Cytotoxicity and Pharmacokinetics of 1-β-D-Arabinofuranosyl-2-thiouracil, a 2-Sulphur Substituted Derivative of Cytarabine

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Received July 13, 1998; accepted October 16, 1998

1-(β-D-Arabinofuranosyl)-2-thiouracil (araSC), a 2-substituted derivative of cytarabine (araC), has been investigated for its cytotoxicity, enzymatic stability, plasma concentration–time profile in mice, and cytokinetics. This derivative showed strong cytotoxicity in several mammalian cell lines, although activity (IC₅₀) was weaker than araC. Greater stability to mouse cytidine deaminase was observed; the half-life in the presence of the enzyme was about 4-times longer than that of araC. The plasma concentration–time profile in mice in vivo showed prolonged retention of araSC when compared with araC. Cytokinetic study using flow cytometry indicated a non-S-phase specific effect of this compound.

Key words 1-β-D-arabinofuranosylcytosine (araC); 1-(β-D-arabinofuranosyl)-2-thiouracil (araSC); cytotoxicity; cytidine deaminase; flow cytometry

1-β-D-Arabinofuranosylcytosine (araC) is a therapeutic agent in the treatment of human hematological malignancies.1,2) It is also used in combination chemotherapy treatment of solid tumors.3) However, it requires a very complex and precise dosage schedule to obtain its maximum therapeutic effect, mainly because the mode of action of araC is specifically associated with the S-phase of the cell cycle.4,5) Furthermore, araC is rapidly inactivated by the enzyme cytidine deaminase resulting in a short retention time in the body.6,7) In order to overcome these difficulties, a variety of araC derivatives have been synthesized and 3 prodrugs, an citabine,8) enocitabine9) and cytarabine ocosfate,10) have been used clinically in Japan.

For broader spectrum and improved pharmacological characteristics, efforts have been made to develop novel deoxycytidine analogues. Introduction of other substituents into the 2'-position of deoxycytidine resulted in active compounds, some of which have been investigated for their clinical use.11,12) Interest in 2-thiopyrimidine nucleosides first began with the isolation of the corresponding odd nucleotides from E. coli t-RNA,13,14) and their general synthesis was reported in the 1960s.15) 1-(β-D-Arabinofuranosyl)-2-thiouracil (araSC, Fig. 1) was firstly synthesized by Ruyle and Shen, and its antiviral activity against vaccinia in tissue culture was reported.16) However, its antitumor activity has not been studied. To identify antitumor activity in other cytosinearabinoside analogues, we examined the in vitro antitumor activity of araSC and its kinetics in mice in comparison to those of araC.

MATERIALS AND METHODS

Chemicals Arabinosylcytosine (araC) was purchased from Yamasa Co. (Choshi, Japan), araSC was prepared from uridine according to the general method reported by Ruyle and Shen.16) Methanol was HPLC grade and purchased from Wako Pure Chemicals Co. (Tokyo, Japan). All other chemicals were of reagent grade.

*Cytotoxic Activity Assay* Suspension cell cultures were maintained in RPMI1640 culture medium with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO₂-95% air. Then, araSC or araC was added to cells (1×10⁶/ml) in a 24-well micro plate (1 ml/well), which were in their exponential phase of growth. Adherent cell cultures were maintained in Dulbecco’s modified Eagle medium (DMEM) culture medium with 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂-95% air. A test compound was added to cells (2×10⁶/ml) in a 96-well micro plate (0.1 ml/well). The increase in cell number of the drug-free culture (control), as well as that of the cultures supplemented with the test compounds, was determined after 72 h of growth.

HPLC Analysis For quantitative analysis of araSC, araC and their deaminated metabolites, a mixture of methanol and 0.02 M phosphate buffer (pH 7.2), pH 7.0 was used as a mobile phase on a C18 reversed-phase (LiChroSpher RP-18(e), 5 μm) 250×4 mm column at a flow rate of 1.0 ml/min. The wavelength of the spectrophotometer was set at 280 nm (for araSC) or 269 nm (for araC) with an attenuation of 0.01 AUFS.

Enzymatic Stability Cytidine deaminase was prepared from a mouse kidney homogenate by denaturation at 60 °C, fractionation with (NH₄)₂SO₄, treatment with alumina gel, and dialysis against 0.02 M acetate buffer (pH 4.5) according to the procedure described by Tomchick et al.17) Enzymatic

Fig. 1. Structures of araC and araSC

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deamination rates of araSC and araC were measured in the presence of a mouse kidney cytidine deaminase preparation (5 mg of protein/1 ml phosphate buffer, pH 7.4) to result in a final concentration of 5 μg/ml. The decrease in concentrations of araSC and araC was followed by HPLC analysis of samples taken periodically from the reaction mixture.

**Plasma Concentration in Mice** Male ddY mice 10 weeks of age and weighing 48-49 g, were fasted for 16 h. 10 mg/kg of each drug dissolved in 2 ml/kg of 70% ethanol were administered via the caudal vein. Blood was collected into a heparinized glass tube by decapitation at specified times after administration. The blood was centrifuged at 17000 × g for 3 min and the resulting plasma was stored at -50°C until HPLC analysis. A 300 μl sample of the plasma was mixed with 300 μl of acetonitrile for deproteinization and the mixture was centrifuged at 17000 × g for 3 min. The resulting supernatant fluid was analyzed by HPLC.

**Protein Binding** Mouse plasma was spiked with the appropriate amounts of araSC or araC solution to provide concentrations of 9 or 40 μg. The samples were incubated for 10 min at 37°C, then ultrafiltration with Ultrafree-MC 5000 NMWL filter unit (Millipore Co., Bedford, MA) was carried out. Free drug concentration was determined by direct injection of the ultrafiltrates into the HPLC system immediately after collection.

**Flow Cytometry** Logarithmically growing A375 cells (2×10⁶/ml) were incubated in the presence of appropriate concentrations of araSC (1, 10, and 100 μg/ml) for a period up to 72 h, then collected by treatment with trypsin (0.25%) and EDTA (0.5 m) solution. The cells were fixed in ice-cold ethanol (70%) and stained with propidium iodide (0.02 mg/ml). DNA content per cell nucleus was determined by measuring red fluorescence (excitation wavelength = 488 nm) using flow cytometry (Epics-XL, Coulter Co., Hialeah, FL, U.S.A.). Data were collected for 104 cells in each sample, and displayed as the total number of cells in each of 1024 channels of increasing fluorescence intensity. The resulting histograms were analyzed for cell cycle distribution, using Multicycle software (Coulter Co.).

**RESULTS AND DISCUSSION**

**Biological Activity** When araSC was initially tested against human leukemic MOLT-4 cells (1×10⁶/well) for 72 h, the IC₅₀ (the concentration required for 50% inhibition of cell growth using the cell-count assay) was 0.005 μg/ml. This value was lower than that of 5-fluorouracil (1 μg/ml) and 5-fluorouracil (0.005 μg/ml) and 5-fluoro-2'-deoxyuridine (0.0045 μg/ml), higher than that of araC (0.002 μg/ml). We then compared the in vitro cytotoxic activities of araSC with those of araC in the several tumor cell lines. As indicated in Table 1, araSC showed potent cytotoxicity in all cell lines tested with IC₅₀ values ranging from 0.013 to 0.05 μg/ml. Although the numbers were higher than those of araC, the difference was not marked (1.0-3.8 folds).

**Enzymatic Stability and Plasma Concentration Profiles in Mice** Since the therapeutic activity of nucleoside antimetabolites depends not only on their in vitro cytotoxicity but retention in the body and/or biological stability, the kinetics and enzymatic susceptibility of araSC were studied and compared with those of araC. Susceptibility to enzymatic deamination was measured in the presence of mouse kidney cytidine deaminase. Though cytidine deaminase activity in human is high in the spleen and the liver, that in mice is restricted to the kidney. A pseudo-first-order reaction was observed and the corresponding deaminated products, araU and araSU, were detected quantitatively. Half-lives obtained from the first-order elimination profiles of araSC and araC were 165 and 42 min, respectively.

Plasma concentration-time profiles of araSC and araC after intravenous administration in mice are shown in Fig. 2. Plasma protein binding of both agents was determined in the presence of mouse plasma. As expected from their hydrophilic structures, both have little affinity to the protein (less than 3%). Although there was little difference in the elimination rate of the initial-phase, the rate of araSC calculated from the terminal-phase was much slower than that of araC. Distribution volumes (V₅₀) for central compartment of araSC and araC in mice were almost the same, 825 and 745 ml/kg, respectively. These observations suggest a larger AUC for araSC than araC, and this may partly account for its stability against enzymatic deamination.

**Cytokinetics** The effect of araSC on the percentage of cells (A375) with S-phase DNA content was investigated by flow cytometry. Although the cells in the logarithmic growth phase were exposed to concentrations of 1, 10, and 100
µg/ml, minimal change was observed in the cell DNA content histograms up to 100 µg/ml (Fig. 3). This finding is different from the reported S-phase specific effect of araC and its derivatives,\(^{(10)}\) in which significantly increased S-phase cells were detected by flow cytometry.

CONCLUSION

A 2-thiopyrimidine nucleoside analogue, araSC, shows potent cytotoxicity and higher enzymatic stability in vitro. The retention in mice in vivo was longer than that of araC. The effect on growing cells was also different from that of the S-phase specific agent, araC. These results indicate that araSC has novel characteristics as an anti-tumor agent. Since araSC is not a prodrug of araC and its mechanism for cytotoxicity seems to be different from that of araC, this agent may be effective against araC resistant tumor cells.

REFERENCES