Cancer Chemother Pharmacol (1989) 24: 80–86

Cancer Chemotherapy and Pharmacology

RNA polymerase II transcripts as targets for 5-fluorouridine cytotoxicity: antagonism of 5-fluorouridine actions by α-amanitin*

Robert Heimer and Alan C. Sartorelli

Department of Pharmacology and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, CT 06510, USA

Summary. The cytotoxicity of 5-fluorouridine (FUrd) results from actions directed at the synthesis of both DNA and RNA. The role of mRNA as a target for FUrd was investigated by selectively decreasing the incorporation of FUrd into RNA polymerase II transcripts of K-562 erythroleukemia cells, which was accomplished by the addition of α-amanitin to cultures of K-562 cells permeabilized with lysolecithin. In these cells α-amanitin at concentrations of 1–5 μg/ml inhibited the incorporation of [^{3}H]-uridine into polyadenylated RNA by up to 45% and decreased the steady-state levels of two specific mRNAs but had no effect on poly A$^-$ RNA synthesis. α-Amanitin decreased the incorporation of FUrd into poly A$^+$ RNA by up to 60%. The decrease in FUrd incorporation produced by α-amanitin was accompanied by an antagonism of the growth inhibitory effects of the fluorinated pyrimidine nucleoside by the mycotoxin, as measured by both growth in suspension culture and colony formation in 0.12% agar. Antagonism between these agents increased as the concentration of α-amanitin was elevated; furthermore, it was sequence-dependent, occurring only when α-amanitin preceded FUrd. These findings provide evidence that the actions of FUrd directed against mRNA are antagonized when FUrd incorporation into mRNA transcripts is decreased and that the effects of FUrd on mRNA produce cytotoxic consequences.

Introduction

The cytotoxicity of the fluoropyrimidines has been attributed to biochemical actions at two levels: the first occurs as a consequence of the inhibition of thymidylate synthase, and the second appears to be due to the incorporation of the analog into RNA. The incorporation of fluoropyrimidines into mammalian ribosomal RNA results in inhibition of the processing of the ribosomal RNA precursor (pre-mRNA) and thus the failure to produce mature ribosomal RNAs [6, 29, 30, 33]. This action has been postulated to be the major cytotoxic event in many types of cells, and significant correlations have been demonstrated between fluoropyrimidine cytotoxicity and incorporation into ribosomal RNA precursors [6, 25, 28].

Additional RNA target sites for fluoropyrimidine cytotoxicity have been investigated. The most likely candidate for at least part of the cytotoxic action of the fluoropyrimidines is messenger RNA. Its consideration as a potential target is partly based on previous studies on the intracellular fate and distribution of the fluoropyrimidines. One study [13] has found that the incorporation of FUra and FUrd into mRNA was correlated with cytotoxicity in HT-29 human colon carcinoma cells. Other investigations have demonstrated that patterns of RNA synthesis were altered by exposure to fluoropyrimidines, with FUra inhibiting the polyadenylation of nuclear RNA without altering transcription in L1210 leukemia [14] and Ehrlich carcinoma [11] cells. FUra has been shown to decrease specific mRNA levels; the induction of globin gene expression in murine erythroleukemia cells was decreased when FUra was added shortly after the addition of an inducing agent [17]. However, fluoropyrimidine incorporation did not impair the translation of cytoplasmic mRNA [4, 12].

These findings suggest that if mRNA is a target, then inhibition of the processing of pre-mRNA is a possible site of fluoropyrimidine action. Recent work on the consequences of FUra exposure has directly addressed this possibility. Both acute exposure of L5178Y leukemia cells [2] and chronic exposure of KB cells [8, 31, 32] to FUra resulted in the accumulation of precursors to dihydrofolate reductase mRNA. Intron-containing and therefore unprocessed sequences increased to a greater extent than exon-containing sequences [31]. In vitro, the processing of synthetically prepared transcripts containing FUra was abnormal, and unspliced intermediates accumulated concomitantly with the loss of normal lariat-containing splicing intermediates [9]. Thus, it has been demonstrated that FUra incorporation into pre-mRNA can effect splicing. Whether a connection exists between the effects of the fluoropyrimidines on processing and cytotoxicity remains to be demonstrated.

* Supported in part by U. S. Public Health Service grants CA-02817 and CA-09085 from the National Cancer Institute

Abbreviations: FUrd, 5-fluorouridine; FUra, 5-fluorouracil; mRNA, messenger RNA; poly A$^+$ RNA, polyadenylated RNA; rRNA, ribosomal RNA; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TCA, trichloroacetic acid, DNase, pancreatic deoxyribonuclease I; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; HSB, oligo-dT cellulose elution buffer containing 0.5 *M* NaCl; LSB, oligo-dT cellulose elution buffer without NaCl; SSC, 0.15 *M* NaCl, 0.015 *M* sodium citrate (pH 7.0); SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline

Offprint requests to: Alan C. Sartorelli, Comprehensive Cancer Center, Yale University School of Medicine, P. O. Box 3333, 333 Cedar Street, New Haven, CT 06510, USA

To gain evidence for such a relationship we have used α-amanitin, a specific inhibitor of RNA polymerase II [5], to decrease specifically the incorporation of FUrd into pre-mRNA transcripts. The mycotoxin α-amanitin is a bicyclic octapeptide that appears to act by binding to the largest subunit of RNA polymerase II [15], thereby preventing the translocation of the polymerase and the nascent RNA on the DNA template [26].

Several criteria must be met by a prospective RNA polymerase II inhibitor if it is to be of use in ascertaining whether incorporation of FUrd into RNA is associated with cytotoxicity. First, the drug must decrease RNA synthesis. Second, the decrease must be restricted to polyadenylated RNA. Third, the drug must decrease FUrd incorporation into poly A^+ RNA. Fourth, to permit the evaluation of the protective effects of the polymerase inhibitor, the effects on poly A^+ RNA must occur at inhibitor concentrations that alone do not substantially decrease the rate of cell growth. α-Amanitin met these criteria once K-562 cells had been permeabilized by treatment with lysolecithin. Thus, in lysolecithin-permeabilized, α-amanitin-treated cells, the total incorporation of [^{3}H]-uridine and [^{3}H]-FUrd were decreased, and the decreases were restricted to poly A^+ RNA. These decreases were demonstrable at concentrations of α-amanitin that caused minimal cytotoxicity. When cultures of permeabilized K-562 human erythroleukemia cells were exposed to α-amanitin prior to FUrd, the cytotoxicity of FUrd was partially antagonized both in suspension and in 0.12% agar-containing cultures. These were small but significant reductions in FUrd cytotoxicity, providing evidence that a component of FUrd cytotoxicity is a consequence of the incorporation of the fluoropyrimidine into pre-mRNA transcripts and suggesting further that the inhibition of mRNA processing by FUrd indeed contributes to the cell death caused by this fluoropyrimidine.

Materials and methods

Cell culture and colony formation. K-562 cells were grown from stocks maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were subcultured into medium containing 50 μ*M* hemin, which had no effect on the rate of growth, cloning efficiency, or response to fluoropyrimidines.

To determine the effects of drugs on growth, cells in suspension culture were exposed as indicated, washed free of drugs, and resuspended in fresh medium, and cell numbers were determined at intervals using a Coulter Model ZB_1 particle counter (Coulter Electronics, Hialeah, Fla). Rates of growth were determined by logarithmic transformation of cell densities and by linear regression. Cloning efficiencies were also determined following drug exposure. To accomplish this, cells were diluted from suspension cultures such that 100, 500, or 1,000 cells were resuspended in 5 ml fresh medium containing 15% fetal bovine serum and 0.12% melted agar in 15-ml culture tubes. After 12–15 days in a 37° C incubator, viable colonies were stained by the overnight addition of a 0.1% solution of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride and counted. The growth in suspension culture and the capacity for colony formation were used to determine the nature of the interactions between α-amanitin and FUrd [3, 27]. The quantification of the interaction in suspension culture made use of logarithmic transformation of cell-density measurements. Thus, the interaction can be computed by an addition of effects. The quantification in the clonogenic assay is the product of the fractional survivals for individual drugs.

Permeabilization of K-562 cells. Lysolecithin was added from a stock solution of 5 mg/ml in 150 m*M* HEPES (pH 7.0), 80 m*M* KCl, 5 m*M* K_2HPO_4, 5 m*M* $MgCl_2$, and 0.5 m*M* $CaCl_2$ to 10^7 cells/ml that had been washed twice and resuspended in PBS. The lysolecithin concentrations ranged from 7.8 to 250 μg/ml. After 2 min on ice, cells were diluted to 10^5 cells/ml by the addition of fresh medium at 37° C. When α-amanitin was used, it was included in the fresh medium.

RNA labeling and isolation. To analyze the effects of α-amanitin on total RNA synthesis, cellular RNA was radiolabeled by the addition of [5-^{3}H]-uridine (18–26 Ci/mmol, Moravek, Brea, Calif) or [^{3}H]-FUrd (18 Ci/mmol, Moravek) to cell cultures for 2 h. Aliquots of cells were removed at various times for up to 2 h, trichloroacetic acid-precipitable material was collected on glass-fiber filters, and radioactivity was determined by liquid scintillation spectrometry. Cytoplasmic and nuclear RNAs were prepared using a modification of the method of Favaloro et al. [10]. Cells were lysed in an isotonic buffer [0.14 *M* NaCl, 10 m*M* TRIS-HCl (pH 8.6), 1.5 m*M* $MgCl_2$] containing 1% of the nonionic detergent Triton X-100 and 5 m*M* vanadyl-ribonucleoside complex (Bethesda Research Labs, Gaithersburg, Md).

Nuclei were separated by underlaying the lysate with lysis buffer containing 24% (w/v) sucrose and centrifuging at 10,000 *g* for 20 min at 4° C. The cytoplasmic fraction was removed from the top of the centrifuge tube, the sucrose was discarded, and the nuclei were lysed in fresh lysis buffer using a 19-gauge needle. Cytoplasmic and nuclear RNAs were isolated by proteinase K digestion, extraction with phenol and chloroform, and precipitation in ethanol. In some cases, total cellular incorporation was measured by omitting the sucrose-gradient separation of nuclei and cytoplasm prior to the proteinase K digestion. RNA was isolated from the precipitates by selective digestion of the DNA with deoxyribonuclease I (DPFF grade from Worthington, Freehold, NJ) in the presence of 2 m*M* vanadyl-ribonucleoside complex [19]. RNA was quantified spectrophotometrically, assuming that a concentration of 40 μg/ml represented 1 absorbance unit at a wavelength of 260 nm. All isolated RNA fractions were stored at −70° C in 70% ethanol containing 0.1 *M* sodium acetate (pH 5.2).

Oligo-dT cellulose affinity chromatography. To determine the activity of the putative RNA polymerase II inhibitor, α-amanitin, its effects on the labeling of poly A^+ RNA were measured. Labeled RNA was diluted in high-salt buffer [HSB: 0.5 *M* NaCl, 10 m*M* TRIS-HCl (pH 8.0), 1 m*M* Na_2EDTA, 0.1% SDS] and 5 μg RNA was heated to 65° C for 5 min and cooled before its application onto an oligo-dT cellulose (Type 7; Pharmacia, Piscataway, NJ) column. Poly A^- RNA was eluted with HSB, and 1-ml fractions were collected and quantified by scintillation spectrofluorometry after the addition of 5 ml aqueous scintillation cocktail (Ecoscint; National Diagnostics, Somerville, NJ) to the fractions. RNA that remained bound to the column

was eluted using buffer from which NaCl was omitted [LSB: 10 m*M* TRIS-HCl (pH 8.0), 1 m*M* Na_2EDTA, 0.1% SDS). LSB eluate fractions were pooled and NaCl was added to give a concentration of 0.5 *M*; this was heated to 65° C for 5 min, cooled, and reapplied to a regenerated oligo-dT cellulose column. The column was washed with HSB, 1-ml fractions were collected, and the radioactivity therein was determined. Poly A^+ RNA was eluted from the column with LSB, 1-ml fractions were collected, and radioactivity was ascertained and reported as a percentage of the total counts in all eluate fractions.

Gel electrophoresis of RNA. To evaluate the effects of α-amanitin on rRNA synthesis, RNA labeled with [^{3}H]-uridine was fractionated using composite agarose-polyacrylamide gels [7, 22] of 0.5% agarose, 1.75% acrylamide (19:1 bis), and TRIS-borate buffer [0.089 *M* TRIS base, 0.089 *M* boric acid, and 2 m*M* Na_2EDTA (pH 7.5)]. Cytoplasmic RNA was resuspended in a TRIS-EDTA buffer [TE: 10 m*M* TRIS-EDTA (pH 8.0) and 1 m*M* Na_2EDTA), and its concentration was determined spectrophotometrically [19]. Lanes on 16 × 16 cm vertical slab gels sandwiched between frosted glass plates (Hoefer Scientific, San Francisco, Calif) were loaded with 20 μg cytoplasmic RNA labeled with [^{3}H]-uridine. Gels were run at a maximum of 100 V until the xylene cyanol FF tracking dye migrated approximately 8 cm. Gels were then fixed in 1 *M* acetic acid, stained with 0.2% methylene blue, and destained. Each lane was sliced into sections that were treated with 0.5 *N* NaOH, and radiolabeled RNA was quantified after the addition of 5.5 ml aqueous scintillation cocktail. Relative mobilities of RNA species were determined based on the mobilities of 4S tRNA and 18S and 28S rRNA species, and a linear relationship was obtained when the logarithm of the molecular weight was plotted as a function of mobility [7].

Dot-blot hybridization of cellular RNA. Cellular RNA was resuspended in TE buffer and denatured in 7.5% formaldehyde by heating to 65° C for 15 min. The RNA was then serially diluted and adsorbed onto nylon membranes (Gene Screen; New England Nuclear, Boston, Mass) using a 96-well manifold. Filters were baked for 1 h at 85° C and prehybridized for 4–6 h at 42° C in a buffer containing 50 m*M* TRIS-HCl (pH 7.5), 1 *M* NaCl, 50% (v/v) deionized formamide, 10% dextran sulfate, 1% SDS, 0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinyl pyrrolidine, 0.1% $Na_4P_2O_7$, and 0.1 mg/ml denatured salmon-sperm DNA. Filter-bound RNA that coded for the c-*abl* and c-*myc* proto-oncogenes was analyzed using a 1.6-kb v-*abl* insert from plasmid pK2 [24] or a 9-kb human c-*myc* insert from plasmid pHSR-1 [1]. Individual cDNA probes, labeled with [^{32}P]-dCTP by nick translation (Bethesda Research Labs, Gaithersburg, Md) and denatured by boiling, were added to the filters at $0.5–2 \times 10^6$ dpm/ml prehybridization buffer. The hybridization was carried out for 16–20 h at 42° C. Filters were then washed three times with 2X SSC and 0.5% SDS for 5 min each. Filters hybridized with a homologous human c-*myc* probe were further washed at 65° C for 60 min in 0.1X SSC + 1% SDS, with the retention of a high signal, whereas those using a viral *abl* probe required washing at 65° C in 2X SSC + 0.1% SDS or at 50° C in 0.5X SSC + 1% SDS to retain a signal.

Results

To evaluate the effects of α-amanitin on mRNA synthesis, K-562 cells were exposed to 5 μg mycotoxin/ml; [^{3}H]-uridine was added 4 h later for a final concentration of 1 μ*M* and its incorporation into total cellular RNA was measured. The rates of incorporation were found to be statistically identical to those in untreated controls (Table 1). Cytoplasmic RNA was extracted after 2 or 24 h of labeling and analyzed by oligo-dT cellulose affinity chromatography. No statistically significant differences were observed between control and α-amanitin-treated cells ($P > 0.05$).

Table 1. Effects of α-amanitin on poly A^+ RNA synthesis

	Control	α-Amanitin
Experiment 1		
Rate of [^{3}H]-uridine incorporation pmol/min per 10^6 cells	0.78	0.86
% poly A^+ RNA	2.0±0.4	2.7±1.0
Experiment 2		
Rate of [^{3}H]-uridine incorporation pmol/min per 10^6 cells	1.95	2.12
% poly A^+ RNA	9.4±1.7	12.4±1.5

K-562 cells were exposed to 5 μg/ml α-amanitin for 4 h prior to the initiation of RNA labeling by the addition of [5-^{3}H]-uridine (20 Ci/mmol). In experiment 1, the final uridine concentration was 0.25 μ*M* and in experiment 2, it was 0.5 μ*M*. Incorporation into TCA-precipitable material was measured at intervals up to 2 h in duplicate cultures and the rate of incorporation during the 1st h labeling was determined. In experiment 1, the labeling was continued for 24 h before cytoplasmic RNA was extracted and analyzed by oligo-dT cellulose affinity chromatography. In experiment 2, cytoplasmic RNA was extracted after 2 h. Measurements of poly A^+ RNA represent averages of at least three separate chromatographic determinations.

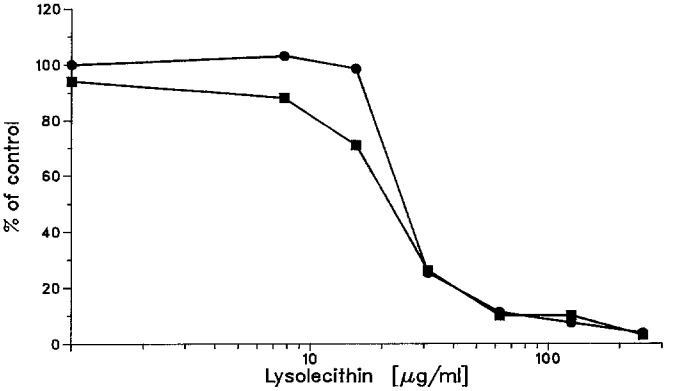

Fig. 1. Effects of lysolecithin on the permeability and growth of K-562 cells exposed to lysolecithin as described in *Materials and methods*. After the resuspension of cells in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μ*M* hemin, an aliquot was removed and mixed with an equal volume of 0.5% trypan blue in PBS, and the exclusion of the dye was scored microscopically (■). The remaining cells were grown in suspension culture, and their rates of growth were determined by logarithmic transformation and plotted as a percentage of the control rate (●). All values are averages of four determinations whose SDs were < 5%

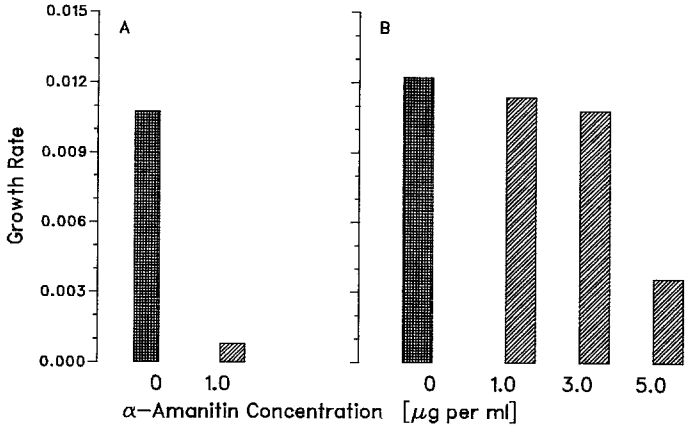

Fig. 2. Growth in suspension culture of lysolecithin-permeabilized K-562 cells exposed to α-amanitin. *A:* Cells permeabilized with 15 μg/ml lysolecithin and continuously exposed to 0 or 1.0 μg/ml α-amanitin. *B:* Cells permeabilized with 15 μg/ml lysolecithin and exposed to 0, 1.0, 3.0, or 5.0 μg/ml α-amanitin for 6 h; cells were then washed free of the α-amanitin and resuspended in fresh RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 μ*M* hemin. Rates of growth, determined by logarithmic transformation, represent the averages of duplicate cultures

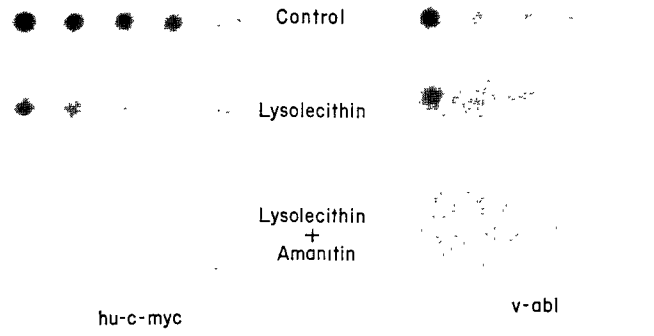

Fig. 4. Effects of α-amanitin on the levels of c-*myc* and c-*abl* mRNA in the lysolecithin-permeabilized K-562 cells. Cytoplasmic RNA was isolated from K-562 cells treated with 15 μg/ml lysolecithin and 3 μg/ml α-amanitin. RNA was resuspended in 10 m*M* TRIS-HCl (pH 8.0) and 1 m*M* EDTA and denatured by incubation for 15 min at 65° C in a buffer containing 1.8 *M* NaCl, 0.18 *M* sodium citrate, 7.5% formaldehyde, 10 m*M* TRIS-HCl (pH 8.0), and 1 m*M* EDTA at an RNA concentration of 0.2 μg/μl. Serial 1:1 dilutions were prepared and 100-μl aliquots containing RNA levels of 0.2–0.0125 μg/ml were adsorbed to nylon membranes using a 96-well manifold. Binding of RNA to the membrane and hybridization of [^{32}P]-dCTP-labeled c-*myc* and v-*abl* cDNA probes were carried out as described in *Materials and methods.* RNA isolated from two separate cultures of cells exposed to both lysolecithin and α-amanitin were evaluated

These findings indicated that α-amanitin could not be used without first making cells more permeable to the drug.

When K-562 cells were treated with lysolecithin, normal rates of growth were observed only at concentrations of $\leqslant 20$ μg/ml (Fig. 1), which were 20-fold lower than the levels previously used to permeabilize Chinese hamster ovary and Novikoff hepatoma cells [20, 23]. The particular sensitivity of K-562 cells to lysolecithin may be due to the fragility of its cell membrane, which has erythroid characteristics. At lysolecithin concentrations of $\geqslant 30$ μg/ml, the exclusion of trypan blue and proliferation were prevented to equal degrees (Fig. 1). At 15 μg lysolecithin/ml, the proliferative capacity of K-562 cells was the same as that in untreated cells, although up to 30% of the cells accumulated trypan blue. Since cells did not appear to be excessively damaged by lysolecithin at this concentration, studies on the actions of α-amanitin were conducted using cells permeabilized with 15 μg/ml lysolecithin.

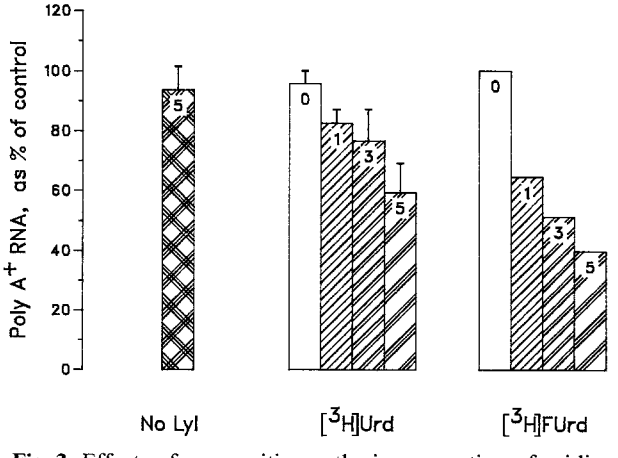

Fig. 3. Effects of α-amanitin on the incorporation of uridine and FUrd into poly A$^+$ RNA. K-562 cells were permeabilized with 15 μg/ml lysolecithin except where indicated (*No Lyl*). After a 2-h exposure to α-amanitin at the concentrations indicated within the bars, cells were labeled for 2 h with [^{3}H]-uridine (18 Ci/mmol) or 1 μ*M* [^{3}H]-FUrd (diluted to 3 Ci/mmol). Cytoplasmic RNA was extracted and poly A$^+$ RNA was quantified using oligo-dT cellulose as described in *Materials and methods.* SDs are reported for observations in triplicate; otherwise, the values plotted represent the average of two experiments. *Lyl*, lysolecithin; *Urd*, uridine

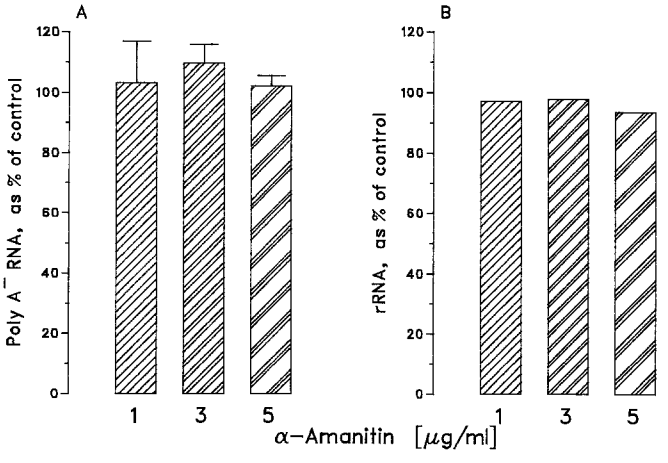

Fig. 5. Effects of α-amanitin on the incorporation of uridine into poly A$^-$ RNA. K-562 cells were permeabilized using 15 μg/ml lysolecithin. After a 2 h exposure to α-amanitin at the indicated concentrations, cells were labeled for 2 h with [^{3}H]-uridine. Cytoplasmic RNA was extracted and poly A$^-$ RNA was isolated as HSB eluates from oligo-dT cellulose columns, pooled, precipitated in ethanol, and analyzed by composite gel electrophoresis. *A:* Effects of α-amanitin on [^{3}H]-uridine incorporation into total poly A$^-$ RNA were determined in quadruplicate and plotted as the average ± SD. *B:* Effects of α-amanitin on [^{3}H]-uridine incorporation into 18S and 28S rRNA are plotted as the average of two determinations

Permeabilized K-562 cells were exposed to α-amanitin at concentrations of 1–5 μg/ml. α-Amanitin was completely toxic at the lowest level tested (1 μg/ml) when exposure was continuous, but when cells were washed free of this agent at the end of a 6 h period of exposure, cells retained high proliferative capacity until the α-amanitin concentration reached 5 μg/ml (Fig. 2). In permeabilized cells, lysolecithin itself had no effect on total poly A^+ RNA synthesis (Fig. 3). The addition of α-amanitin for 2 h prior to a 2-h [^{3}H]-uridine labeling period decreased the synthesis of poly A^+ RNA in a concentration-dependent manner. Increasing the labeling period to 21 h did not further decrease poly A^+ RNA synthesis, which remained at approximately 65% of the control level (data not shown). α-Amanitin decreased the levels of both c-*myc* and c-*abl* mRNA in lysolecithin-permeabilized cells as revealed by dot-blot hybridization (Fig. 4). These decreases occurred with no change in the labeling of poly A^- RNA or of 18S or 28S rRNA by α-amanitin (Fig. 5), which attested to the specificity of this agent as an inhibitor of RNA polymerase II.

α-Amanitin decreased the incorporation of [^{3}H]-FUrd into poly A^+ RNA by 35.5%–62% (Fig. 3); these decreases were larger than those seen using [^{3}H]-uridine and may result from FUrd-induced inhibition of rRNA synthesis. This action would, in effect, decrease the amount of label incorporated into poly A^- RNA and increase the poly A^+ RNA fraction in all cells exposed to the 1 μ*M* [^{3}H]-FUrd. Nevertheless, in lysolecithin-permeabilized cells, α-amanitin decreased the incorporation of [^{3}H]-FUrd into poly A^+ RNA over the same concentration range at which the synthesis of poly A^+ RNA was decreased and at which α-amanitin cytotoxicity was manifested.

Cells permeabilized with lysolecithin and pretreated with α-amanitin for 4 h were used to investigate whether FUrd cytotoxicity was antagonized. FUrd was added for 2 h and cells were then washed with fresh medium and cultured in fresh drug-free medium. Growth in both suspension culture and 0.12% agar was investigated; the growth rates in suspension culture are shown in Table 2. Pretreatment with α-amanitin resulted in subadditive cytotoxicity for all combinations ($P < 0.05$), even when negative growth rates were seen upon combination of both agents at high concentrations (Fig. 6). Thus, α-amanitin both decreased FUrd incorporation into mRNA and antagonized FUrd-induced cytotoxicity in suspension culture. Colony formation in agar was determined for permeabilized cells treated with 1 μg/ml α-amanitin, which by itself decreased survival by about 30%, and with FUrd at 0.5, 1.0, and 2.0 μ*M*. α-Amanitin also antagonized the FUrd-induced inhibition of colony formation at each concentration of FUrd (Fig. 7). The antagonisms observed were statistically significant in pair-wise comparisons of observed vs expect-

Table 2. Effects of α-amanitin and FUrd on Rates of growth in suspension cultures of lysolecithin-permeabilized K-562 cells

FUrd (μ*M*)	α-Amanitin (μg/ml)			
	0	1.0	3.0	5.0
0	0.01223	0.01227	0.01082	0.00358
0.6	0.00827	0.00980	0.00769	0.00095
1.0	0.00627	0.00706	0.00561	0.00061
2.0	0.00456	0.00565	0.00373	−0.00304
4.0	0.00235	0.00391	0.00154	−0.00439

Duplicate cultures of K-562 cells were established for each condition. Cells were permeabilized with 15 μg/ml lysolecithin and exposed to α-amanitin for 2 h before the addition of FUrd for 2 h. Cells were washed free of drugs and resuspended in fresh medium at a density of approximately 25×10^3 cells/ml. Densities were determined after 47 and 91 h, the densities were transformed logarithmically, and the data were analyzed by linear regression. The slopes of the generated lines correspond to the rates of growth such that $\log_{10}2$ slope = doubling time

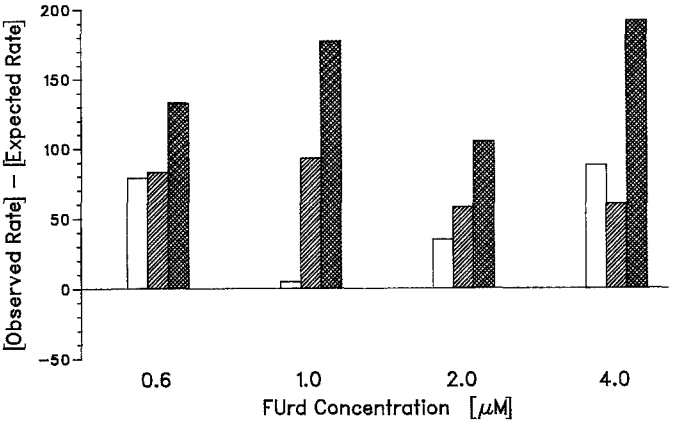

Fig. 6. Effects of the combination of α-amanitin and FUrd on the growth of K-562 cells, which were permeabilized with 15 μg/ml lysolecithin and exposed for 4 h to α-amanitin at 1 (□), 3 (▨), or 5 (■) μg/ml prior to the addition of FUrd for 2 h. The subsequent rates of growth for cultures exposed to only one drug (data in Table 2) were used to determine the rates expected for an additive interaction. This expected rate was subtracted from the observed rate; differences > 0 indicate antagonistic interactions

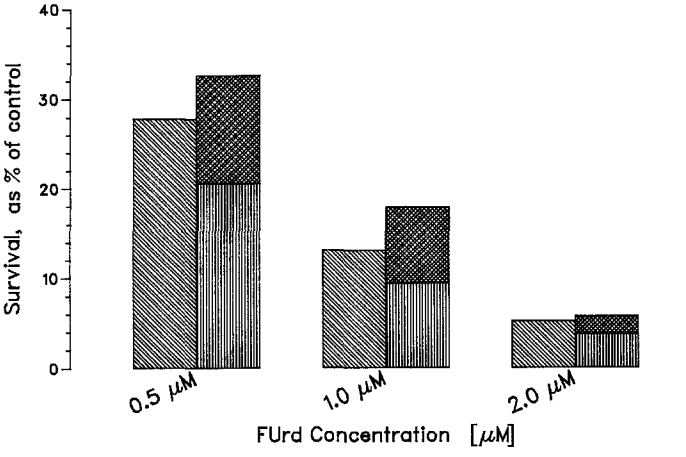

Fig. 7. Effects of combinations of α-amanitin and FUrd on the soft-agar cloning of K-562 cells. Lysolecithin-permeabilized K-562 cells were exposed to 1 μg/ml α-amanitin for 2 h prior to their exposure to FUrd for 2 h. Survival of cells exposed to drug(s) was computed as a percentage of the number of colonies found for permeabilized cells that were not exposed to FUrd or α-amanitin. The *left-hand bar* of the three pairs at 0.5, 1.0, and 2.0 μ*M* FUrd represents the survival of cells exposed to FUrd alone (▧). The *right-hand bar* represents the survival observed following exposure to combinations of drugs (■) superimposed on the survival expected for an additive effect (▥). The data represent the averages of three cloning experiments

ed survival in three separate experiments ($P < 0.05$). In fact, the survival of cells exposed to both drugs was, on average, greater than that of cells exposed only to FUrd. In contrast, when cells were treated with FUrd prior to permeabilization with lysolecithin and exposure to α-amanitin, cytotoxicity in suspension cultures was additive (data not shown). Thus, antagonism was sequence-specific and consistent with the reduction in FUrd incorporation into mRNA that was produced by α-amanitin.

Discussion

To explore the impact of FUrd on both mRNA synthesis and cytotoxicity, the effects of pretreatment with α-amanitin, an inhibitor of pre-mRNA transcription, were investigated in K-562 cells. α-Amanitin proved to be effective as a specific RNA polymerase II inhibitor only if cells were made permeable to this agent. Thus, α-amanitin at concentrations as great as 5 μg/ml failed to decrease poly A^+ RNA synthesis in intact K-562 cells, probably because the accumulation of this mycotoxin was insufficient. The bicyclic amatoxin octapeptides bind to the same membrane polypeptides that bind bile salts and are then transported across the plasma membrane in the presence of a Na^+ gradient [18]. Competition studies suggest that this saturable pathway is required for the intracellular accumulation of these agents [21]. The findings in this report suggest the absence of this transport mechanism in K-562 cells, thus rendering these cells naturally impermeable to α-amanitin. However, in lysolecithin-permeabilized cells α-amanitin appeared to act specifically on RNA polymerase II transcription. No changes were observed in the labeling of poly A^- RNA over the same range of concentrations that decreased the synthesis of polyadenylated RNA by about 50%. The specific reductions in the incorporation of [^{3}H]-FUrd into RNA were greater than those of [^{3}H]-uridine.

α-Amanitin pretreatment also antagonized the cytotoxicity of FUrd, measured both in growth assays in suspension culture and in colony-forming assays in 0.12% agar. In suspension culture, an increase in the concentration of α-amanitin increased the degree to which FUrd cytotoxicity was antagonized. Thus, when cells were pretreated with 1, 3, or 5 μg/ml α-amanitin, FUrd-induced cytotoxicity was antagonized; such antagonism was dependent on the sequence of drug administration, with α-amanitin having to precede FUrd. When cells were pretreated with α-amanitin at 1 or 3 μg/ml, the antagonism of FUrd cytotoxicity was small. Reversal of cytotoxicity averaged only 10% in cloning experiments when the concentration of α-amanitin was 1 μg/ml and 8.5% and 12.6% in suspension cultures when the concentration of α-amanitin was 1 and 3 μg/ml, respectively. These reversals were observed at levels of α-amanitin that decreased the incorporation of uridine into poly A^+ RNA by only 20% and decreased FUrd incorporation by as much as 50%. At 5 μg/ml α-amanitin, the reversal of FUrd toxicity to K-562 cells was increased to 24.1% and FUrd incorporation into poly A^+ RNA was reduced by more than 60%; however, it was difficult to interpret these findings because of the negative growth rates of cells exposed to both 5 μg/ml α-amanitin and higher concentrations of FUrd. Nevertheless, the results demonstrate that α-amanitin can antagonize the cytotoxicity of FUrd. These small but consistent and statistically significant reversals support the hypothesis that a minor part of the toxicity of FUrd to K-562 cells is exerted through the incorporation of FUrd into mRNA transcripts. Previous studies have demonstrated strong correlations between cytotoxicity and the inhibition of rRNA synthesis [6, 16, 25]. The results reported herein in no way contradict this, but rather imply that the RNA-directed actions of FUrd are multifarious. The findings provide evidence that the observations of Dolnick and co-workers [7, 8, 21, 32] and Armstrong et al. [2] are consequential to our understanding of the cytotoxic actions of the fluoropyrimidines. The major RNA-directed effect may well be the inhibition of ribosomal RNA maturation, but the observations in this report add support to the premise that FUrd has an mRNA-directed action.

References

1. Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM (1983) Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80: 1707
2. Armstrong RD, Lewis M, Stern SG, Cadman EC (1986) Acute effects of 5-fluorouracil on cytoplasmic and nuclear dihydrofolate reductase messenger RNA metabolism. J Biol Chem 261: 7366
3. Berenbaum MC (1981) Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 35: 269
4. Carrico CK, Glazer RI (1979) Effect of 5-fluorouracil on the synthesis and translation of polyadenylic acid-containing RNA from regenerating rat liver. Cancer Res 39: 3694
5. Cochet-Meilhac M, Chambon P (1974) Animal DNA-dependent RNA polymerases: 11. Mechanism of the inhibition of RNA polymerase B by amatoxins. Biochim Biophys Acta 353: 160
6. Cohen MB, Glazer RI (1985) Cytotoxicity and the inhibition of ribosomal RNA processing in human colon carcinoma cells. Mol Pharmacol 27: 308
7. Dingman CW, Peacock AC (1968) Analytical studies on nuclear ribonucleic acid using polyacrylamide gel electrophoresis. Biochemistry 7: 659
8. Dolnick BJ, Pink JJ (1983) 5-Fluorouracil modulation of dihydrofolate reductase RNA levels in methotrexate-resistant KB cells. J Biol Chem 258: 13299
9. Doong S-L, Dolnick BJ (1988) 5-Fluorouracil substitution alters pre-mRNA splicing in vitro. J Biol Chem 263: 4467
10. Favaloro J, Treisman R, Kamen R (1980) Transcription maps of polyoma virus-specific RNA: analysis by two-dimensional nuclease S1 gel mapping. Methods Enzymol 65: 718
11. Glazer RI, Hartman KD (1981) Analysis of the effect of 5-fluorouracil on the synthesis and translation of polysomal poly (A) RNA from Ehrlich ascites cells. Mol Pharmacol 19: 117
12. Glazer RI, Hartman KD (1983) In vitro translation of messenger RNA following exposure of human colon carcinoma cells in culture to 5-fluorouracil and 5-fluorouridine. Mol Pharmacol 23: 540
13. Glazer RI, Lloyd LS (1982) Association of cell lethality with incorporation of 5-fluorouridine into nuclear RNA in colon carcinoma cells in culture. Mol Pharmacol 21: 468
14. Glazer RI, Peale AL (1979) The effect of 5-fluorouracil on the synthesis of nuclear RNA in L1210 cells in vitro. Mol Pharmacol 17: 270
15. Greenleaf AL (1983) Amanitin-resistant RNA polymerase II mutations are in the enzyme's largest subunit. J Biol Chem 258: 13403
16. Herrick D, Kufe DW (1984) Lethality associated with incorporation of 5-fluorouracil into preribosomal RNA. Mol Pharmacol 26: 135

17. Iwata T, Watanabe T, Kufe DW (1986) Effects of 5-fluorouracil on globin mRNA synthesis in murine erythroleukemia cells. Biochemistry 25: 2703
18. Kroencke KD, Fricker G, Meier PJ, Gerock W, Wieland T, Kurz G (1986) α-Amanitin uptake into hepatocytes. Identification of hepatic membrane transport systems used by amatoxins. J Biol Chem 261: 12562
19. Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor
20. Miller MR, Castellot JJ, Pardee AB (1978) A permeable animal cell preparation for studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S phase initiation. Biochemistry 17: 1073
21. Munter K, Mayer D, Faulstich H (1986) Characterization of a transporting system in rat hepatocytes. Studies with competitive and non-competitive inhibitors of phalloidin transport. Biochim Biophys Acta 860: 91
22. Peacock AC, Dingman CW (1967) Resolution of multiple ribonucleic acid species by polyacrylamide gel electrophoresis. Biochemistry 6: 1818
23. Samal B, Ballal NR, Busch H (1980) Initiation of transcription in permeabilized Novikoff hepatoma cells. Cell Biol Int Rep 4: 175
24. Srinivasan A, Reddy EP, Aaronson SA (1981) Abelson murine leukemia virus: molecular cloning of infectious integrated proviral DNA. Proc Natl Acad Sci USA 78: 2077
25. Takimoto CH, Cadman EC, Armstrong RD (1986) Precursor-dependent differences in the incorporation of fluorouracil in RNA. Mol Pharmacol 29: 637
26. Vaisius AC, Wieland T (1982) Formation of a single phosphodiester bond by RNA polymerase B from calf thymus is not inhibited by α-amanitin. Biochemistry 21: 3097
27. Valeriote F, Lin H-s (1975) Synergistic interaction of anticancer agents: a cellular perspective. Cancer Chemother Rep 59: 895
28. Wilkinson DS, Crumley J (1976) The mechanism of 5-fluorouridine toxicity in Novikoff hepatoma cells. Cancer Res 36: 4032
29. Wilkinson DS, Pitot HC (1973) Inhibition of ribosomal ribonucleic acid maturation in Novikoff hepatoma cells by 5-fluorouracil and 5-fluorouridine. J Biol Chem 248: 63
30. Wilkinson DS, Cihak A, Pitot HC (1971) Inhibition of ribosomal ribonucleic acid maturation in a rat liver by 5-fluoroorotic acid resulting in the selective labeling of cytoplasmic messenger ribonucleic acid. J Biol Chem 246: 6418
31. Will CL, Dolnick BJ (1986) 5-Fluorouracil augmentation of dihydrofolate reductase gene transcripts containing intervening sequences in methotrexate-resistant KB cells. Mol Pharmacol 29: 643
32. Will CL, Dolnick BJ (1987) 5-Fluorouracil augmentation of dihydrofolate reductase RNA containing contiguous exon and intron sequences in KB7B cells. J Biol Chem 262: 5433
33. Willen R (1970) Polyacrylamide-agarose electrophoretic pattern of the RNA labeling in liver cytoplasm and total liver of 5-fluorouracil treated rats. Hoppe-Seylers Z Physiol Chem 35: 1141

Received July 25, 1988/Accepted October 18, 1988