3}H]uridine in the presence or absence of granaticin (2.13 μg/ml/hr). In similar experiments (Table II), KB cells were labeled with [^{3}H]uridine and exposed to granaticin

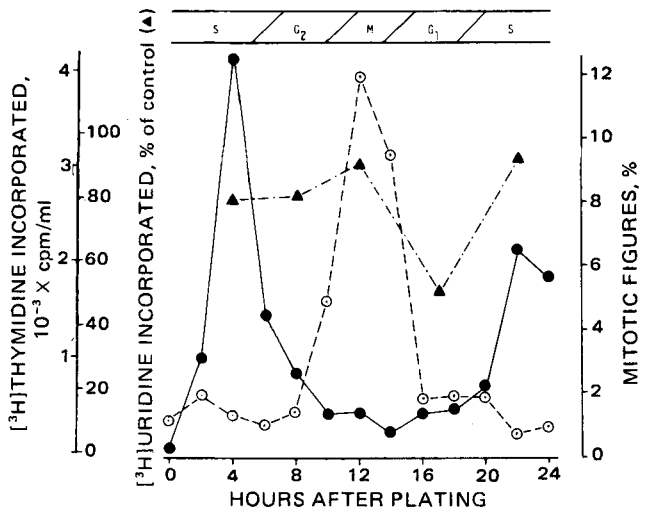

Figure 4—*Traverse of KB cells through the cell cycle after a double-thymidine block. The thymidine was removed at zero hr after plating. Cell-cycle phases were identified through the incorporation of [^{3}H]-thymidine into DNA (●) and through counting of the mitotic figures (⊙) after staining with hematoxylin. The effect of granaticin (6.3 μg/ml for 30 min) on RNA synthesis in KB cells in different phases of the cell cycle was measured by the incorporation of [^{3}H]uridine (▲) (0.4 μCi/ml) reported as percentage of control incubations. Typical values for control incorporations of [^{3}H]uridine into RNA were: S-phase, 780 cpm/mg of protein; G_2-phase, 450 cpm/mg of protein; M-phase 120 cpm/mg of protein; and G_1-phase, 1320 cpm/mg of protein. Each point represents the average of three measurements.*

Table III—Effect of Granaticin on the Uptake and Conversion of [^{3}H]Uridine into Uridine Monophosphate, Uridine Diphosphate, and Uridine Triphosphate [a]

Granaticin, μg/ml	Incubation, hr	Uridine, %	Uridine Monophosphate, %	Uridine Diphosphate, %	Uridine Triphosphate, %
0	0	87.9	6.0	4.0	2.1
0	2	74.0	12.2	10.5	3.3
0	4	72.0	11.4	12.2	4.7
2.75	0	84.7	7.2	5.1	3.0
2.75	2	74.0	11.0	11.3	3.7
2.75	4	70.9	12.9	11.6	4.4

[a] [^{3}H]Uridine (1.1 μCi/ml, 22 μCi/μmole) was added to 1.6–2.4 × 10^5 KB cells/ml and incubated with and without 2.75 μg of granaticin/ml for the time indicated. Separation of uridine and its mono-, di-, and triphosphate ester was by TLC.

concentrations of 3.2 and 5 μg/ml for 1 and 2 hr. The results extend and support the data in Figs. 2 and 3.

Summation of the radioactivity associated with the sucrose gradient fractions representing 45S, 32S, 28S, and 18S RNA, in the control incubation and in incubations of KB cells with granaticin, showed that higher granaticin concentrations (3.2 and 5 μg/ml) have a more drastic effect on the maturation of ribosomal RNA than incubations with 2.16 μg of granaticin/ml of medium (Table II). In addition to granaticin concentration, the incubation time of granaticin with KB cells affected maturation of ribosomal RNA. After a 2-hr incubation at 2.16 or 3.2 μg of granaticin/ml of medium, the inhibition of the formation of functional ribosomal RNA was more pronounced than inhibition after a 1-hr incubation (Table II). At 5 μg of granaticin/ml of medium, no radioactivity was found in 32S, 28S, and 18S ribosomal RNA after a 1-hr incubation (Table II). In all experiments where contact time or granaticin concentration was varied, the maturation of 45S RNA was inhibited to a greater extent than the synthesis of 45S RNA (Table II).

No inhibitory effect of granaticin at a concentration of 2.75 μg/ml for 2 or 4 hr was observed on intracellular accumulation of [^{3}H]uridine and on the subsequent conversion of [^{3}H]uridine to uridine monophosphate, uridine diphosphate, and uridine triphosphate (Table III).

Possible cell cycle specificity of granaticin was investigated in synchronized KB cells. The degree of synchronization was determined by [^{3}H]thymidine incorporation and by counting mitotic figures. A typical profile is reproduced in Fig. 4. Synchronized KB cultures were used to measure [^{3}H]uridine incorporation in the different cell-cycle phases in the presence and absence of granaticin (Fig. 4). Similarly synchronized cell cultures were used to measure the cell survival after treatment with granaticin in the different cell-cycle phases (Fig. 5). [^{3}H]Uridine incor-

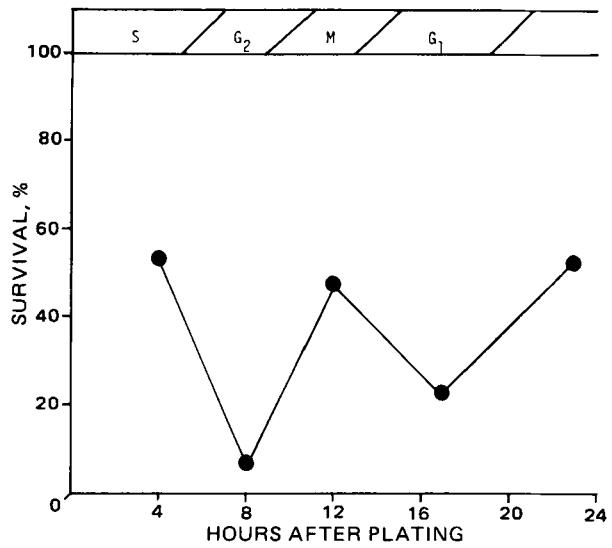

Figure 5—*Effect of granaticin on the survival of synchronized KB cells. Synchronized S-phase KB cells were incubated for 1 hr with 6.3 μg/ml granaticin at 4, 8, 12, 17, and 23 hr after removal of the thymidine block, diluted 1:1000, and grown in monolayers for 7 days. Thereafter, stained colonies were counted and represented as percentage of control incubations without granaticin. Each point represents the average of three measurements.*

poration was inhibited 50% by the addition of granaticin to cells in the G_1-phase (Fig. 4). Little or no effect of granaticin on RNA synthesis was observed in the mitotic and early S-phase and only minimal inhibition (20%) in the late S- and G_2-phases (Fig. 4). However, the effect of granaticin on the cell survival in the different phases was much more pronounced. Almost no cells survived in the G_2-phase (Fig. 5). Similarly, only 25% of the G_1-phase treated cells survived. Survival in the S- and M-phases was ~50% (Fig. 5).

DISCUSSION

In previous studies with bacterial systems granaticin inhibited RNA synthesis *in vivo* (8, 9) and in cell-free incubations (22). In addition, granaticin inhibited reverse transcriptase (10). In both cases, interaction of granaticin with the template was proposed. Although the most recent result with *Bacillus subtilis* (9) indicated that granaticin inhibition was due to the failure to charge leucyl-tRNA, it was not clear if granaticin exerted its inhibitory properties through binding to the tRNA molecule or to the proteins involved in this process. Inhibition of the reverse transcriptase was attributed to the interaction of granaticin with the template (RNA) for this enzyme (10). This conclusion was drawn from sequential addition experiments and, therefore, does not prove interaction of granaticin with the template.

The present results show that RNA synthesis is affected by granaticin at concentrations where protein DNA synthesis is not inhibited. Subsequent analysis of the synthesized RNA showed that maturation of the 45S ribosomal RNA precursor is inhibited. This inhibition was dependent on the granaticin concentration in the incubation mixtures as well as on the duration of incubation. These results may indicate that granaticin binds to 45S RNA and therefore maturation cannot occur. However, similar results would have been obtained if granaticin interacted with one or more of the enzymes (23) involved in the processing of 45S RNA to functional ribosomal RNA.

An indication that granaticin at higher concentrations interacts with other vital processes in mammalian cells was apparent from a comparison of the results of the cell cycle inhibition experiments (Figs. 4 and 5). If inhibition of RNA synthesis was the only site of action of granaticin, then the inhibition of RNA synthesis (Fig. 4) and cell survival (Fig. 5) should have yielded comparable values. Inhibition of RNA synthesis by granaticin in cells in the different phases of the cell cycle was not of the same magnitude as the inhibition of cell growth in the same phases. The magnitude of inhibition in each phase should have been the same if inhibition of RNA synthesis was the only site of action of granaticin toxicity. This type of inhibition was not found. Although 7% of the granaticin-treated G_2-cells did survive (Fig. 5), only 18% inhibition of RNA synthesis was observed in cells treated with granaticin in the same phase (Fig. 4). It should be emphasized that these experiments were done with a granaticin concentration of 6.3 μg/ml. At concentrations of 2 μg/ml, comparable to the inhibition studies in Figs. 2 and 3, this difference in cell survival and inhibition of RNA formation was not apparent.

A number of other compounds inhibit maturation of ribosomal RNA (24). The compounds studied in greatest detail are nucleoside analogs which, in addition to inhibiting the processing of ribosomal RNA, also inhibited *de novo* precursor synthesis (24). Although granaticin cannot be classified as a purine or pyrimidine analog and, therefore, should not inhibit *de novo* nucleoside synthesis, the effect of granaticin on the uptake and phosphorylation of [^{3}H]uridine to the corresponding nucleotides was tested and found to be negative. Camptothecin inhibited ribosomal RNA synthesis (25); however, in this case it appeared to inhibit transcription and not the maturation of preribosomal RNA.

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