Selective Activation of 5′-Deoxy-5-fluorouridine by Tumor Cells as a Basis for an Improved Therapeutic Index

R. Douglas Armstrong and Robert B. Diasio

ABSTRACT

The intracellular metabolism of a new fluoropyrimidine, 5′-deoxy-5-fluorouridine (5′-dFUr), was compared with the metabolism of 5-fluorouracil (FUr), 5-fluorouridine (FUr), and 5-fluoro-2′-deoxyuridine (FdUr) in freshly isolated bone marrow cells and Ehrlich ascites tumor cells. Following exposure to tumor cells, all four fluoropyrimidines were metabolized to identical products (i.e., FUr, 5-fluorouridine 5′-monophosphate, 5-fluorouridine 5′-diphosphate, 5-fluorouridine 5′-triphosphate, and 5-fluoro-2′-deoxyuridine 5′-monophosphate), all produced an incorporation of FUr into RNA (FUr > FUr > FdUr > 5′-dFUr), and all completely inhibited thymidylate synthetase activity by 1 hr. However, in bone marrow cells, very different patterns were observed. 5′-dFUr accumulated in the cells, but there were no measurable metabolism, no incorporation of FUr into RNA, and no inhibition of thymidylate synthetase activity. In contrast, both FUr and FUr were metabolized and produced an incorporation of FUr into RNA (2.7 pmol FUr per µg RNA and 4.8 pmol FUr per µg RNA at 2 hr, respectively) in bone marrow. Only a minor inhibition of thymidylate synthetase activity was detected. FdUr was also metabolized by bone marrow cells, produced a low level of FUr incorporation into RNA (0.23 pmol FUr per µg RNA at 2 hr), and produced a complete inhibition of thymidylate synthetase activity. Since 5′-dFUr is not directly cytotoxic itself, its superior therapeutic index compared to other fluoropyrimidines may largely reflect the selective activation of 5′-dFUr by sensitive tumor cells as opposed to bone marrow cells, which can activate FUr, FUr, and FdUr.

INTRODUCTION

The sensitivity of normal host tissues, such as the bone marrow, to cytotoxicity caused by fluoropyrimidines limits the administration of an effective therapeutic dose for eradication of otherwise fluoropyrimidine-sensitive cancers (6, 12). As a result, many attempts have been made to synthesize analogs of FUr, the prototype fluoropyrimidine, to either increase the antineoplastic activity or to increase the therapeutic index (12). Recently, a new synthetic fluoropyrimidine, 5′-dFUr, (Chart 1) has been demonstrated (2, 3, 7, 8) to have antineoplastic activity superior to FUr, FUr, FdUr, or the FUr prodrug, fluorafur, when tested against several rodent tumors. 5′-dFUr also exhibited a marked increase in the therapeutic index compared to the other fluoropyrimidines (3, 8). Study results have shown that 5′-dFUr produces less leukopenia (3) and is also less immunosuppressive (11) than other fluoropyrimidines.

The present studies attempt to determine a basis for the apparent increased therapeutic index of 5′-dFUr with regard to myelosuppression. The intracellular metabolism, the incorporation of FUr into RNA, and the thymidylate synthetase activity were characterized in both mouse bone marrow cells and sensitive (2) Ehrlich ascites tumor cells following their exposure in vitro to either 5′-dFUr, FUr, FUr, or FdUr.

MATERIALS AND METHODS

Chemicals. [6-3H]FUr (18 mCi/µmol), [6-3H]FdUr (18 mCi/µmol), and [6-H]FUr (18 mCi/µmol) were purchased from Moravek Biochemicals, City of Industry, Calif. 5′-[2-14C]FdUr (104 µCi/mg), 5′-dFUr, and FUr were generously supplied by Hoffman-LaRoche Inc., Nutley, N. J. Liquid scintillation-counting cocktail and supplies were purchased from Research Products International Corp., Elk Grove Village, Ill. All other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Liquid Chromatography. A Hewlett-Packard Model 1084 A high-performance liquid chromatograph, equipped with an automatic injection system, was utilized for all fluoropyrimidine separations. Samples were separated by use of a 5-µm (30 cm × 0.4 cm) Spheri-5, C-18 reverse-phase column (Brownlee Labs, Santa Clara, Calif.). Samples were eluted with 5 mM TBHPS, containing 31.2 mM potassium phosphate and 1.8 mM potassium hydroxide (pH 7), and with 2.5 mM TBHPS in methanol (designated methanol-TBHA). High-performance liquid chromatograph runs were carried out at 1 ml/min isocratically from 0 to 20 min, followed by a linear gradient from 20 to 60 min (to 25% methanol-TBHA), and then a linear gradient to 75% methanol-TBHA from 60 to 80 min. Column temperature was maintained at 25° during each run. Column eluate was collected as 1-ml fractions in 7-ml scintillation-counting vials by an LKB RediRac fraction collector. Five ml of Triton-based toluene scintillation fluor were added to each fraction, and radioactivity was measured by a Beckman LS-8000 liquid scintillation counter. This methodology enabled resolution of FUr (7.0 min), FdUr (22 min), 5′-dFUr (32 min), FUMP (24 min), FdUMP (27 min), FUDP (44.5 min), FdUDP (46 min), FUTP (60 min), and FdUTP (60.5 min).

Tumor Cells. Ehrlich ascites tumor cells (obtained from the Laboratory of Chemical Pharmacology, NIH, Bethesda, Md.) were used for the studies reported in this paper. The line was maintained in male CF−1 mice, with cells used for experimentation 8 to 10 days following inoculation of approximately 4 × 10⁶ cells.

Intracellular Metabolism Study. Ehrlich ascites tumor cells were harvested from the peritoneal cavity of CF−1 mice immediately prior to each experiment and washed twice with ice-cold 0.85% NaCl solution. The washed cell pellet for each incubation contained an average of 1.4 × 10⁶ cells. Bone marrow was obtained from the tibias and femurs of male CF−1 mice (approximately 40 mice per experiment) by flushing the isolated, open-ended bones with an iced minimal essential medium.
buffer (5). The isolated marrow was washed 3 times and consisted of an average of 2.1 x 10⁶ cells. The isolated tumor or bone marrow cells were warmed to 37°C and then mixed with 10 ml of an incubation medium containing a minimal essential medium with glucose and 30 μM fluoropyrimidine. When isotope was used, the specific activity of all drugs was adjusted with unlabeled drug to 25.7 μCi/μmol. This mixture was contained in a round-bottomed incubation flask held in a 37°C water bath. The mixture was stirred constantly with a Teflon paddle, and the pH was maintained at 7.2 to 7.4 by passing warmed and humidified 95% O₂-5% CO₂ over the cell suspension. At specific time intervals from the start of the incubation, 1-ml aliquots were removed and added to 5 ml of an iced 100 mM dipyridamole-0.85% NaCl solution. This was immediately centrifuged at 2000 x g for 25 sec, and the supernatant was discarded. The cell pellet was disrupted by addition of 1 ml of 5% trichloroacetic acid. Following centrifugation, 1 ml of the supernatant was removed and adjusted to pH 6.0 with 3 M NaOH and 0.1 M sodium phosphate buffer. A 200-μl sample was then examined for intracellular acid-soluble fluoropyrimidines using the described liquid chromatographic methodology.

Determination of Radiolabeled Fluoropyrimidine Associated with RNA. As described elsewhere (17), RNA extractions were completed on the trichloroacetic acid precipitate obtained from the incubation procedure described above. RNA was quantitated by the orcinol reaction (15).

Determination of Thymidylate Synthetase Activity. Analogous to the incubation conditions described above, cells were incubated in either the absence of drug (controls) or the presence of 30 μM unlabeled FUra, FUrd, FdUrd, or 5'-dFUrd. One-ml aliquots were removed from the incubation mixture, injected into an ice-cold centrifuge tube containing 0.85% NaCl solution, and immediately centrifuged, and the supernatant was discarded. Three hundred μl of iced 0.85% NaCl solution, and immediately centrifuged at 2000 x g for 5 min. The cellular debris was sepa rated by centrifugation at 40,000 x g for 30 min. The supernatant was removed and examined for thymidylate synthetase activity, as FUra, FUrd, and FdUrd were all metabolized by both tumor and bone marrow cells. [3H]FUra formed ribonucleotides in both bone marrow and tumor cells. [3H]FUrd was phosphorylated by both tumor and bone marrow cells to the ribonucleotides and also was cleaved to FUra. Exposure of either bone marrow or tumor cells to [3H]FdUrd resulted in expansion of

RESULTS

Intracellular Metabolism. The acid-soluble fluoropyrimidines were characterized in both bone marrow and Ehrlich ascites tumor cells following their exposure to either 30 μM 5'-[14C]dFUrd, [3H]FUra, [3H]FUrd, or [3H]FdUrd. Representative profiles of the intracellular fluoropyrimidine pools obtained from 20-min incubations with bone marrow or tumor cells are shown in Chart 2. 5'-[14C]dFUrd accumulated in both bone marrow and tumor cells. However, 5'-dFUrd was not measurably metabolized by the bone marrow cells, whereas in tumor cells, 5'-dFUrd was metabolized to FUra and the ribonucleotides (i.e., FUMP, FUDP, and FUTP). Only 5'-dFUrd exhibited this selectivity, as FUra, FUrd, and FdUrd were all metabolized by both bone marrow and tumor cells. [3H]FUrd formed ribonucleotides in both bone marrow and tumor cells. [3H]FUrd was phosphorylated by both tumor and bone marrow cells to the ribonucleotides and also was cleaved to FUra. Exposure of either bone marrow or tumor cells to [3H]FdUrd resulted in expansion of

Chart 2. The percentage of acid-soluble fluoropyrimidines detected in either mouse bone marrow (open bars) or Ehrlich ascites tumor cells (striped bars) following a 20-min exposure to either 30 μM 5'-[14C]dFUrd, [3H]FUra, [3H]FUrd, or [3H]FdUrd in vitro and as described under "Materials and Methods."
small FUra, FdUMP, and ribonucleotide pools. In general, it is apparent that, for each fluoropyrimidine, metabolism occurred more rapidly in the Ehrlich ascites tumor cells than in bone marrow cells.

Incorporation of FUra into RNA. The incorporation of FUra into the RNA of either bone marrow or Ehrlich ascites tumor cells during exposure to either 30 μM 5'-[14C]dFUrd, [3H]FUrd, or [3H]FUra or [3H]FdUrd is depicted in Chart 3, A (bone marrow) and B (tumor). There was measurable FUra incorporation in bone marrow cells incubated with [3H]FUra, [3H]FUrd, or [3H]FdUrd, although the rates of incorporation were very different (i.e., FUrd > FUra > FdUrd). There was no detectable incorporation of FUra into RNA in bone marrow cells incubated for 2 hr with 5'-[14C]dFUrd. In Ehrlich ascites tumor cells, FUra incorporation was measurable with all 4 fluoropyrimidines. [3H]FUrd exhibited the fastest rate of FUra incorporation, followed by [3H]FUra and [3H]FdUrd, which had similar rates. 5'-[14C]dFUrd produced the slowest rate of FUra incorporation into RNA tumor cells, although a large level of incorporation was detected by 60 min. Reflecting the patterns exhibited in the intracellular metabolism, FUra incorporation resulting from each of the fluoropyrimidines occurred more in the tumor cells than in the bone marrow cells.

Thymidylate Synthetase Activity. The thymidylate synthetase activity in cells during incubation with either 30 μM 5'-dFUrd, FUra, FUrd, or FdUrd is shown in Chart 4, A (bone marrow) and B (Ehrlich ascites tumor cells). Incubation of 5'-dFUrd with bone marrow cells did not produce any inhibition of thymidylate synthetase activity. FUra and FdUrd produced only a delayed, minor inhibition of thymidylate synthetase activity in bone marrow cells. Exposure of tumor cells to either 30 μM 5'-dFUrd, FUrd, or FUra produced essentially a complete inhibition of thymidylate synthetase activity by 1 hr, although the rate of onset of enzyme inhibition varied for each agent (i.e., FUrd > FUra > 5'-dFUrd) and was much slower than that exhibited by FdUrd. FdUrd produced a rapid and complete inhibition of thymidylate synthetase activity in both bone marrow and tumor cells. Although FdUMP was not detected in tumor cells as an acid-soluble metabolite of 5'-dFUrd, FUra, or FUrd, the inhibition of thymidylate synthetase activity suggests that FdUMP was produced.

**DISCUSSION**

Cytotoxicity resulting from the fluoropyrimidines is known to be mediated, either singly or in combination, by (a) an incorporation of FUra into RNA following the metabolic production of FUTP (9) or (b) an inhibition of thymidylate synthetase activity by FdUMP (6). The results reported in this paper suggest that both of these events occur in tumor cells when exposed to either 5'-dFUrd, FUrd, FUra, or FdUrd. However, in bone marrow cells, very different patterns were observed. 5'-dFUrd was not measurably metabolized by bone marrow cells (Chart 2). This was demonstrated by the absence of acid-soluble metabolites as well as by the lack of either incorporation of FUra into RNA or detectable inhibition of thymidylate synthetase activity in bone marrow cells exposed to 5'-dFUrd for 2 hr. Since 5'-dFUrd has no apparent direct cytotoxicity itself (1, 7), bone marrow cells should be resistant to cytotoxicity from 5'-dFUrd. 5'-dFUrd alone exhibits this selectivity for activation by tumor cells as opposed to bone marrow cells.
Similarly, FURA can be converted directly into FUMP through 5'-phosphoribosyl-1-pyrophosphate-transferase with subsequent conversion to FUTP andFdUMP (6). As a result, FURA, Furd, and FdUrd can all produce one or both of the active metabolites independently of phosphorylase activity. These patterns were exhibited in the studies reported here. It is uncertain why FURA is detected from both FURd and FdUrd, which suggests possible phosphorylase activity, and not from 5'-dFUrd. It may simply reflect the affinity difference between 5'-dFUrd and these agents for the low level of phosphorylase present. A second possibility is that FURd andFdUrd are cleaved by nucleoside ribosyltransferase and deoxyribosyltransferase (EC 2.4.2.5 and EC 2.4.2.6), respectively. 5'-dFUrd has not been shown to be a substrate for either of these enzymes.

While it is suggested that 5'-dFUrd appears not to be activated by bone marrow cells in vivo, other factors become involved in vivo. 5'-dFUrd may be metabolized by other tissues which can result in the formation of FURA in the blood (2). The FURA thus formed can enter bone marrow cells and proceed to produce either FUTP orFdUMP. However, a greater degree of tissue selectivity for 5'-dFUrd should still result, compared to the other fluoropyrimidines, since tumor cells will still directly activate 5'-dFUrd to FURA, while the formation of FURA in bone marrow remains dependent upon the metabolism of 5'-dFUrd at some secondary site. The FURA prodrug, florafor, is similar to 5'-dFUrd in that it also is not apparently metabolized by bone marrow cells. However, in contrast to 5'-dFUrd, tumor cells appear to be incapable of directly metabolizing florafor (12). Formation of FURA is thought to be dependent on breakdown of florafor by other tissue, notably the liver (12). Therefore, 5'-dFUrd would exhibit a selective advantage over florafor as well.

It has been suggested that bone marrow toxicity may result from FURA-RNA and not thymidylate synthetase inhibition (10). The results of the studies reported in this paper would support this concept. Neither FURd nor FURA, which are the most toxic fluoropyrimidines (6, 11, 12), produced inhibition of thymidylate synthetase activity in bone marrow cells. However, FURd and FURd did exhibit the highest rates of incorporation of FURA into RNA from bone marrow cells (Chart 3). FdUrd, which is generally less toxic than FURA or FURd in mice (5, 12), had the lowest detectable rate of incorporation of FURA into RNA and was the only fluoropyrimidine examined that produced a complete inhibition of thymidylate synthetase activity by 2 hr. Further studies utilizing equitoxic doses of each of these drugs are needed with examination of thymidylate synthetase activity and the rate of incorporation of FURA into RNA to determine whether incorporation of FURA into RNA correlates with hematicological toxicity, as would be implied by this study.

In conclusion, the superior therapeutic index of 5'-dFUrd, compared to other fluoropyrimidines administered to mice, may largely be the result of the selective activation of 5'-dFUrd by the sensitive tumor cells as opposed to the bone marrow. This selective activation of 5'-dFUrd by tumor cells represents an important and potentially valuable advance in fluoropyrimidine pharmacology. Similar studies are underway to determine the toxicity of 5'-dFUrd in human bone marrow.

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