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MODE OF ACTION OF THE ANTITUMOR COMPOUND GIRODAZOLE (RP 49532A, NSC 627434)

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Abstract—Girodazole (RP 49532A) or 3-amino-1-[4-(2 amino-1*H*-imidazolyl]-propanol, 2HCl is an experimental antitumor compound which inhibits protein synthesis in cell cultures and in cell free systems. The compound has been evaluated for its capacity to inhibit specific assays of initiation, elongation and termination of protein synthesis. Girodazole inhibited the release of nascent peptides from polyribosomes in rabbit reticulocyte lysates indicating that the major effect of the compound is on the protein synthesis termination step.

Girodazole (RP 49532A, NSC 627434) or 3 amino-1-[4-(2 amino-1*H*-imidazolyl]-propanol, 2HCl† is an antitumor compound originally isolated from the marine sponge *Pseudaxinyssa cantharella* [1]. The chemical structure of Girodazole is shown in Fig. 1. The compound is cytotoxic *in vitro* against several tumor cell lines and active against murine grafted tumors, including P388 and L1210 leukemias, MA16/C mammary adenocarcinoma and M5076 histiocytosarcoma [2]. Although its chemical structure may suggest alkylating properties toward nucleic acids, the compound does not appear to interact with either DNA or RNA [2]. In contrast, Girodazole inhibits protein synthesis in cell cultures and in cell free systems; inhibition of protein synthesis markedly correlates with the cytotoxic activity of the compound [2]. Several inhibitors of eukaryotic protein synthesis are known. These include emetine [3], homoharringtonine [4], anguidine [5], bruceantin [6, 7], lycorine [8], sparsomycin [9] and the most recent antitumor marine compounds mycalamide-A and -B and onnamide [10]. With the possible exception of the latter compounds [10], whose mode of action is unknown, and of lycorine, which acts on termination, eukaryotic protein synthesis inhibitors act preferentially on initiation or elongation. To better understand the mode of action of Girodazole, we investigated its effect on initiation, elongation and termination reactions in protein synthesis. In this article, we report evidence that, in agreement with our previous observations [2], the compound interacts preferentially with the termination step.

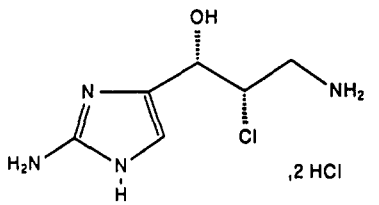

Fig. 1. Chemical structure of Girodazole.

MATERIALS AND METHODS

Materials. Girodazole (RP 49532A, NSC 627434) was obtained from the Chemical Reserve of Rhône-Poulenc Rorer. Anguidine and bruceantin were provided by N.C.I. All other inhibitors were from Sigma. [3,4,5-^{3}H]Leucine (50 Ci/mmol) was obtained from C.E.A. (Saclay, France). [^{14}C]-Leucine (342 mCi/mmol), [^{14}C]phenylalanine (250 Ci/mmol), [8(*n*)-^{3}H]puromycin (5 Ci/mmol) labelled amino acids were from Amersham (U.K.). Glass fiber filters (GF/C) were from Millipore and all other chemicals and enzymes, unless specified, were obtained from Sigma.

Standard protein synthesis assay. Reticulocyte lysates were prepared from New Zealand rabbits according to standard procedures [11]. They were stored as aliquots in liquid N_2 until used.

Standard reaction mixtures to measure protein synthesis contained in a 200-μL volume [6]: 100 μL lysate, 10 mM Tris–HCl pH 7.8, 74 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 50 μM hemin, a mixture of 19 amino acids, excluding leucine, each at 100 μM, 15 mM phosphocreatine, 500 U/mL creatine kinase, 6 mM β-mercaptoethanol and 40 μM [3,4,5-^{3}H]leucine at 630 μCi/μmol. Inhibitors, concentrated 20-fold, were added in a volume of 10 μL. Following incubation at 30°, aliquots (usually 10 μL) were removed, mixed with 1 mL 1 M NaOH/1.5% H_2O_2 and incubated for 10 min at 37°. Three millilitres of cold 25% trichloroacetic acid (TCA) were added and the precipitate obtained after 10 min was collected on glass fiber filters. Filters were washed five times with 5% TCA, twice with ethanol, dried at 50° and radioactivity was counted by liquid scintillation in a Beckman apparatus.

Ribosomes sedimentation studies. Protein synthesis was carried out as above using 7.3 μM [^{14}C]leucine

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† Abbreviations: Girodazole, 3 amino-1-[4-(2 amino-1*H*-imidazolyl]-propanol, 2HCl; TCA, trichloroacetic acid; EF, elongation factor.

at 342 mCi/mmol. At each time point, aliquots of 100 μL were mixed with 200 μL of ice-cold buffer A (10 mM Tris–HCl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$) and layered on the top of 10-mL sucrose gradients (10–20% in buffer A) [6] prepared in SW41 Beckman tubes. Tubes were then centrifuged at 4° for 90 min at 140,000 *g*. The absorbance (O.D.) at 254 nm was determined during collection of the gradient using a UVcord detector (Pharmacia). Aliquots (about 10 drops, variable volume) were processed for the measurement of radioactivity as described above. For pulse-chase experiments, cold leucine was added at 730 μM during the chase phase.

Puromycin reaction. Incorporation of [^{3}H]puromycin into nascent peptides was determined by published methods [5] with some modifications. The reaction mixture (200 μL) contained 100 μL reticulocyte lysate, 50 mM Tris–HCl pH 7.4, 0.3 M KCl, 4 mM $MgCl_2$, 5 μM [^{3}H]puromycin (5 Ci/mmol) and the test substance. Following incubation at 30° for 5 min, the amount of peptidyl-puromycin formed was assessed after TCA precipitation on glass fiber filters. Filters were washed three times with TCA and five times with ethanol before scintillation counting.

Elongation reaction. The elongation reaction in the poly-U-directed protein synthesis was carried out with calf thymus elongation factors (EFs), *Artemia salina* ribosomes, *Escherichia coli* phenylalanine tRNA and phenylalanine tRNA synthetase kindly provided by Drs Crechet and Parmeggiani (Ecole Polytechnique, Saclay, France) [12–14]. tRNA charging was done for 10 min at 37° in a volume of 840 μL containing 35 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 80 mM KCl, 1 mM dithiotreitol, 2 mM phosphoenol pyruvate, 1 μg pyruvate kinase, 100 μg of tRNA, 4 μg phenylalanine tRNA synthetase, 1.5 μM [^{14}C]phenylalanine (250 Ci/mol). Sixty microlitres of 80S ribosomes at 626 O.D./mL and 75 μL of poly-U at 2 mg/mL were added and samples were further incubated for 10 min. Elongation in the presence or absence of Girodazole was initiated with 5 μL of EF_1 and 2 μL of EF_2 at 105 μg/mL and 220 μg/mL, respectively, in 25 mM Tris pH 7.4, 1 mM dithiothreitol, 10% glycerol. At different times, samples of 20 μL were spotted on filters and precipitated with 10% TCA. Filters were then boiled in 5% TCA, washed with a 1:1 ethanol–ether mixture, dried and counted for radioactivity.

RESULTS

Time course of inhibition of protein synthesis

It has been shown previously that inhibitors of protein synthesis can be differentiated on the basis of the time lag required for inhibition to occur [5, 6]. Girodazole was compared to two compounds, anguidine and bruceantin which require completion of ongoing elongation before protein synthesis becomes affected [5–7]. The results are shown in Fig. 2. Incorporation of [^{3}H]leucine into proteins was markedly affected as early as 2 min following

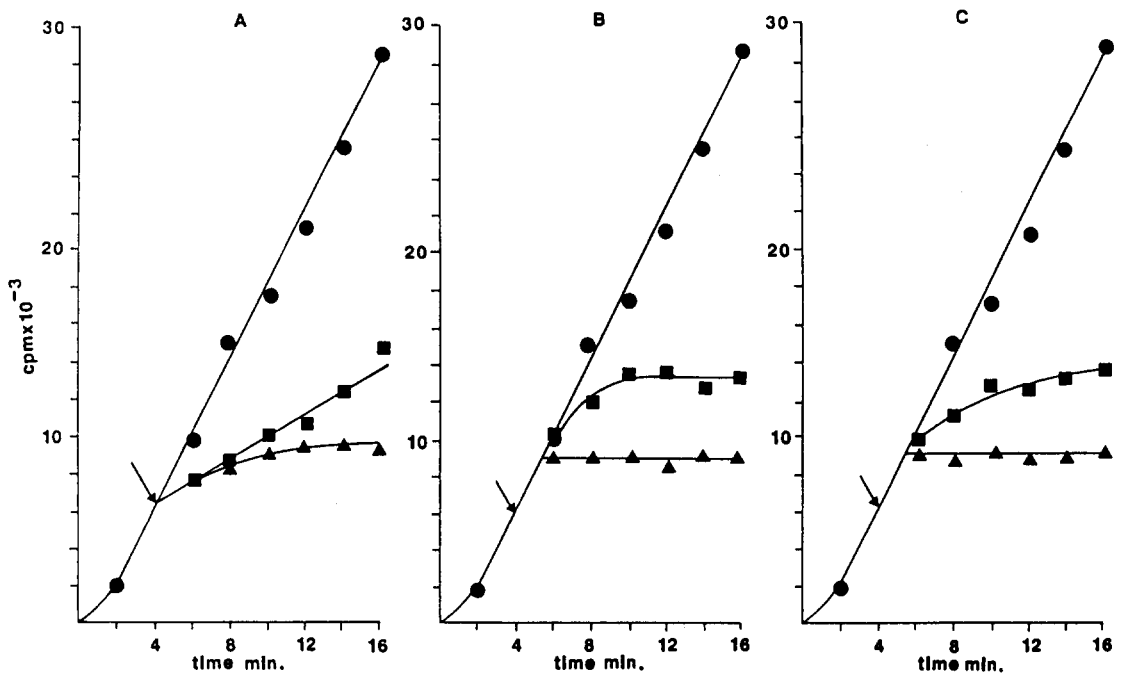

Fig. 2. Time course of globin synthesis by reticulocyte lysate: effect of Girodazole, anguidine and bruceantin. The reaction mixture was incubated, as described in Materials and Methods, in the presence of [^{3}H]leucine for 4 min (arrow); then Girodazole (1A), anguidine (1B) or bruceantin (1C) was added each at the concentration of 1 (■) or 10 μM (▲). Control tubes (●) received a corresponding volume of H_2O. At the indicated times, 10 μL were removed for determination of protein synthesis.

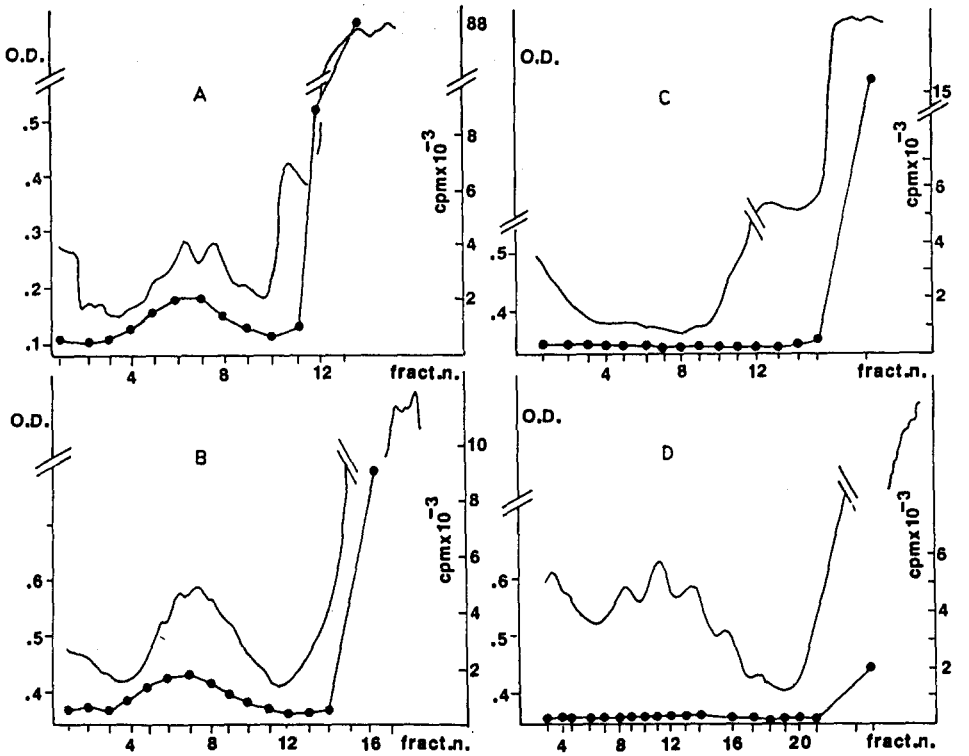

Fig. 3. Sedimentation profile of reticulocyte lysates engaged in globin synthesis: effect of Girodazole, bruceantin and emetine. Protein synthesis was carried out for 7 min, as described in Materials and Methods, with [^{14}C]leucine, in the absence (A) or in the presence of 100 μM Girodazole (B), bruceantin (C) or emetine (D). Following centrifugation on sucrose gradient, the optical density ($O.D._{254nm}$, arbitrary units —) and the TCA-precipitable radioactivity (●) of the indicated fractions of variable volume were determined. Polyribosome peaks occur around fraction 7 (A, B). The direction of centrifugation is from right to left.

addition of Girodazole at 1 or 10 μM (Fig. 2A). In contrast, inhibition of protein synthesis by anguidine or bruceantin was not apparent before 4 min (Fig. 2B, C).

Polyribosome structure

Polyribosomes undergo extensive breakdown into ribosomes in the presence of initiation inhibitors (such as anguidine) but are relatively stable in the presence of elongation inhibitors (such as emetine) [5, 6]. Bruceantin and its analogues, which stop elongation after one round of peptide synthesis, behave as initiation inhibitors, inducing polyribosome degradation [6, 7]. The effect of Girodazole (100 μM) on the sedimentation of polyribosomes and ^{14}C-labelled peptides as compared to that of bruceantin and emetine (each at 100 μM) is shown in Fig. 3. Control samples (Fig. 3A) displayed a ^{14}C-labelled polyribosome profile and a substantial amount of radioactivity released as free proteins in the upper fractions. Girodazole (Fig. 3B) did not modify the polyribosome profile, while bruceantin (Fig. 3C), as previously shown by others [6], caused extensive polyribosome breakage. Polyribosome structures were also maintained in the presence of emetine (Fig. 3D). Under such experimental conditions (7-min incubation), Girodazole did not decrease the amount of radioactivity associated with polyribosomes, while 90% inhibition was seen in the case of the top fractions. Bruceantin allowed completion of ongoing peptide synthesis as indicated by the radioactivity released in the upper fractions. The same result was obtained with anguidine (data not shown). Radioactivity in all fractions was virtually abolished by emetine, despite the presence of polyribosomes.

The effect of Girodazole on the structure of polyribosomes, and on the nascent (polysome-associated) and released peptides was examined at different times (1, 2, 4 or 7 min) following addition of the compound. The results of different experiments are summarized in Table 1.

In control samples, the amount of [^{14}C] radioactivity associated with polyribosomes increased with time, the maximal capacity for synthesizing peptides being achieved by approximately 4 min. During this time period, a significant inhibition of nascent peptides was observed in the presence of Girodazole at 10 or 100 μM. The highest inhibition (68%) was observed at 4 min but by 7 min no inhibition was observed and even higher counts were detected at the polyribosome level. The effect of Girodazole on

Table 1. Effect of Girodazole on total protein synthesis, and polyribosome-associated and -released peptides

Expt	Time (min)	Girodazole concn (μM)	Total radioactivity (cpm)	Inhibition (%)	Polyribosome-associated radioactivity (cpm)	Inhibition (%)	Released radioactivity (cpm)	Inhibition (%)
I	2	0	26,954		6582		20,372	
		10	6548	76	2554	62	3984	81
	4	0	38,866		10,780		28,086	
		10	8844	78	4563	58	4281	85
	7	0	60,630		9164		51,466	
		10	13,270	79	7868	15	5402	90
II	1	0	7393		3081		4312	
		100	4872	35	1920	38	2952	32
	4	0	32,694		13,443		19,251	
		100	6799	80	4424	68	2369	88
III	7	0	106,482		11,410		95,072	
		100	21,988	80	12,290	0	9694	90
IV	7	0	118,509		9371		109,138	
		100	19,761	84	10,602	0	9159	92

Reticulocyte lysates were incubated for protein synthesis with [^{14}C]leucine, as described in Materials and Methods, in the absence or in the presence of Girodazole. At the time indicated, they were centrifuged on a 10–20% sucrose gradient. Fractions (15–20) were collected and counted individually for radioactivity. Counts corresponding to polyribosomes (lower fractions) and to released peptides (upper fractions) were added separately. Data on Expt No. III were obtained from the corresponding sedimentation profile shown in Fig. 3.

peptide release displayed a different pattern. Firstly, this inhibition was higher than that observed for nascent peptides and secondly, it increased with time, reaching high levels (90–92%) by 7 min.

Protein elongation reactions

To gain more information on the effect of Girodazole on elongation, two sets of experiments were performed: the transpeptidation reaction and the poly-U-directed polyphenylalanine biosynthesis. The transpeptidation reaction was examined by using the puromycin technnique and anisomycin as a reference compound [5]. Lysates were incubated with [^{3}H]puromycin and the amount of peptidyl-[^{3}H]puromycin formed was determined in the absence or in the presence of inhibitors. The amount of peptidyl-[^{3}H]puromycin in control samples was 10,200 cpm. Anisomycin at 100 μM inhibited this reaction by 50%. In contrast, Girodazole had virtually no effect on the transpeptidation reaction, as 0 and 7% inhibition were obtained at the concentrations of 1 and 100 μM, respectively. Purified components (phenylalanine tRNA, phenylalanine tRNA synthetase, EF_1, EF_2, 80S ribosomes) were used to carry out poly-U translation. The reaction was linear for 50 min and strictly elongation factor dependent. [^{14}C]Polyphenylalanine synthesis was virtually unaffected by Girodazole. TCA-precipitable radioactivity at 50 min was 13,200 cpm in the control reaction and 12,200 and 11,200 cpm at 10 and 100 μM Girodazole, respectively. The lack of inhibition on polyphenylalanine biosynthesis was also confirmed with a nuclease-treated reticulocyte lysate and supplemented with exogenous poly-U (data not shown).

Assessment of termination by measurement of protein release

As indicated in Fig. 3 and in Table 1, the main effect of Girodazole was observed on the release of newly made proteins from polyribosomes, indicating a preferential inhibition by the compound of the termination step. To better characterize this step, nascent peptides were labelled for 2 min with [^{14}C]-leucine and then chased in the absence or in the presence of 100 μM Girodazole. Results are shown in Fig. 4. In control samples, labelled peptides were released into the upper gradient following a chase of 2 and 6 min (Fig. 4A, B, C). However, in Girodazole-treated samples, this step was completely blocked as even at 6 min a significant amount of radioactivity was still associated with the polyribosomal fraction (Fig. 4D, E). As expected from its mechanism of action, bruceantin did not interfere with peptide release (Fig. 4F). A similar experiment was carried out in the presence of different concentrations of Girodazole (Fig. 5). Peptide release following a 6-min chase was not affected by 100 μM anguidine (Fig. 5C), a reference initiation inhibitor [5]. Inhibition of peptide release in the presence of Girodazole (Fig. 5D, E, F) was concentration dependent. Seventy-one per cent of the polyribosome-associated radioactivity was released in control samples, 53% at 1 μM, 26% at 10 μM and only 3% at 100 μM, corresponding to an inhibition of 26, 64 and 96%, respectively.

DISCUSSION

Girodazole is an antitumor compound whose mechanism of action involves inhibition of protein synthesis. It has been shown previously that this inhibition is not the result of an interaction of the compound with mRNA, which would impair its capacity to be translated [2]. In fact, despite its chemical structure, the compound does not appear to alkylate either RNA or DNA [2]. To better

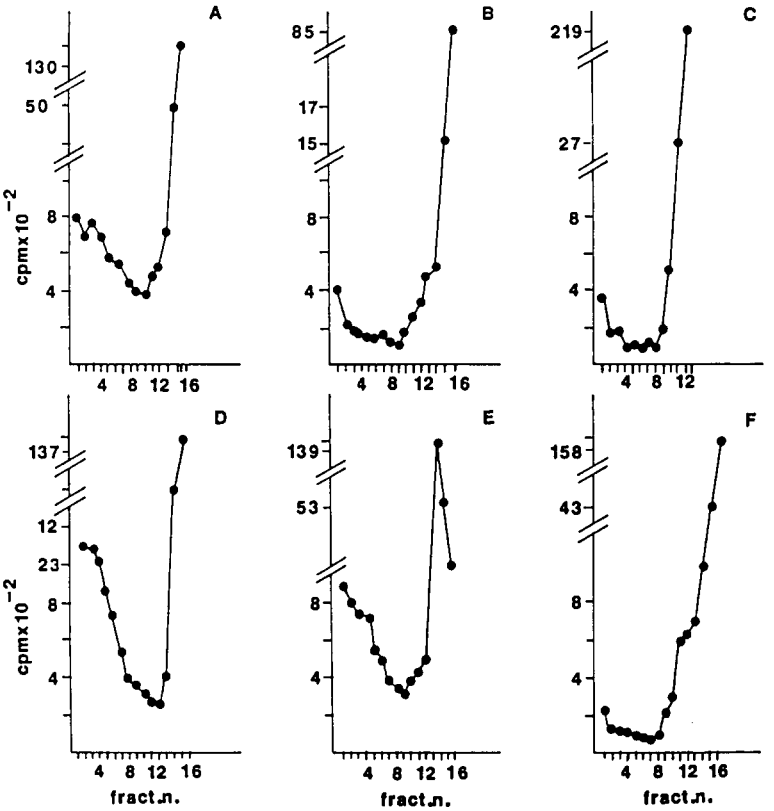

Fig. 4. Effect of Girodazole and bruceantin on the release of ^{14}C-labelled peptides from polyribosomes. Six standard protein synthesis reaction mixtures were incubated for 2 min with [^{14}C]leucine. Control tubes (no inhibitor) were immediately mixed with cold buffer A (time = 0, A) to assess the amount of newly made peptides or after a chase of 2 min (B) or 6 min (C). Girodazole (100 μM) was added during a 2-min (D) and a 6-min (E) chase and bruceantin (100 μM) during a 6-min chase (F). After centrifugation on sucrose gradients, fractions were collected and counted as described in Materials and Methods. The positioning of nascent (polysome-associated) and released peptides was determined from the profile of the radioactivity and $O.D._{254}$ tracing (not shown). The direction of centrifugation is from right to left.

elucidate the mode of action of Girodazole, experiments on the initiation, elongation and termination steps of protein synthesis were performed. The activity of Girodazole was compared to reference compounds which inhibit elongation immediately (emetine, anisomycin) [3, 6] or after one round of peptide synthesis (bruceantin) [6, 7] as well as to the initiation inhibitor anguidine [5]. Results presented in this paper and previously [2] argue against an effect of Girodazole on initiation. First, Girodazole rapidly inhibited protein synthesis, in contrast to anguidine and bruceantin which required a lag of a few minutes (Fig. 2). Second, in contrast to these compounds, Girodazole did not induce polyribosome breakdown (Fig. 3). Furthermore, it has been shown [2] that in a reticulocyte lysate, Girodazole inhibited preferentially high molecular mass products indicating that inhibition does not require completion of nascent chains as in the case of bruceantin [6].

Although an effect of Girodazole on elongation could be suggested by its inhibition of nascent peptides (polysome-associated radioactivity) (Table 1), this appears unlikely. First, inhibition of nascent chains was transient and lower than the overall inhibition of protein synthesis. Second, Girodazole did not inhibit the transpeptidation and the synthesis of poly-U directed polyphenylalanine which are typical reactions of elongation, not requiring initiation or termination factors.

The most likely mode of action of Girodazole is inhibition of termination. This was indicated from the dramatic inhibition in the release of the newly formed peptides from polyribosomes in the pulse-chase experiments (Figs 4 and 5). While anguidine and bruceantin had no effect, Girodazole inhibited protein release by 26, 64 and 94% at 1, 10 and 100 μM, respectively.

It is likely that inhibition of termination may slow down nascent protein synthesis, particularly during the time required for polyribosomes to reach full translation capacity ($\leq$4 min, under our experimental

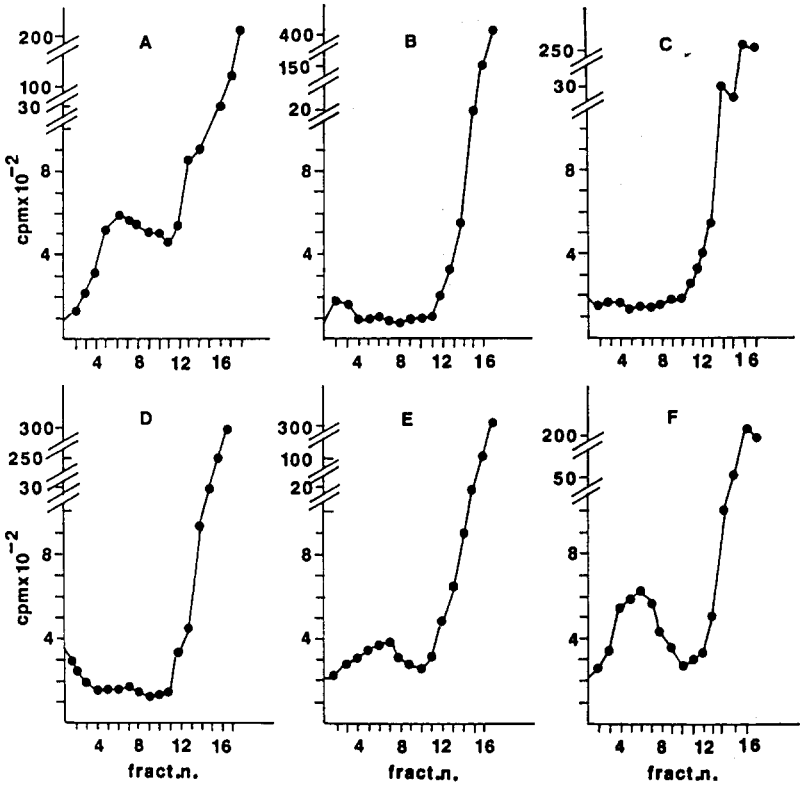

Fig. 5. Effect of Girodazole and anguidine on the release of ^{14}C-labelled peptides from polyribosomes. Six standard reaction mixtures were incubated for 2 min with [^{14}C]leucine. Control tubes (no inhibitor) were immediately mixed with cold buffer A (time = 0, A) or after a chase of 6 min (B). Inhibitors were added during the 6-min chase: 100 μM anguidine (C) and Girodazole at 1 μM (D), 10 μM (E) and 100 μM (F). After centrifugation on sucrose gradients, fractions were collected and counted as described in Materials and Methods. The positioning of nascent (polysome-associated) and released peptides was determined from the profile of the radioactivity and $O.D._{254}$ tracing (not shown). The direction of centrifugation is from right to left.

conditions). This may explain the apparent effect on elongation observed by 2–4 min in the experiments described in Table 1.

The mode of action of eukaryotic protein synthesis inhibitors has often been difficult to establish as the effect may sometimes vary with the concentration and the test system used (6–8). The use of biochemical assays [15] with purified components has provided unequivocal results for some protein synthesis inhibitors [13]. In this work, the use of purified components has ruled out an effect of Girodazole on elongation but similar biochemical studies are needed to characterize the inhibition of termination at the molecular level.

It is interesting to note that Girodazole is the first inhibitor of termination possessing antitumor activity in experimental models in animals [2]. Girodazole has been proposed for clinical trials [2] and results from Phase I will be published elsewhere.

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