ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 11–16

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

In vitro toxicity of N^3-methyl-5′-deoxy-5-fluorouridine, a novel metabolite of doxifluridine: a bioanalytical investigation

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Received 24 February 1997; received in revised form 13 May 1997

Abstract

The cytotoxicity of N^3-methyl-5′-deoxy-5-fluorouridine (N^3-Me-5′-dFUR), a novel metabolite of the anticancer pro-drug 5′-deoxy-5-fluorouridine (5′-dFUR), has been evaluated by in vitro experiments with cultures of different cancer cell lines. The new metabolic product was found to be non-toxic in all the cell growth experiments performed. The absence of cytotoxicity could be explained by the observation that the metabolite was not recognized as a substrate by thymidine phosphorilase, the enzyme responsible for 5-fluorouracil (5-FU) release from doxifluridine, as ascertained by high-performance liquid chromatography/ultraviolet (HPLC–UV) analysis of the incubation mixture. The biomethylation process leading to N^3-Me-5′-dFUR could be considered as a possible detoxification pathway, altering the drug bioavailability, in competition with 5′-dFUR cleavage to the active 5-FU.

Keywords: Cancer; Cell toxicity; Drug metabolism; Liquid chromatography

1. Introduction

Fluorinated pyrimidines and their nucleosides have a significant anticancer activity. Among these compounds, 5-fluorouracil (5-FU) is one of the most active anticancer drugs [1,2], widely used for the treatment of some solid tumors of the breast, colon and rectum. Unfortunately, it also causes undesired toxic effects. In order to improve the therapeutic index of 5-FU, the pro-drug 5′-deoxy-5-fluorouridine (doxifluridine, 5′-dFUR) has been synthesized [3]. Using the deoxy-ribofuranosil moiety as a carrier into the neoplastic tissue, 5′-dFUR is cleaved into the active 5-FU preferentially in tumor cells under the action of an intracellular thymidine phosphorilase [4–7].

After its release from the pro-drug, 5-FU can be anabolized by two possible pathways. It can be reversibly converted, by uridine phosphorylase, to 2′-deoxy-5-fluorouridine (2′-dFUR) which is phosphorylated, by thymidine kinase, to 2′-deoxy-5-fluorouridine-5′-monophosphate (2′-dFUMP) which blocks the thymidilate synthetase system. The alternative anabolic pathway involves the

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0731-7085/98/$19.00
PII S0731-7085(97)00164-7

conversion of 5-FU to 5-fluorouridine monophosphate (FUMP) either directly by a phosphoribosyl transferase or stepwise via 5-fluorouridine (FUR); FUMP is successively diphosphorylated and reduced to 2′-deoxy-5-fluorouridine diphosphate (2′-dFUDP) which is finally dephosphorylated to 2′-dFUMP. 5-FU catabolism occurs mainly in liver where it is degraded by dihydrouracil dehydrogenase (DUD) to 5,6-dihydro-5-fluorouracil ($5\text{-}FUH_2$), which then undergoes further biotransformation with the final release of ammonia, urea and carbon dioxide. $5\text{-}FUH_2$ seems to produce inhibition of thymidilate synthetase activity in Ehrlich ascites tumor cells [8] suggesting that it may also contribute to 5-FU toxicity.

Recently, evidence of in vivo biomethylation of 5′-dFUR with formation of a novel metabolite of the pro-drug has been presented for the first time by Zambonin and Palmisano [9]. Plasma extracts of cancer patients undergoing doxifluridine chemotherapy were analyzed by gas chromatography–mass spectrometry and found to contain a metabolic product of 5′-dFUR with a retention time different from that of the known metabolites mentioned above and absent in blank control plasma samples.

Based on the observed electron impact fragmentation pattern, the metabolic product was tentatively identified as N^3-methyl-5′-deoxy-5-fluorouridine (N^3-Me-5′-dFUR). Definite confirmation of the proposed structure was achieved by comparison of the mass spectra and chromatographic characteristics of the suspected metabolite with those of a synthetically prepared reference standard.

In the present work, the cytotoxicity of N^3-Me-5′-dFUR has been evaluated by in vitro experiments with cultures of different cancer cell lines. The new metabolite was found to be non-toxic in all the performed cell growth experiments.

The absence of cytotoxicity could be explained by the observation that the metabolite was not recognized as a substrate by thymidine phosphorilase, the enzyme responsible of 5-FU release from doxifluridine.

The biomethylation process leading to N^3-Me-5′-dFUR could be considered as a possible detoxification pathway, altering the drug bioavailability, in competition (see Fig. 1) with 5′-dFUR cleavage to the active 5-FU.

2. Experimental

2.1. Materials

Human hepatoma (HuH-7) [10], colon carcinoma (Caco-2) and breast carcinoma (MDA-MB231) were cultured in Dulbecco's medium (DMEM); osteoclastoma cell lines (GCT-51) [11] were cultured in Iscove's modified Dulbecco's medium (IMDM). The media were supplemented with 10% fetal bovine serum (FCS, Gibco, Uxbridge, UK), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} amphotericin B and 50 IU ml^{-1} mycostatin (Eurobio, Paris, France), at 37°C, in a water-saturated atmosphere with 5% CO_2 and fed by medium replacement every 2–3 days.

Thymidine phosphorilase from *Escherichia coli* (EC 2.4.2.4, 900 units ml^{-1}) was obtained from Sigma (St. Louis, MO).

Isopropanol, methanol, (Carlo Erba, Milan, Italy) and ethyl acetate (Merck, Darmstadt, Germany) were high-performance liquid chromatography (HPLC) grade. Other chemicals were analytical grade reagents. The HPLC mobile phase was filtered through a 0.45 mM membrane (Whatman, Maidstone, UK) before use. 5-FU and 5-bromouracil (5-BrU) were obtained from

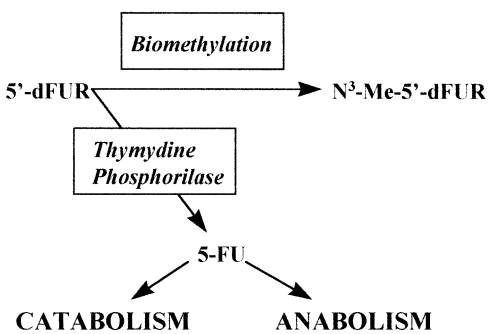

Fig. 1. Simplified metabolic scheme of 5′-dFUR. The biomethylation process of 5′-dFUR to N^3-Me-5′-dFUR is in competition with the pro-drug cleavage to 5-FU catalyzed by the enzyme thymidine phosphorilase.

Sigma (St. Louis, MO). 5′-dFUR was kindly provided by Roche SpA (Milan, Italy). The synthesis of N^3-Me-5′-dFUR was accomplished as already described [9] by methylation of 5′-dFUR with CH_3I.

2.2. Growth experiments

The kinetics of cell growth in the presence of 5-FU, 5′-dFUR or N^3-Me-5′-dFUR was followed for each cell line. The growth assays were performed on cells plated on 96-well microtiter plates, in the presence of 10% FCS. After trypsinization, cells were counted, diluted at a density of 15×10^3 ml^{-1}, and 200 μl of cell suspension, containing 3000 cells, were placed in each microtiter well in a humidified atmosphere containing 95% air and 5% CO_2.

After 24 h the experiments were started, treating the cultures with increasing concentrations of 5′-dFUR or N^3-Me-5′-dFUR ranging from 10^{-8} to 10^{-4} M for different incubation periods. The number of cells was determined by the Titertek technique [12] at the established time intervals after seeding. Briefly, cells were fixed with 3% paraformaldehyde for 20 min at room temperature, followed by rinsing with PBS, air-dried and stained with 0.5% crystal violet for 15 min, followed by extensive rinsing. The dye was released from the cells by the addition of 0.1 M Na–citrate in 50% ethanol. The optical density of the released stain solution was read in a Titertek colorimeter at 540 nm.

2.3. Incubation experiments

The incubation of 5′-dFUR with each cell line was performed as follows. Cells, plated in 25-cm^2 Falcon flasks (Oxnard, CA) at a density of 500 000 cells per flask, were cultured in DMEM with 10% FCS, supplemented or not with 10^{-4} M 5′-dFUR for different periods. Media supplemented with the pro-drug and control media were collected after an incubation of 10, 30, 60 min or 2, 8, 24 and 48 h.

The incubation reaction of thymidine phosphorilase with 5′-dFUR and N^3-Me-5′-dFUR, respectively, was accomplished as follows: 0.2 μmol of the nucleoside (5′-dFUR or N^3-Me-5′-dFUR) were added to a phosphate buffer solution (0.5 M, pH 7.4) containing 0.9 units of the enzyme, 0.02% of sodium azide and bovine serum albumin (BSA), and uracil at a concentration level of 2 mM. The reaction mixture was then incubated for 20 min at 25°C.

2.4. Chromatographic and detection conditions

The HPLC system consisted of a Waters (Milford, MA) 600-MS multisolvent delivery system equipped with a Reodyne injector with a 20 μl loop and a 5 μm Supelcosil LC-18-S ODS column (250×4.6 mm i.d.) (Supelco, Bellefonte, PA). A 5-μm Supelguard LC-18-S precolumn (20×4.6 mm i.d.) (Supelco) was used to protect the analytical column. The detector was an HP 1040A photodiode array spectrophotometer (Hewlett-Packard, Palo Alto, CA) interfaced to an HP 85 computer equipped with an HP dual disk drive and an HP 7470A plotter.

The mobile phase, the gradient program and the chromatographic conditions have been described previously [13,14].

2.5. Sample collection and pretreatment

Samples from culture media and enzymatic reactions were collected without any further manipulation. Sample pretreatment consisted of deproteinization and liquid–liquid extraction (LLE) procedures, already described in Refs. [9,13,14]. Aliquots of 250 μl of the considered sample and, when used, 20 μl of the internal standard (5-bromouracil, 5-BrU) solution were mixed in a tapered tube and 500 μl of a saturated ammonium sulphate solution were added, followed by a brief vortex mixing. A total of 5 ml of an ethyl acetate–isopropanol (90:10, v/v) mixture were successively added and the resulting mixture was vigorously shaken for 5 min, centrifuged at 4000 rpm for 5 min and the organic phase carefully transferred into a tapered tube. The extract so obtained was evaporated to dryness at room temperature under a gentle stream of nitrogen, reconstituted with 250 μl of mobile phase and 20 μl injected.

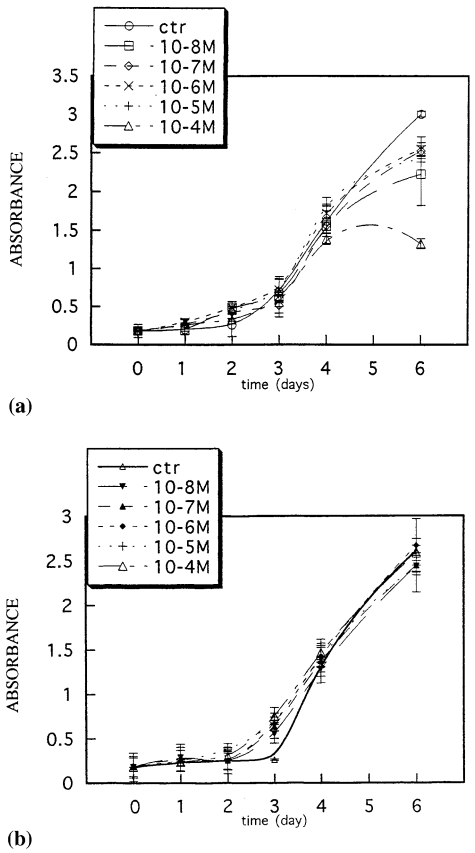

Fig. 2. Kinetics of Caco cell growth in the presence of 5′-dFUR (A) and N^3-Me-5′-dFUR (B) at concentration levels ranging from 10^{-8} to 10^{-4} M followed from day 1 to day 6. Ctr, control.

3. Results and discussion

Time course in cell growth experiments was followed by collecting samples from day 1 to day 6. Results shown in Fig. 2(A) demonstrate in the Caco cell line a decrease of cell growth in the presence of the pro-drug after 6 days of incubation at all the concentrations tested compared to the control. The most effective dosage was 10^{-4} M 5′-dFUR, with which a decrease of approximately 50% of cell growth was obtained. Similar experiments showed that N^3-Me-5′-dFUR did not affect cell growth compared to the control (Fig. 2(B)). Practically identical results were obtained in all the performed experiments with all the tested cell lines (HuH7, MDA-MB231 and GCT-51). In order to verify the suitability of the cell lines used for the chemosensitivity studies, cell growth experiments were also performed with the active drug 5-FU. Fig. 3 compares the results obtained after 6 days of incubation of the Caco cell line with 5-FU, 5′-dFUR and N^3-Me-5′-dFUR, respectively. As apparent, the presence of a potent cytotoxic drug such as 5-FU caused a strong decrease of cell growth.

Furthermore, the effects of thymidine phosphorilase, the enzyme responsible of 5-FU release from doxifluridine, on 5′-dFUR and N^3-Me-5′-dFUR were also evaluated. The two compounds were separately incubated with the enzyme, as described in Section 2; then, the reaction mixtures were analyzed by HPLC–UV. Fig. 4(A) and (B) shows the relevant chromatograms. As expected, doxifluridine is cleaved by thymidine phosphorilase to the active 5-fluorouracil. In contrast, N^3-Me-5′-dFUR did not appear to be a substrate for thymidine phosphorilase and remained unaltered, thus giving a possible explanation for its ineffectiveness on the considered cell systems.

All the above findings suggested that the described biomethylation, in competition with pro-drug cleavage to the active 5-FU, could be the result of a detoxification process operated by the neoplastic cells as an attempt to minimize 5′-dFUR toxicity.

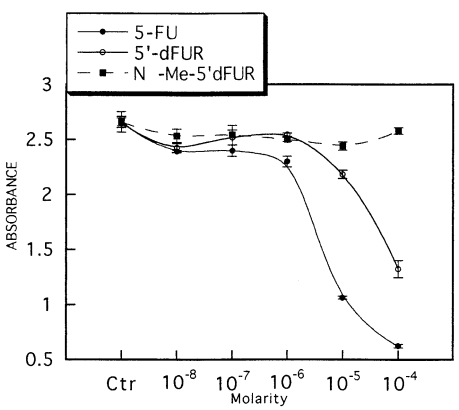

Fig. 3. Kinetics of Caco cell growth after 6 days of incubation with 5-FU, 5′-dFUR and N^3-Me-5′-dFUR, respectively, at concentration levels ranging from 10^{-8} to 10^{-4} M.

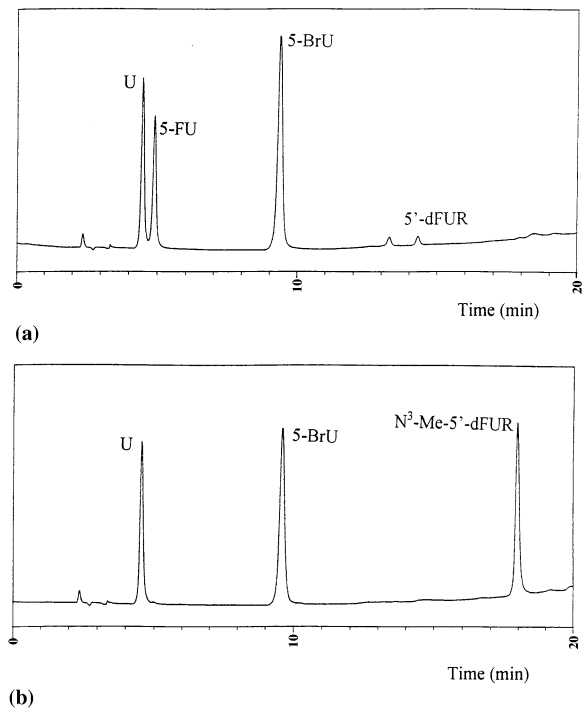

Fig. 4. Chromatograms relevant to the extracts of the reaction mixture of the incubation of thymidine phosphorilase with 5′-dFUR (A) and N^3-Me-5′-dFUR (B), respectively. Absorbance axis: 35 mAU full scale. Chromatographic conditions as in Section 2.

In vitro experiments were performed by incubating cultures of different cancer cell lines with 5′-deoxy-5-fluorouridine to see whether biomethylation of the pro-drug could be observed in vitro.

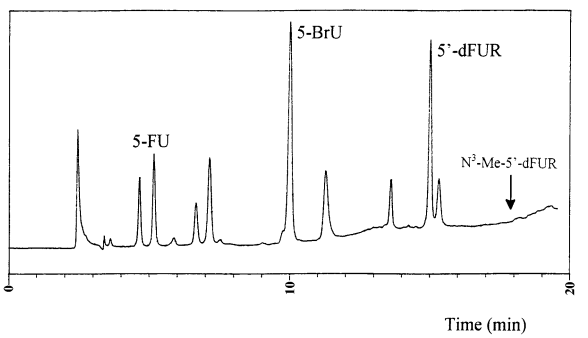

Fig. 5. Chromatogram relevant to the extract of the Caco cells culture media taken after 48 h of incubation with 5′-dFUR. Absorbance axis: 18 mAU full scale. Chromatographic conditions as in Section 2.

Fig. 5 reported, for instance, the HPLC–UV chromatogram of an extract obtained from the Caco cells culture media showing no evidence of N^3-Me-5′-dFUR formation.

The biomethylation process was not observed in any in vitro metabolization experiment. A possible explanation of this last evidence could be that the in vivo formation of N^3-Me-5′-dFUR appears only after a long period of pro-drug administration at certain dosage regimen (i.e. 4 g m^{-2} i.v. infusion over 1 h), that is consistent with the previously described hypothesis of detoxification.

Thus, the analysis of new serum samples collected from patients treated with doxifluridine would be useful to a better understanding of all the aspects of the observed in vivo biomethylation reaction. Work in this direction is being planned for the near future.

Acknowledgements

This work was carried out with financial support from the Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan) and MURST. Thanks are due to Silvio Mastrolitti for experimental help during his undergraduate research training.

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