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ORIGINAL ARTICLE

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Evaluation of plasma 5-fluorouracil nucleoside levels in patients with metastatic breast cancer: relationships with toxicities

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Abstract This paper describes the relationship between 5-fluorouracil (FUra)–derived toxicities and plasma levels of the FUra anabolites 5-fluorouridine (FUrd) and 5-fluoro-2′-deoxyuridine (FdUrd) monitored in patients receiving continuous infusions of FUra (1000 mg/m^2 per 24 h) over 5 days preceded by the administration of cisplatin (100 mg/m^2). A total of 63 courses of this treatment were given as second-line chemotherapy to 17 patients with metastatic breast cancer. The active FUra anabolites FUrd and FdUrd were monitored twice daily in the plasma by high-performance liquid chromatography. Data were analyzed using multiple analysis of variance (ANOVA). Only a low proportion of patients exhibited measurable plasmatic levels of FUrd (43%) and FdUrd (70%). The areas under the plasma concentration-time curves (AUC) determined over 120 h for FUrd (AUC_{FUrd}) and for FdUrd (AUC_{FdUrd}) were found to be statistically significantly different for chemotherapy cycles with and those without myelosuppression. Chemotherapy cycles without neutropenia were associated with low AUC_{FUrd} values (mean ± SEM, 2.9 ± 0.7 μg ml^{-1} h) and high AUC_{FdUrd} values (14.1 ± 2.7 μg ml^{-1} h), respectively, whereas courses with myelosuppression (WHO grades 2–4) showed inverse profiles with high AUC_{FUrd} values (16.3 ± 2.3 μg ml^{-1} h) and low AUC_{FdUrd} values (3.1 ± 1.0 μg ml^{-1} h), respectively. A statistically significant difference in AUC_{FdUrd} values was also observed between cycles with and those without mucositis ($P = 0.0027$), with AUC_{FdUrd} values being 22.6 ± 5.6 and 7.8 ± 1.9 μg ml^{-1} h, respectively. Whereas hematotoxicity could be correlated with both AUC_{FUrd} and AUC_{FdUrd} values, mucositis was associated with high AUC_{FdUrd} levels. Moreover, a negative correlation was found between the AUCs determined for FUrd and FdUrd ($P = 0.002$), indicating that activation of FUra via FUrd or via FdUrd may involve competitive processes. Therefore, to follow the development of the major FUra-derived toxicities, measurement of FUrd and FdUrd plasma levels appeared very attractive.

Key words 5-fluorouracil · Anabolites · 5-fluorouridine · 5-fluoro-2′-deoxyuridine · Breast cancer · Toxic side effects

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Introduction

The biochemical importance of the anabolites of 5-fluorouracil (FUra), including the nucleosides 5-fluorouridine (FUrd) and 5-fluro-2′-deoxyuridine (FdUrd) and the nucleotide 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), has recently been reviewed [20]. The exploration of their metabolic pathways is of great interest since the mechanism(s) through which FUra kills neoplastic cells and/or normal cells in healthy tissues with manifestation of limiting toxicity remains uncertain and under debate. One explanation might be that FdUMP inhibits the enzyme thymidylate synthetase (TS) after phosphodeoxyribosylation of FdUrd. TS methylates deoxyuridine monophosphate (dUMP) to 2-deoxythymidine-5′-monophosphate (dTMP), which is an important step in the de novo synthesis of deoxythymidylate, 2-deoxythymidine-5′-triphosphate (dTTP). 5,10-methylenetetrahydrofolate (Me-THF) then forms a ternary complex with TS and dUMP, or FdUMP. The rate of inhibition of TS by FUra and the

stability of the complex depend on the amount of enzyme and on the cellular concentrations of Me-THF, dUMP, and FdUMP, respectively [2].

Numerous cellular events need to be considered when studies are designed to correlate drug or metabolite pharmacokinetics and response to FUra. The cytotoxic effect can also be assessed by the incorporation of 5-fluorouridine-5′-triphosphate (FUTP) into RNA or by the incorporation of 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP) into DNA. There is no evidence that any of these mechanisms is more important than one of the others. The activity of key enzymes is the different pathways may determine the mechanisms of action through which they work [20] and may vary between tumoral and normal tissue and even between tumors of different origins.

Continuous venous infusion (CVI) chemotherapy has recently been advocated so as to reduce the toxic side effects of antitumor drugs without impairing their antitumor effectiveness [33]. This was claimed in particular for FUra given by CVI over 5 consecutive days to patients with gastrointestinal carcinoma [23]. This schedule was also combined with *cis*-diamminedichloroplatinum (CDDP) for patients with head and neck or breast cancer [13]. The dose-limiting toxicities of 5-day CVI of FUra are mostly hematotoxicity, mucositis, and nausea/vomiting [12, 15]. Whereas numerous studies showed a correlation between the plasma FUra concentrations achieved and the myelosuppressive effect of the therapy courses [10, 18], the occurrence of the other toxic side effects was not found to be correlated with high plasma concentrations of FUra [30].

Studies of cellular pharmacokinetics and pharmacodynamics met practical difficulties since surgical biopsies were needed before and after the administration of the drug [26]. To overcome these drawbacks, the intracellular concentration of FUra was determined by noninvasive techniques such as nuclear magnetic resonance spectroscopy (NMRS) [35]. Nevertheless, this technique currently lacks the appropriate sensitivity for the determination of intracellular metabolites [17].

Several groups reported the existence of correlations between the individual capacity to eliminate FUra or its metabolites and the plasmatic concentrations of these molecules and the therapy-related toxicites [7, 22, 24, 29]. The importance of the role of the nucleosides FUrd and FdUrd with regard to side effects in mice was demonstrated by Houghton et al. [11]. FUrd and FdUrd were reported to induce toxicity at considerably different dose levels [19].

The present study reports on monitoring of plasma concentrations of FUra, FUrd, and FdUrd in patients with recurrent metastatic breast cancer and discusses the value of this procedure for predicting the development of FUra-derived toxicities, representing herewith a novel pharmacological approach to monitoring of FUra toxicity in clinical studies.

Patients and methods

Patients' characteristics

A total of 17 women with metastatic breast cancer relapsing after our standard chemotherapy protocol for this disease agreed to participate in this study after having been informed of its aims and nature. The patients' characteristics are described in Table 1. The study started after it had received approbation from the Ethics Committee of the Alexis Vautrin Cancer Center.

Table 1 Patients' characteristics *B* bone, *L* liver, *N* nodes, *O* omentum, *S* skin, *Lu* lung, *Pe* peritoneum, *Pl* pleura, *N* normality, *Ab* abnormality

Patients	Age (years)	Number of cycles per patient	Type of metastasis	Liver function	Total dose (per cycle)	
					CDDP (mg)	FUra (mg)
1	60	2	B	N	150	7500
2	50	5	B, Pe, O	N	125	7000
3	63	5	Pe, N	N	150	7500
4	52	2	None	N	150	4500
5	54	3	B, Pe, Pl	N	150	7500
6	54	5	L	Ab	150	7500
7	43	3	N, B, S	N	150	7500
8	40	3	L	Ab	150	7500
9	69	6	L	Ab	150	7500
10	50	6	P	N	140	7000
11	64	3	B, L, S	Ab	160	8000
12	49	6	B, L, Lu	N	150	5000
13	62	1	None	N	150	7500
14	50	6	None	N	170	8000
15	54	3	S	N	150	7500
16	51	3	B	N	150	7500
17	52	3	N	N	100	7500

Chemotherapy protocol

Patients received CDDP on day 1 (Cisplatyl; Roger Bellon, Paris, France) at a dose of 100 mg/m^2 diluted in 250 ml of 0.9% NaCl solution by intravenous infusion at a rate of 1 mg/min, starting at 5 p.m. Intravenous prehydration consisted of a 3-h infusion of 1 l of 5% dextrose containing 6 g of NaCl, 3 g of KCl, and 500 ml of 20% mannitol. Therapy with FUra (5-Fluoro-uracile; Roche, Neuilly, France) started at 8 p.m. on day 1 and lasted for 120 h. FUra was infused intravenously at a constant rate with a volumetric pump (VMM; Vial Medical, France). The dose of FUra (1000 mg/m^2) to be delivered over 24 h was diluted in 1 l of 5% dextrose. The whole therapy was repeated every 21–28 days for six cycles.

Hematology tests and hepatic and renal function tests were determined within 1 week of (typically or the day prior to) the initiation of chemotherapy. All patients had normal renal function as gauged by serum creatinine levels, but four patients had altered liver function as gauged by serum γ-glutamyltransferase, transaminase, and bilirubin values (Table 1). Toxicities were quantified according to the World Health Organization (WHO) grades [34].

Sample pretreatment

Blood samples (5 ml) collected into tubes containing ethylenediaminetetraacetic acid (EDTA) were taken at 6 p.m. on day 1 before the start of the FUra infusion, at 8 a.m. and 5 p.m. on days 2–5, and at 8 a.m. on day 6. Subsequently, the tubes were centrifuged at 4°C at 3000 g for 10 min and the plasma was stored at −20°C until analysis. FUra, FUrd, and FdUrd plasma levels were determined for each patient and each course prior to the infusion and at regular intervals during the 120-h continuous infusion period. Aliquots of plasma (0.5 ml) were transferred into polypropylene tubes. After the addition of 3 ml acetonitrile the tubes were gently mixed and vortexed. Following centrifugation (10 min at 3000 *g*), the organic layer was pipetted into a 5-ml tube and evaporated to dryness at 45°C under a stream of nitrogen. The dried residue was dissolved in 200 µl of ethyl acetate-isopropanol (95: 5, v/v), vortexed for 30 s, and then eluted through a C18 Sep-Pack cartridge (Millipore HA, Molsheim, France) with 2 ml ethyl acetate-methanol (80: 20, v/v). The eluate was evaporated to dryness for 30 min as described above. The final residue was dissolved in 200 µl of deionized water and filtered through a 0.45-µm membrane (Millipore HV4). Aliquots of 30 µl were introduced into the chromatographic system by the autosampler.

Chromatography

The high-performance liquid chromatography (HPLC) method used has previously been described in detail [1]. The HPLC system consisted of a computer-monitored System Gold PC apparatus (Beckman, Gagny, France), a WISP 512 automatic injector (Waters Millipore, Molsheim, France) and a Beckman ULTRASPHERE ODS 5-µm microbore column (150 × 2 mm inside diameter). The mobile phase was KH_2PO_4, 2×10^{-2} *M*, pH 5.0: methanol (95: 5, v/v). Compounds of interest were monitored at a flow rate of 0.3 ml/min and the mobile phase was not recycled. Detection was performed at 280 nm. The determination limits of the assay were 2 (FUra), 10 (FUrd), and 10 ng/ml (FdUrd).

Pharmacokinetic analysis

Areas under the curve (AUC), representing total drug exposures during each cycle, where calculated by the trapezoidal rule without extrapolation.

Statistical analysis

AUCs determined for FUra, FUrd, and FdUrd and the degree of toxic side effects were analyzed for correlation using one-way analysis of variance(ANOVA) with Scheffé's procedure for multiple comparisons with a 95% confidence limit. The correlations between AUCs calculated for FUrd and FdUrd were checked using the Spearman rank correlation coefficient (r) .

Results

Treatment tolerance

The limiting toxicity of this therapy regimen was leukopenia in 40% of patients and neutropenia in 43%, followed by mucositis in 30%, vomiting in 35% and alopecia in 32%. In all, 19% of the cycles presented leukopenia (WHO grade > 1) and 35%, neutropenia (WHO grade > 1) . Overall, 63 cycles were evaluable for mucosal toxicity. Vomiting was observed in 35% of the cycles, and diarrhea occurred in 10% and was of moderate degree only. Alopecia, although observed in 51% of patients, was unexpected with this chemotherapy regimen.

Hematologic toxicities

With regard to myelosuppression, statistically significant differences in the AUC values determined for FUrd and FdUrd were found between cycles with and those without hematotoxicity, but no significant difference was calculated for AUC_{FUra} (Table 2). Chemotherapy cycles without hematotoxicity were associated with low AUC_{FUrd} values and high AUC_{FdUrd} values, whereas for myelosuppression the inverse profile was observed.

Mucositis

The 120-h plasma concentration of FUra, FUrd, and FdUrd were characterized by large intersubject variation. No statistically significant difference was found between AUC_{FUra} values and AUC_{FUrd} values obtained for patients with and without mucositis. Conversely, a statistically significant difference in AUC_{FdUrd} was observed between patients without ($n = 41$) or with ($n = 22$) mucositis ($P = 0.0027$, Table 2). However, a very high value for AUC_{FdUrd} (63.6 µg ml^{-1} h) was calculated for one patient who did not develop mucositis at the end of her first cycle but in whom WHO grade 3 mucositis appeared only during the four subsequent courses. It can be seen that this patient had an abnormality in her liver function test (patient 9, Table 1). On the basis of the ratio between the AUCs determined for FdUrd and FUrd, a statistically significant difference ($P = 0.001$, Table 2) was observed between the cycles without mucositis (grades 0, 1) and the others (grades 2–4).

Table 2 Interrelationships evaluated between the AUC values determined for FUra and its anabolites and the toxicitied oberved. *P* values were determined by ANOVA with Scheffe's procedure (*n* number of cycles evaluated, *NS* no significant difference)

Toxicities	AUC ($\mu g\ ml^{-1}$ n)			
	FUra (mean ± SE)	FUrd (mean ± SE)	FdUrd (mean ± SE)	FdUrd/FUrd (mean ± SE)
Grades 0, 1 ($n = 43$)	35.9 ± 2.0	2.9 ± 0.7	14.1 ± 2.7	11.7 ± 2.6
Neutropenia Grades 2–4 ($n = 20$)	NS	$P = 0.0001$	$P = 0.0083$	$P = 0.0095$
	34.2 ± 3.6	16.3 ± 2.3	3.1 ± 1.0	1.3 ± 1.0
Grades 0, 1 ($n = 41$)	34.7 ± 1.9	7.7 ± 1.4	7.8 ± 1.9	5.4 ± 1.7
Mucositis (Grades 2–4) ($n = 22$)	NS	NS	$P = 0.0027$	$P = 0.001$
	38.2 ± 4.6	4.7 ± 2.1	22.6 ± 5.6	20.9 ± 6.0

Pharmacokinetics

FUra analysis

AUC_{FUra} levels were evaluable for 16, 15, and 10 patients consecutively over 1, 2, and 3 cycles of therapy, respectively. Interindividual value dispersion was observed in all cycles, but the analysis of individual profiles from one cycle to the next revealed only a slight global increase in systemic drug exposure. The mean values varied from 34.5 ± 13.1 to 37.6 ± 16.3 to 44.3 ± 12.3 $\mu g\,ml^{-1}\,h$ for cycles 1, 2, and 3, respectively.

FUrd analysis

FUrd was detected in only 8 patients (43%). AUC_{FUrd} values ranged from 1.3 to 39.9 $\mu g\,ml^{-1}\,h$ (Fig. 1). It is noteworthy that patient 11, who had the highest AUC_{FUrd} value, also presented an abnormality in the liver function test (Table 1).

FdUrd analysis

FdUrd was detected in 12 patients (70%). Interindividual AUC_{FdUrd} value dispersion was also observed in all cycles, and the analysis of individual profiles revealed a variation of plasma concentration from cycle to cycle.

Relationship between FUra, FUrd, and FdUrd

High AUC_{FUrd} values ($> 30\ \mu g\,ml^{-1}\,h$) were detected only in patients presenting simultaneously low levels of FUra ($< 30\ \mu g\,ml^{-1}\,h$; Fig. 1A). No significant correlation was found between AUCs determined for FdUrd and FUra (Fig. 1B), but a statistically significant inverse correlation was demonstrated between AUC values calculated for FUrd and FdUrd ($r = -0.39$, $P < 0.002$; Fig. 1C).

Discussion

Considering the complicated cellular events determining the metabolic pathway of FUra involved in the pharmacodynamics of this drug [5, 20], it is apparent that the measurement of plasma concentrations of FUra alone can have only limited applicability for the individualization of treatment with this substance and that, unfortunately, the measurement of FUra levels in plasma is a poor indicator of tissue anabolite levels and the clinical response [8, 35]. To date, there have been few studies interested in the following of FUra-anabolite plasma levels. Malet-Martino et al. [17] observed high amounts of fluorine in biological samples using ^{19}F-NMR studies, and the plasmatic levels were well correlated with total fluorine levels in the tumor for all doses and over all infusion times. This suggested that anabolic activation of FUra produced steady-state fluorine concentrations corresponding to the sum of all levels of the fluorinated anabolites (FUrd and FdUrd in particular), but the NMRS technique currently lacks the appropriate sensitivity for determination of plasma metabolites [17].

In the present study, we show that in some cases the plasmatic circulating rates, i.e., the FUrd and/or FdUrd AUCs, can be close to or even higher than the plasmatic concentrations of FUra (Fig. 1). Stein et al. [28] have demonstrated that the cellular levels of anabolites consist of both FUra nucleosides and FUra nucleotides but that serum levels of anabolites comprise only FUra nucleosides. They also demonstrated that continuous infusion of FUra both produced higher levels of the anabolites than did bolus infusion and maintained constant levels throughout the infusion period. Cellular fluorinated nucleosides easily diffuse through the plasma membrane into the blood. In their

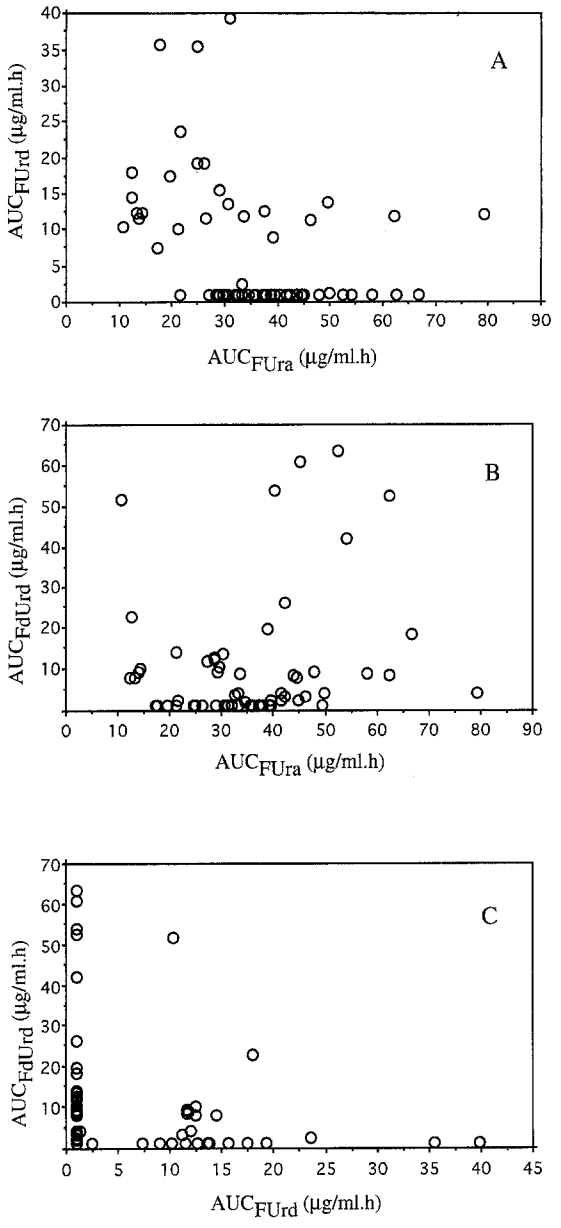

Fig. 1A–C Correlation plots between the respective AUC values determined for FUra, FUrd, and FdUrd in 7 patients who received consecutive cycles of chemotherapy. **A** High levels of FUrd were detected only in patients presenting simultaneously with low levels of FUra ($< 30\ \mu g\ ml^{-1}\ h$. **B** AUC_{FdUrd} fluctuated considerably from 2 to 64 $\mu g\ ml^{-1}\ h$, and no significant correlation was found between AUCs determined for FdUrd and FUra. **C** A statistically significant inverse correlation ($r = -0.39$, $P < 0.002$, Spearman rank correlation was demonstrated between AUCs found for FUrd and FdUrd

study, at 2 h after bolus infusion of FUra, levels of FUra anabolites exceeded FUra levels in serum. More interestingly, after continuous infusion, serum anabolite concentrations were found to be considerably greater than those of FUra [28].

The critical tissue and plasma levels of the anabolites of FUra, FUrd, and FdUrd required for clinical efficacy are not known. Blood levels of FUra do not accurately reflect tissue anabolite levels, and obtaining tumor samples during chemotherapy is very difficult. However, the following of plasmatic levels of anabolites could represent a new approach and could be an indicator for minimization of the toxic effects. Measurement of plasma FUra nucleoside levels could help to achieve this goal. In our study, FUrd and FdUrd plasma levels were determined by HPLC in 43% and 70% of cases, respectively. Monitoring of plasma concentrations of FUra, FUrd, and FdUrd in patients proved to be useful for predicting the development of some toxicities (Table 2). Therapy cycles without hematotoxicity were always accompanied by low AUC_{FUrd} and high AUC_{FdUrd} values, whereas courses with myelosuppression (WHO grades 2–4) showed an inverse profile (Table 2). This was also confirmed by the negative correlation found between the AUC values determined for FUrd and FdUrd, indicating that the activation of FUra to FUrd within the tumor might involve competitive processes (Fig. 1C).

On the whole, 63 cycles of therapy were evaluable for assessment of the correlation between drug levels and mucositis that was not associated with hematotoxicity. This observation was confirmed by the AUC values obtained for the anabolites (Table 2). More than 80% of FUra is eliminated through the metabolic route, mostly at the hepatic level. However, the existence of an extrahepatic metabolism has been demonstrated, since the systemic clearance of FUra is higher than the hepatic flow. Thus, a catabolic and anabolic pathway outside the liver have been demonstrated for FUra [16, 17]. Its elimination includes two major routes: renal clearance of unchanged drug (10% of the dose) and catabolic breakdown, mainly by the liver. Fleming et al. [6] have recently reported obtaining a significant correlation between FUra clearance and the activity of dihydropyrimidine dehydrogenase (DPD), the key regulating enzyme for FUra catabolism measured in lymphocytes. DPD activity in lymphocytes may reflect the activity observed in hepatic tissue, but the greatest DPD activity in human tissues has been observed in the liver [6]. Thus, it would be very exciting to use patients' pretreatment lymphocyte values to evaluate their ability to produce FUrd and/or FdUrd.

The end products of FUra, α-fluoro-β-alanine and α-fluoro-β-ureidopropionic acid, are also subjected to renal clearance, emphasizing the importance of normal renal function for FUra therapy [3]. CDDP partially inhibits the hepatic catabolism of FUra and is well known to cause renal toxicity. In this case, the FUra anabolic pathway gains importance. In our study, patients did not develop major renal toxicity, but in four patients an abnormality in liver function tests was noted (Table 1) and corresponded to cycles with high AUC_{FUrd} or AUC_{FdUrd} values.

The decrease in FUra clearance and the increase in anabolite (FUrd and FdUrd) levels can have a dramatic impact on the development of toxic side effects subjected to the steep dose-response relationship as

demonstrated by Houghton et al. [11] and could probably explain the high rate of mucositis observed during this chemotherapy regimen. As far as gastrointestinal toxicity is concerned, the three fluorinated pyrimidines considered in our study have been reported to produce toxicity in mice and even lethality at different dose levels after a single administration [11]. The order of toxicity was FUrd > FUra > FdUrd, and dose-related lesions appeared in most gastrointestinal tissues at sublethal doses. These data suggest that a common mechanism is involved in cell killing in the gastrointestinal tissues of mice that is independent of the nature of the fluorinated pyrimidine given [9, 31].

Possible explanations for the high FdUrd and/or FUrd levels observed could be a decreased uptake of these anabolites [21, 25] ; a lower hepatic extraction of FUra [4], which could be translated into an increase in the anabolic pathway; or an accumulation of plasma FdUrd and/or FUrd over long FUra infusion periods, because of a decrease in cell proliferation and utilization of anabolites [19, 28]. Thus, the activation routes of FUra could account for an important part of the metabolism of this molecule. A genetic variability in the expression of enzymes implied in the anabolic route could probably explain the large interindividual variations.

Nevertheless, it remains a matter of speculation as to whether the hematotoxicity associated with significantly higher FUrd levels is basically mediated via the FUrd–inhibited RNA synthesis and the mucositis associated with increased FdUrd levels is primarily dependent on FdUrd–inhibited DNA synthesis or whether a more complex association may exist.

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