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Note

Quantitation of 5-fluorouracil incorporation into RNA by high-performance liquid chromatography without the use of radioactive precursors

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The mechanism of action of the antineoplastic drug 5-fluorouracil (FUra) has been studied in vivo in non-human systems and in human cells in culture [1]. Fluorodeoxyuridine monophosphate (FdUMP) inhibits the enzyme thymidylate synthetase, blocking the de novo formation of TMP The ribonucleotide triphosphate (FUTP) is incorporated into RNA where it causes aberrations in RNA processing and function [2,3]. FUra is also incorporated into DNA, although it has not been established that this route contributes to toxicity [4,5]. In any given tumor system, one or the other of these mechanisms may contribute predominantly to cytotoxicity.

Our studies using radioactive FUra to measure the incorporation of drug into RNA [6–9] and the work of others [10–13] have led to an appreciation of the correlation between FUra in RNA and cytotoxicity. However, there are still questions concerning the mechanism by which FUra exerts its cytotoxicity in human tumors Knowledge of mechanism of action of any drug guides decisions as to choice to modulating and rescue agents in the development of multidrug combinations In identifying the importance of FUra in RNA in the CD8F1 mammary and CD2 Colon 26 murine systems, radioactive FUra was used to measure incorporation [6–8]. Some human studies have used trace amounts of radioactive FUra to follow FUra metabolism [14–16]. However, routine use

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of ^{3}H or ^{14}C for incorporation studies in humans is not practical. Although nuclear magnetic resonance can detect FUra, its degradation products and free FUra nucleotides, it may not be sensitive enough to quantitate FUra incorporated into RNA in vivo [17]. Thus, there is a need for a method of quantitating the incorporation of FUra into RNA without the use of radioactive precursors.

In this note we report the development of a method for identifying and quantitating 2′,3′-FUMP (the 2′ and 3′ mixed isomers of fluorouridine monophosphate) in alkaline hydrolystates of murine tumor RNA using high-performance liquid chromatography (HPLC) and UV spectrophotometry. This new procedure gave comparable results to the traditional method of measuring acid-precipitable, alkali-labile radioactivity.

EXPERIMENTAL

Animals

The experiments detailed in this paper utilized CD8Fl mice bearing first-generation transplants of the CD8Fl spontaneous murine mammary carcinoma [18]. They were allowed food and water ad libitum.

Source of drugs and radiolabeled compounds

Normal pyrimidine and purine nucleotides, purine bases, fluorouridine (FUrd), fluorodeoxyuridine (FdUrd) and bacterial acid phosphatase (BAP) were obtained from Sigma (St. Louis, MO, U S.A), FUra was obtained from Hoffman-La Roche (Nutley, NJ, U.S.A.), [6-^{3}H]FUra from Moravek Biochemicals (Brea, CA, U.S.A.) and 2′,3′-FUMP from Sierra Bioresearch (Tucson, AZ, U.S.A.). All drugs were made up in 0.85% sodium chloride solution so that the desired concentration was contained in 0.1 ml per 10 g animal body weight, and the solutions were injected intraperitoneally

Preparation of alkaline hydrolysates of tumor RNA

Tumor-bearing CD8Fl mice received 100 mg/kg FUra containing [^{3}H]FUra (final specific activity 0.077 mCi/mmol) intraperitoneally. After a 2-h labeling period, the animals were sacrificed, and the tumors were removed and homogenized in 8 volumes of TNE buffer (0.01 *M* Tris–HCl, 0.15 *M* NaCl, 0.001 *M* EDTA, pH 7.8), extracted twice with phenol–cresol (100 14, v/v), saturated with TNE (2 volumes of homogenate to 1 volume of phenol–cresol) and extracted once with chloroform–isoamyl alcohol (24 1, v/v) (2 volumes of extract to 1 volume of chloroform–isoamyl alcohol). Nucleic acids were recovered by the addition of 2 volumes of ethanol. The first ethanol precipitate was redissolved and reprecipitated. The second ethanol precipitate was dissolved in 1.5 ml TNE. Duplicate 0.1-ml aliquots were taken for determinations of total and alkali-stable, acid-precipitable radioactivity. The acid precipitates were collected on Whatman GF/A glass fiber filters, the filters were dried, and ra-

dioactivity was determined in a Packard liquid scintillation counter using OCS (Amersham) scintillation cocktail. The alkali digest for HPLC analysis was prepared by combining 0.9 ml of nucleic acid solution and 0.1 ml of 4 *M* sodium hydroxide, incubating for 2 h at 37 °C and adding 0.2 ml of concentrated (11.65 *M*) perchloric acid. DNA was collected by centrifugation and quantitated by the Burton diphenylamine color reaction [19]. The acidified alkaline hydrolysate was then placed in a boiling water bath for 15 min in order to convert purine nucleotides to purine bases [20]. After cooling, the hydrolysate was neutralized by extracting with 3 volumes of a mixture of tri-*n*-octylamine–freon (1·2, v/v).

High-performance liquid chromatography

Samples were analyzed on a Waters 840 HPLC system using a Waters C_{18} radial compression cartridge (Resolve C_{18}, 5-μm particles, 100 mm×8 mm I.D.). The buffer consisted of 0.05 *M* KH_2PO_4 and 5 m*M* tetrabutyl ammonium hydrogensulfate (Aldrich), pH 2.7, at a flow-rate of 2.0 ml/min. UV-absorbing compounds in the column effluent were monitored with a Waters Model 481 LC spectrophotometer at a wavelength of 254 nm, 0.001 a.u.f.s. An injection volume of 500 μl was used. In some experiments, fractions of the columns effluent were collected directly into scintillation vials for determination of ^3H radioactivity (ACS II scintillation cocktail from Amersham) RNA hydrolysates from non-FUra-treated tumor containing known amounts of 2′,3′-FUMP were used as quantitation standards.

RESULTS AND DISCUSSION

[^3H]FUra-containing nucleic acid was prepared from tumors taken from treated mice as described under Experimental. The pellet obtained after ethanol precipitation was then dissolved in 0.4 *M* sodium hydroxide in order to hydrolyse the RNA fraction to a mixture of 2′- and 3′-monophosphate nucleotides. After removal of DNA with perchloric acid, the acidified sodium hydroxide digest was placed in a boiling water bath for 15 min prior to neutralization [15]. The resulting mixture (consisting primarily of adenine, guanine, 2′,3′-CMP, 2′,3′-UMP and 2′,3′-FUMP) was then chromatographed, and the results are presented in Fig. 1. Fig. 1A is a sample of an alkali digest from control tumor RNA. The two purine bases and 2′,3′-CMP tend to co-chromatograph, followed by 2′,3′-UMP. Fig. 1B is the alkali digest of RNA from a tumor treated with FUra at 100 mg/kg. Note the appearance of a small peak with a longer retention time than 2′,3′-UMP. This peak was identified as 2′,3′-FUMP by the fact that it co-chromatographed with authentic 2′,3′-FUMP, its absence in control tumors and the presence of ^3H radioactivity in the column effluent at this time (Fig. 2A). (Using marker compounds, we determined that FUra, FUrd, FdUrd and 5′-FUMP do not co-chromatograph with the putative 2′,3′-

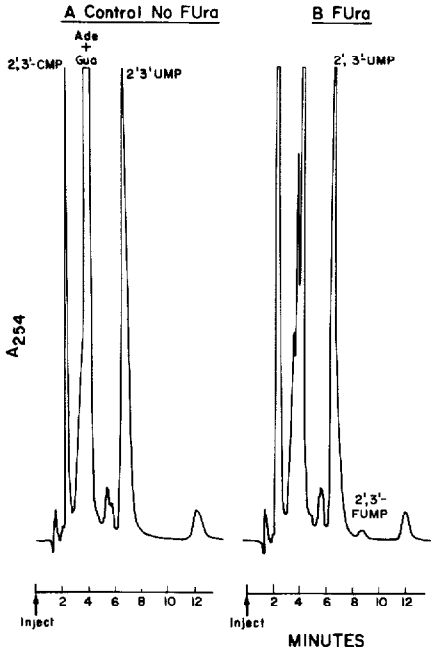

Fig 1 HPLC analysis of alkali-digested FUra-containing RNA CD8F1 tumor RNA was labeled in vivo (100 mg/kg [^{3}H]FUra) The [^{3}H]FUra-containing RNA was prepared from tumor by phenol extraction and ethanol precipitation RNA was degraded by treatment with alkali (0 4 *M* NaOH, 37°C, 2 h) DNA was precipitated by the addition of perchloric acid The acidified alkali digest was heated (100°C, 15 min) prior to neutralization with octylamine-freon Conversion of purine nucleotides to free bases by this step was neccessary since 2′,3′-FUMP tended to co-chromatograph with and be obscured by the much larger 2′,3′-GMP peak The mixture was analyzed by ion-pairing HPLC, 0 05 *M* KH_2PO_4 in 5 m*M* tetrabutyl ammonium hydrogensulfate, pH 2 7, 2 0 ml/min, room temperature (A) Control (no FUra treatment), (B) FUra-treated

FUMP peak in this system; data not presented) The identity of this peak was further confirmed by collecting the column effluent corresponding to the peak, raising the pH to 4.5 with sodium hydroxide and digesting with bacterial acid phosphatase After this treatment, greater than 90% of the resulting ^{3}H radioactivity co-chromatographed with a 5-FUrd marker (Fig. 2B).

In order to determine if this new procedure could be used to quantitate incorporation of FUra into RNA, we prepared [^{3}H]FUra-containing RNA from tumor tissue of mice treated with 100 mg/kg [^{3}H]FUra. Nucleic acids were prepared as described by sequential extraction with phenol and then chloroform and ethanol precipitation. Portions of each sample were used for determination of total acid-precipitable and alkali-stable, acid-precipitable radioactivity. The remainder was analyzed for 2′,3′-FUMP content by HPLC. The results, presented in Table I, show that there is good agreement between the two methods, each indicating an average incorporation of 5 nmol of 2′,3′-FUMP

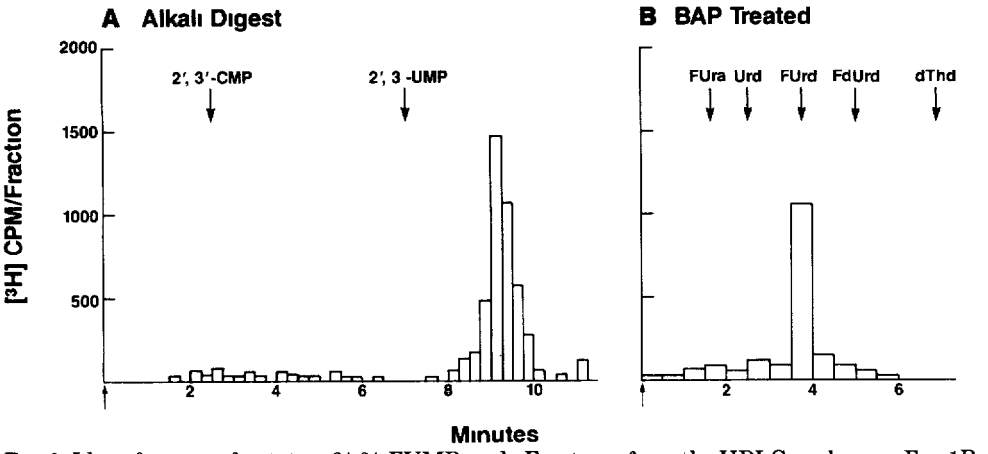

Fig 2 Identification of putative 2′,3′-FUMP peak Fractions from the HPLC analysis in Fig 1B were collected and analyzed for ^{3}H radioactivity (A) Radioactivity profile in the alkali digest of [^{3}H]FUra-labeled tumor RNA (B) Effect of treatment with bacterial acid phosphatase (BAP) A portion of the major peak of radioactivity from A was further digested with BAP and rechromatographed on the same HPLC system For both A and B, the retention time of known standards is indicated

TABLE I

COMPARISON OF ACID PRECIPITATION AND HPLC FOR MEASUREMENT OF RNA FUMP

Tumor-bearing CD8F1 mice received 100 mg/kg [^{3}H]FUra After a 2-h labeling the animals were sacrificed and nucleic acids from the individual tumors were prepared by phenol and chloroform extraction and ethanol precipitation Aliquots of the purified nucleic acids were then utilized for comparison of RNA FUMP levels determined by acid precipitation and HPLC

Tumor sample	Amount of 2′,3′-FUMP incorporated per mg DNA (nmol)	
	Acid	HPLC
1	5 5	6 2
2	4 1	3 9
3	5 1	5 1
4	4 3	4 3
Mean ± S D	4 8±0 3	4 9±0 5

per mg of DNA in this group of four tumors The alkaline hydrolysates contained approximately 10 nmol 2′,3′-FUMP and 2 mg DNA per ml. The limit of detection is approximately 1 log less with this particular HPLC system and detector.

These experiments show that HPLC analysis can be used to identify the presence of 2′,3′-FUMP in murine tumor RNA and to quantitate the level of

incorporation. The amount of 2′,3′-FUMP in the hydrolysate was quantitated from the area of the 2′,3′-FUMP peak in the chromatogram and can be expressed as pmol or nmol per gram of tissue, per mg of protein or per mg of DNA

The usefulness of these procedures in clinical application will, of course, depend on the levels of (FUra)RNA (RNA containing incorporated FUra) in human tissues being somewhat comparable to those in murine tissues Clinically, a single test of this new method using nucleated bone marrow cells from a FUra-treated patient has demonstrated that the procedure is capable of quantitating FUMP residues. The further evaluation of biopsy specimens from FUra-treated patients is ongoing.

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