The Interaction of Granaticin with Nucleic Acids and Pyruvate Decarboxylase

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Abstract □ The interaction of granaticin with two different yeast ribonucleic acids, dialyzed transfer RNA and calf thymus DNA was studied. Contrary to previous reports, no binding of granaticin to DNA or RNA was observed. The visible spectrum of granaticin on direct mixing or equilibrium dialysis of granaticin with RNA or DNA was unchanged. Furthermore, granaticin did not displace acridine orange from DNA in competitive binding studies using fluorescence polarization. However, granaticin was shown to inhibit pyruvate decarboxylase. From the K_i for granaticin (3.8 m*M*) it was concluded that granaticin is as efficient as other 1,4-naphthoquinones in inhibiting pyruvate decarboxylase.

Keyphrases □ Granaticin A—absence of interaction with DNA and RNA, interaction with proteins from the inhibition of pyruvate decarboxylase □ DNA—interaction with granaticin A □ RNA—interaction with granaticin A □ Pyruvate decarboxylase—inhibition by granaticin A in the interaction between granaticin A and protein

Granaticin, isolated from *Streptomyces olivaceus* (1), is a quinone antibiotic which is active against Gram-positive bacteria (2). Also known as litmomycin (3), granaticin has been studied for its interaction with various biochemical species. Previous work proposed that granaticin (*a*) interferes with the charging process of transfer RNA, thereby inhibiting the synthesis of leucyl transfer RNA (4), (*b*) interferes with leucyl transfer RNA synthetase (5), (*c*) inhibits the synthesis of RNA or the function of RNA polymerase, which is not DNA dependent (6), (*d*) inhibits reverse transcriptase by binding to the template RNA (7), and (*e*) appears to inhibit maturation of ribosomal RNA (8). These biological effects of granaticin could be due to the interaction of granaticin with template macromolecules (*i.e.*, DNA and RNA) or with catalytic macromolecules, (*i.e.* proteins).

The inhibition of the reverse transcriptase was attributed to the interaction of granaticin with the RNA template (7). Inhibition of leucyl transfer RNA formation (4) and maturation of RNA may have been due to binding of granaticin to the RNA or to the reaction of granaticin with a protein factor required in the formation of RNA (8).

The purpose of this study was to investigate the binding of granaticin to two different RNA preparations and DNA and to measure the effect of granaticin on enzyme activity.

EXPERIMENTAL

Binding Studies—A stock solution of granaticin A[1] (27.9 μM) was prepared in 0.005 *M* phosphate buffer, pH 7.2, by stirring in the dark for 3 days at 2–37°. The solution was gravity filtered before use. Yeast transfer RNA type III (19.0 mg)[2] (9) was dissolved in 250 ml of 0.005 *M* phosphate buffer, pH 7.2. A fixed amount of granaticin stock solution (15 ml) was directly mixed with varying amounts of RNA solution (2, 5, 8, 10, 15, and 20 ml) and diluted to 100 ml with phosphate buffer. Visible spectra of these samples were recorded[3] scanning from 625–460 nm. The baseline was established by using phosphate buffer in both the sample and reference cells. Control absorption curves were generated using granaticin stock solution (15 ml) diluted to 100 ml with phosphate buffer and RNA solution (2–20 ml, as described above) diluted to 100 ml with phosphate buffer. The same procedure was used to test the interaction of granaticin with RNA type XI[2] (a mixture of messenger and ribosomal RNA) and calf thymus DNA[2].

In a slightly different experimental approach, 1 and 2 ml of an RNA solution (type XI and type III)[2] containing 20 mg of RNA in 10 ml of 0.005 *M* phosphate buffer, pH 7.2, was placed in a dialysis bag[4], sealed, and immersed in 50 ml of a granaticin solution (27.9 μM) in phosphate buffer. The solution was stirred in the dark for 72 hr at 2–37°. Thereafter, visible spectra (625–460 nm) of the RNA–granaticin solution inside the dialysis bag and the granaticin solution outside the dialysis bag were recorded[3]. In control incubations, phosphate buffer replaced the RNA solutions inside the dialysis bag, and in a second incubation the granaticin solution outside the dialysis tubing was replaced with phosphate buffer. Controls were treated the same as the samples.

Competitive Binding Studies[5]—Granaticin, 0.025–25 μg/ml in 0.01 *M* cacodylate[2] buffer, pH 6.7, was used in the competitive binding assay inhibiting the binding of acridine orange[2] to calf thymus DNA type I[2]. This binding is accompanied by an increase in fluorescence polarization. Granaticin and acridine orange (100 μl of a 10-μM solution) were mixed in a cell, and the initial fluorescence polarization (P_0) was measured[6] using excitation and emission filters at 492 and 520 nm, respectively. DNA (equivalent to 35% saturation) was then added to the cell and the final polarization (P) was determined after mixing. Results were expressed as a percentage of maximum binding; maximum binding was expressed as the P–P_0 obtained without granaticin (11).

Enzyme Assays—Pyruvate decarboxylase[2] (2-oxo acid carboxyl-lyase, EC 4.1.1.1, 13 U/mg protein) was assayed in a coupled assay with alcohol dehydrogenase[2] (alcohol:NAD$^+$ oxidoreductase EC 1.1.1.1, 300 U/mg protein) by measuring the oxidation of reduced nadide[2] at 340 nm (12). A typical incubation mixture contained reduced nadide (1.2 μmoles), alcohol dehydrogenase (0.3 U), pyruvate[2] (0–150 μmoles), pyruvate decarboxylase (0.29 U), and granaticin (0, 0.33, 0.42, and 0.52 μmoles) in a total volume of 3.0 ml of 0.2 *M* citrate[2] buffer, pH 6.0. Granaticin was dissolved in 0.2 *M* citrate buffer, pH 6.0 by stirring in the dark for 4 days at 2°. The reagents, except pyruvate decarboxylase, were mixed in a cell and the base line was recorded from 300–380 nm. The spectrophotometer[3] was then set at 340 nm and pyruvate decarboxylase was added, rapidly mixed, and the change in absorbance at 340 nm recorded. Temperature was maintained at 30°.

To ensure that the observed effect of granaticin in the enzyme assay was not due to the inhibition of alcohol dehydrogenase by granaticin, this enzyme was assayed with or without granaticin and without pyruvate decarboxylase. The reaction mixture contained reduced nadide (1.2 μmoles), acetaldehyde[2] (0–114 μmoles), granaticin (0, 0.33, 0.42 and 0.52 μmole, solution prepared as described previously), and alcohol dehydrogenase (0.3 U) in a total volume of 3.0 ml and 0.2 *M* citrate buffer, pH 6.0. The reagents, except the enzyme, were mixed in a cell and the base line recorded from 300–380 nm. The reaction was started by adding alcohol dehydrogenase and the change in absorbance at 340 nm was recorded. Temperature was maintained at 30°.

RESULTS AND DISCUSSION

It was proposed that granaticin binds to transfer RNA (4) and to the template RNA in the reverse transcriptase assay (7); however, these results were based on indirect observations. Therefore, binding of granaticin

[1] Granaticin A was a gift of Dr. H. G. Floss, Department of Medicinal Chemistry and Pharmacognosy, Purdue University.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Cary model 17 recording spectrophotometer.
[4] Fisher Scientific Co., Spectra/Por 1 Membranes: 6000 to 8000 MWCO.
[5] Analysis was performed by Dr. Carol L. Richardson, Principal Scientist, Meloy Laboratories, Inc., Springfield, Va.
[6] Model Fluoro I, Meloy Laboratories, Inc., Springfield, Va.

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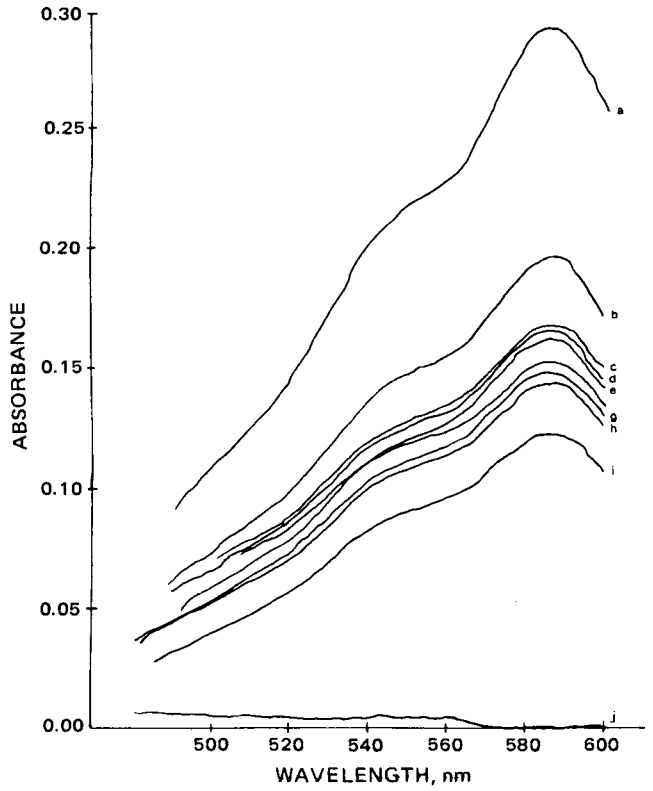

Figure 1—*Visible spectrum of granaticin and RNA type III in a direct mixing experiment. Key: a, 15 ml granaticin + 85 ml buffer; b, 10 ml granaticin + 90 ml buffer; c, 10 ml granaticin + 2 ml RNA type III + 88 ml buffer; d, 10 ml granaticin + 7 ml RNA type III + 83 ml buffer and 10 ml granaticin + 10 ml RNA type III + 80 ml buffer; e, 10 ml granaticin + 5 ml RNA type III + 85 ml buffer; f, 10 ml granaticin + 30 ml RNA type III + 60 ml buffer; g, 10 ml granaticin + 15 ml RNA type III + 75 ml buffer and 10 ml granaticin + 25 ml RNA type III + 65 ml buffer; h, 10 ml granaticin + 20 ml RNA type III + 70 ml buffer; i, 7 ml granaticin + 93 ml buffer; j, buffer only.*

to yeast transfer RNA type III and a mixture of ribosomal RNA and messenger RNA type XI through direct addition and dialysis experiments was attempted. In neither experiment did the absorption spectrum of granaticin exhibit any changes on addition of the RNA. Part of the visible absorption spectrum of granaticin is shown in Fig. 1 (curve a). Neither the peak at 580 nm, nor the shoulder at 550 nm in the granaticin spectrum were changed significantly on addition of RNA. The same results were obtained when granaticin and RNA were mixed or dialyzed for up to 3 days at temperatures that varied from 2 to 37° in different experiments. These results indicate that granaticin does not bind to RNA molecules *in vitro*.

In identical experiments where calf thymus DNA was substituted for RNA, no *in vitro* binding of granaticin to DNA was observed.

The inability of granaticin to interact *in vitro* with nucleic acids is supported by the results of the competitive binding assay (11). Granaticin showed no evidence of displacement of acridine orange from DNA.

Since the inhibitory action of granaticin on reverse transcriptase (7) and on the charging of transfer RNA (4) might be due to the interaction with proteins involved in these processes, and no interaction between RNA or DNA with granaticin was observed, the possibility that granaticin binds to protein (potential nucleophilic reactants) was investigated. The enzyme selected for these experiments was pyruvate decarboxylase, since compounds similar in structure to granaticin, naphthoquinone, and anthraquinone, inhibit this enzyme (13). Pyruvate decarboxylase was assayed at optimum conditions (12) and was coupled to the reduction of acetaldehyde, the product of the pyruvate decarboxylase reaction, by alcohol dehydrogenase resulting in the oxidation of reduced nadide.

Lineweaver–Burk plots ($1/V$ *versus* $1/[S]$) yielded parallel lines which indicated that granaticin is an uncompetitive inhibitor of pyruvate decarboxylase. In a control experiment where only alcohol dehydrogenase was assayed, granaticin had no effect on the enzyme. The K_i for granaticin was calculated to be 3.8 mM. Granaticin is, therefore, as good an inhibitor of pyruvate decarboxylase as 1,4-naphthoquinone (k_i = 4.2 mM) (12), whereas anthraquinone, at twice the concentration, did not inhibit pyruvate decarboxylase (13). Interestingly, granaticin had a half-wave potential of −0.685v (14) compared with a half-wave potential of −0.603v for naphthoquinone (15), but different from the half-wave potential of −0.85v for anthraquinone (16). These values would indicate a bioreductive alkylation (17) of a nucleophilic center in a protein. Additional experiments are required to prove this hypothesis.

It appears that at least one of the inhibiting actions of granaticin on living cells is the interaction of granaticin with proteins and not with RNA or DNA as reported previously (4, 7). However, it should be emphasized that the experimental procedures used in the present report utilized direct binding studies, whereas previous reports (4, 7) used indirect biochemical measurements to conclude that granaticin interacted with RNA and DNA.

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