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Changes in folate concentration in Yoshida sarcoma after administration of leucovorin or cisplatin

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Abstract Both leucovorin (LV) and cisplatin (*cis*-dichlorodiammine platinum II, CDDP) act as modulators of 5-fluorouracil (5-FUra) by increasing the intracellular concentration of reduced folate. We measured intracellular folate levels following the administration of LV or cisplatin in tumor-bearing rats to determine the optimal schedules for their use as 5-FUra modulators. Donryu rats were inoculated with Yoshida sarcoma cells on the right flank. Seven days after tumor inoculation, the animals were injected with LV or CDDP. The kinetic and dose-related changes in intracellular folate concentration were analyzed by means of a binding assay. Folate levels in the tumor tissues were significantly higher than baseline 1 and 2 h after administration of LV and remained significantly high until 8 h after administration. Folate levels in the tumor tissues were significantly higher than baseline 1 and 2 h after cisplatin administration, then decreased to a rather low level 8 h after, and to a significantly lower level than baseline 24 h after administration. The folate levels in the tumor tissue increased in proportion to the dose of LV, but did not increase when the dose of cisplatin was increased from 1 mg/kg to 8 mg/kg. Repeat high-dose administration of LV and repeat low-dose administration of cisplatin are advocated when they are used as modulators of 5-FUra.

Key words Biochemical modulation · Leucovorin
Cisplatin

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Introduction

Complete removal of malignant cells is the ideal method of treating gastric or colorectal carcinomas. However, recurrent disease is not rare even when extended procedures are employed, including resection of the organ origin, broad lymph node dissection, and combined resection of neighboring organs. The search for effective chemotherapy for these tumors has been in progress for a long time. However, carcinomas originating from the alimentary tract show poor response to conventional chemotherapy.

5-Fluorouracil (5-FUra) is an antimetabolite, which is now most frequently used in the treatment of carcinoma derived from the gastrointestinal tract. 5-FUra acts against tumor growth mainly by competitive inhibition of thymidylate synthase (TS), which is the key enzyme of de novo synthesis of pyrimidine nucleotides. However, some potential mechanisms of resistance have been observed after administration of 5-FUra [1, 2]. These mechanisms of resistance can be overcome by increasing the intracellular folate level. Leucovorin (5-formyltetrahydrofolate, LV) and cisplatin (*cis*-dichlorodiammine platinum II, CDDP) can modulate 5-FUra by increasing intracellular folate [3–5].

In this study, we analyzed the intracellular levels of 5,10-methylenetetrahydrofolate ($CH_2H_4PteGlu$) and tetrahydrofolate ($H_4PteGlu$) in tumor-bearing rats following the administration of LV or cisplatin. The objectives of this study were to compare their effects on intracellular folate levels and to determine the optimal administration schedules for these agents as modulators of 5-FUra.

Materials and methods

Tumor models

Male Donryu rats were obtained from Nippon SLC, Shizuoka, Japan. Yoshida sarcoma cells, obtained from T. Sasaki (Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa

Table 1 Changes in tumor folate level (pmol/g tissue; mean ± SD) following administration of leucovorin (*$CH_2H_4PteGlu$* methylenetetrahydrofolate)

Time after administration					
	Baseline	1 h	2 h	8 h	24 h
Total folate	3.6±0.6	7.8±1.8**	8.0±2.1*	5.9±0.9*	5.1±0.7*
$CH_2H_4PteGlu$	2.5±0.3	6.0±0.9**	5.7±0.9**	3.8±0.4**	2.6±0.3

*P <0.05; **P <0.01 vs baseline

Table 2 Changes in tumor folate level (pmol/g tissue; mean ± SD) following administration of cisplatin

Time after administration					
	Baseline	1 h	2 h	8 h	24 h
Total folate	2.3±0.3	4.7±0.9**	3.7±0.6*	2.0±0.4	1.3±0.2**
$CH_2H_4PteGlu$	1.4±0.1	2.9±0.6*	2.3±0.5*	1.0±0.4	0.8±0.2**

*P <0.05; **P <0.01 vs baseline

University), were passaged in Donryu rats by i.p. inoculation at weekly intervals. The Donryu rats were 4 weeks of age on arrival; they were quarantined for 1 week, then inoculated s.c. with 1×10^6 Yoshida sarcoma cells on the right flank. All animals were allowed access to food and water ad libitum.

Chemicals

d,l-LV and cisplatin were gifts from Nippon Lederle, Tokyo, Japan and Bristol-Myers Squibb, Tokyo, Japan, respectively. [6-^{3}H]fluorodeoxyuridine ([6-^{3}H]FdUrd) was purchased from New England Nuclear (Boston, Mass.). Other chemicals were purchased from Sigma Chemicals (St. Louis, Mo.).

Kinetic study of intracellular folate levels in Yoshida sarcoma following administration of LV or cisplatin

Seven days after the tumor inoculation, the animals were fasted for 24 h then injected with LV (100 mg/kg; expt 1) or cisplatin (4 mg/kg; expt 2) via the caudal vein. At 1, 2, 8, and 24 h after injection, the rats were anesthetized with 40 mg/kg sodium pentobarbital (i.p.) prior to removal of the tumor. Untreated rats were entered in each experiment as the source of the baseline values. Every group contained four rats.

Dose-related changes in intracellular folate levels in Yoshida sarcoma following administration of LV or cisplatin

Seven days after the tumor inoculation, the animals were fasted for 24 h, then injected with LV (25, 50, 100, or 200 mg/kg; expt 3) or cisplatin (0.2, 0.5, 1, 2, 4, or 8 mg/kg; expt 4) via the caudal vein. Two hours after injection, the rats were anesthetized with 40 mg/kg sodium pentobarbital (i.p.) prior to removal of the tumor. Untreated rats were entered in each experiment as the source of the baseline values. Every group contained five rats.

Preparation of tumor for folate assay

The tumor samples (250 mg) were homogenized with a fourfold amount of 0.2 M Tris-HCl solution (pH 7.4) containing 20 mM 2-mercaptoethanol, 15 mM cytidine 5′-monophosphate (CMP), and 0.1 M sodium fluoride, after which they were centrifuged (105000 *g*, 60 min). The supernates were used for the measurement of folate level.

Preparation of [6-^{3}H]FdUMP

[6-^{3}H]FdUMP was synthesized enzymatically from [6-^{3}H]FdUrd in the laboratories of Toyama Chemical (Toyama, Japan). The solution was diluted with water to give 3.7×10^6 dpm/ml radioactivity, and a concentration of 114 pmol/ml.

Cofactor solution A and B

Cofactor solution A consisted of 50 mM potassium phosphate buffer solution (pH 7.4) containing 9 mM formaldehyde, 20 mM 2-mercaptoethanol, 15 mM CMP, 0.1 M NaF, 16 mM ascorbic acid, and 2% fetal calf serum albumin. Cofactor solution B was the same solution with the formaldehyde omitted.

Methylenetetrahydrofolate and tetrahydrofolate assay

The folate levels were assayed by determination of ternary complex composed of intrinsic TS, intrinsic $CH_2H_4PteGlu$, and extrinsic tritiated FdUMP. The supernates prepared for folate assay were each incubated with 0.1 ml [6-^{3}H]FdUMP and 0.05 ml of cofactor solution A or B at 30° C for 20 min. The reaction was stopped with 10% trichloroacetic acid. $CH_2H_4PteGlu$, and $H_4PteGlu$ concentrations were measured as described previously [6]. We measured both the monoglutamate and the polyglutamates of methylenetetrahydrofolate as components of TC.

Statistical analysis

All results were expressed as means ± SD. Comparisons of values were analyzed by Student's or Welch's *t*-test. Statistical significance was considered to be achieved at P <0.05.

Results

Kinetic analysis of intracellular folate levels in Yoshida sarcoma following administration of LV or cisplatin

The sum of $CH_2H_4PteGlu$ and $H_4PteGlu$ (total folate) in the tumor tissue was increased about twofold compared with baseline at 1 and 2 h after administration of LV. These two values were significantly different from the baseline. The changes in $CH_2H_4PteGlu$ levels following administration of LV were similar to those in total folate levels. Subsequently, folate levels had declined 8 h after LV administration, but still remained significantly high. By 24 h after LV administration, $CH_2H_4PteGlu$ had declined to a level that was not significantly different from baseline, but total folate level

Table 3 Tumor folate level (pmol/g tissue; mean ± SD) following administration of various doses of leucovorin

	Dose of leucovorin (mg/kg)				
	Baseline	25	50	100	200
Total folate	2.6±0.3	3.6±0.2**	4.3±0.9*	5.1±0.1**	5.5±0.9**
$CH_2H_4PteGlu$	1.7±0.3	2.4±0.2**	2.7±0.5*	3.2±0.6**	3.6±0.5**

*P <0.05; **P <0.01 vs baseline

Table 4 Tumor folate level (pmol/g tissue; mean ± SD) following administration of various doses of cisplatin

	Dose of cisplatin (mg/kg)						
	Baseline	0.2	0.5	1.0	2.0	4.0	8.0
Total folate	2.7±0.3	2.9±0.3	3.7±0.6*	5.1±0.8**	4.8±0.5**	5.2±0.7**	4.8±0.9**
$CH_2H_4PteGlu$	1.6±0.5	2.0±0.5	2.5±0.6	3.2±0.5**	3.0±0.4**	3.0±0.9*	2.9±0.5*

*P <0.05; **P <0.01 vs baseline

still remained significantly high (expt 1; Table 1). Total folate and $CH_2H_4PteGlu$ levels in the tumor tissue were significantly higher than baseline at 1 and 2 h after administration of cisplatin. Folate levels at these times were significantly higher than baseline. However, 24 h after administration of cisplatinm total folate and $CH_2H_4PteGlu$ had declined to significantly low levels (expt 2; Table 2).

Dose-related changes in intracellular folate levels in Yoshida sarcoma following administration of LV or cisplatin

Intracellular folate levels after LV were increased in a dose-dependent manner. At doses above 25 mg/kg, folate levels were significantly higher than baseline. Total folate and $CH_2H_4PteGlu$ levels increased 2.1-fold following administration of 200 mg/kg of LV (expt 3; Table 3). Following administration of cisplatin, total folate and $CH_2H_4PteGlu$ also increased, showing dose dependency at doses less than 1 mg/kg. The values for total folate and $CH_2H_4PteGlu$ were significantly different from baseline at doses more than 0.5 and 1.0 mg/kg, respectively. However, the levels did not increase when the dose of cisplatin was increased from 1 mg/kg to 8 mg/kg (expt 4; Table 4).

Discussion

In recent years, combination chemotherapy has been expanded to include biochemical modulation, which employs a drug (modulator) to increase the pharmacological effects of the anticancer drug (effector) by selectively enhancing the activity of the effector [7, 8]. 5-FUra is the drug most commonly used as an effector. Such combination arms with 5-FUra and its modulators lead to substantial improvement in the treatment of colonic and gastric carcinoma [7, 9]. Many agents have been reported as modulators of 5-FUra. Uracil inhibits the degradation of 5-FU to F β-alanine and enhances its anti-tumor activity [10]. Methotrexate acts as a modulator of 5-FUra by increasing the concentration of phosphoribosylpyrophosphate in the tumor [11, 12]. Alpha-2a-interferon may also demonstrate a modulator effect on 5-FUra [13], but the therapeutic advantages of its concomitant use with 5-FUra have been questioned [14, 15].

5-Fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), a metabolite of 5-FUra, competitively inhibits TS by forming a ternary complex (TC). This TC is composed of FdUMP, TS and CH_2H_4PtGlu. To achieve a cytocidal effect of 5-FUra it is necessary to obtain a high TS inhibition rate (TSIR). If the total TS levels and FdUMP supply and deoxyuridine monophosphate (dUMP) levels are the same, TSIR appears to depend on reduced folate levels and increases in proportion to it. Intracellular folate deficiency, low FdUMP, high dUMP, and high TS levels might reduce TSIR, resulting in innate resistance to 5-FUra, which can be overcome by an increase in intracellular folate [1]. Moreover, the intracellular level of $CH_2H_4PteGlu$ affects the stabillity of TC [6]. Consequently, a high intracellular concentration of $CH_2H_4PteGlu$ is necessary to achieve satisfactory results with 5-FUra chemotherapy. Both LV and cisplatin act as modulators of 5-FUra by increasing the intracellular folate level.

We measured the ternary complex by adding a sufficient amount of tritiated FdUMP to cytosol as the folate level. This value is generally correlated with the endogenous folate pool, but might actually be affected by the endogenous TS level. However, we have confirmed that the TS level is unchanged 2 h after administration of 5-FUra in solid Yoshida sarcoma (data not shown). Furthermore, our purpose was to investigate not the actual amount of folates, but the kinesis and dose-related changes of the intracellular folate level after LV or cisplatin. In this study, we used a homogeneous tumor model consisting of a Donryu rat with Yoshida sarcoma. This experimental model proved suitable for our purpose.

The major clinical success in modulation of 5-FUra has been achieved with the use of LV [4, 16–18]. However, the optimal dose and schedule for LV as a modulator of 5-FUra has remained obscure. Many prospective randomized clinical trials have been performed to establish the most efficacious regimen. The Gastrointestinal Tumor Study Group reported a prospective and randomized study of

343 previously untreated patients with measurable metastatic colorectal carcinoma [19]. The response rate was significantly increased from 12% to 30% by the addition of high-dose LV (500 mg/m^2) to 5-FUra, but low-dose LV did not increase the response rate over 5-FUra alone. The study of the Mayo Clinic-North Central Cancer Treatment Group, which included 280 patients with metastatic colonic carcinoma, gave rise to the conclusion that both high-dose and low-dose LV lengthened the survival time of the patients [16]. Since LV alone has no antimetabolic effect, a synergistic effect of LV on 5-FUra depends on the increase in folate levels following LV administration. In our study, folate levels in the tumor tissue were significantly higher than baseline at 1 and 2 h after administration of LV and remained high until 8–24 h after administration. The increase in intracellular folate levels correlated with the dose of LV, reaching a 2.1-fold increase following administration of 200 mg/kg of LV. Therefore, the optimal dose of LV as a modulator of 5-FUra in rats will be 200 mg/kg, or more, from the viewpoint of augmentation of intracellular folates. However, most of the folate increase following LV will be in monoglutamate form. The correlation between the synergistic effect of LV on 5-FUra and the increase of the intracellular folate pool should be examined in future studies.

Cisplatin is also a modulator of 5-FUra. Although cisplatin itself is cytotoxic through binding with DNA bases, its synergism with 5-FUra is believed to be related to some other mechanism [20]. Recently, we investigated the possible biochemical modulation of 5-FUra by cisplatin in rodent tumor models in vivo [21]. We found that i.p. administration of cisplatin (5 mg/kg) significantly enhanced 5-FUra cytotoxicity by inhibiting intracellular L-methionine metabolism and consequently increasing the reduced folate pool in Yoshida sarcoma cells. Moreover, we found that i.p. administration of cisplatin on day 1 and continuous infusion of 5-FUra from day 1 to day 6 had a synergistic effect against tumor growth in Yoshida sarcoma-bearing rats.

We measured the augmentation of intracellular folate level using solid Yoshida sarcoma in the present study. The time-course of folate level change in a solid tumor will presumably be different from that in ascitic cells [21]. The folate levels in the tumor tissue were significantly increased over baseline at 1 and 2 h after the injection of cisplatin. The maximum inhibition of amino acid accumulation in L1210 murine leukemia cells occurred following 10–30 min of preincubation with cisplatin [20, 22]. These processes corresponded in time to the increase in reduced folate in the tumor tissue. We found here that folate declined to a rather low level 24 h after cisplatin administration. Folate levels were also increased in proportion to the dose of cisplatin at doses less than 1 mg/kg, but they did not show any correlation at doses above 1 mg/kg. We reported dose-dependent inhibition of L-methionine uptake by ascitic tumor cells after i.p. administration of cisplatin [21]. However, we consider that the limitation of folate augmentation revealed in this study is due to limitation of intracellular homocysteine and the *S*-adenosyl-methionine pool. Consequently, even if cisplatin causes dose-dependent inhibition of methionine transport, the increase in intracellular folate following methionine deficiency will not be correlated with the dose of cisplatin administered and will have some upper limit. Methionine transport into L1210 cells is inhibited by aqueous derivatives of cisplatin at concentrations as low as 10 μM in vitro, and there is little further inhibition at concentrations of 25 to 50 μM [20]. The biosynthesis of folate following the deficiency of intracellular methionine can also be brought about by a low-dose injection of cisplatin in vivo. The optimal schedule for cisplatin as a modulator of 5-FUra seemed to be repeat low-dose administration.

The concomitant use of 5-FUra and a modulator may improve the response rate. However, the side-effects of such combination chemotherapy are far from negligible [17, 23]. The findings in the present study have demonstrated that repeat high-dose administration of LV and repeat low-dose administration of cisplatin are optimal for use in the modulation of 5-FUra with regard to intracellular folate change. Further studies are mandatory to clarify the clinical response and side effects of such regimens.

References

1. Spears CP, Gustavsson BG, Berne M, Frösing R, Bernstein L, Hayes AA (1988) Mechanism of innate resistance to thymidylate synthase inhibition after 5-fluorouracil. Cancer Res 48: 5894
2. Omura K, Kawakami K, Ishida F, Hashimoto T, Matsu T, Adachi I, Watanabe Y (1993) Changes of the number of FdUMP binding sites of thymidylate synthase and folate pools in human colorectal carcinomas following the administration of tegafur and uracil: preliminary report. Nippon Geka Gakkai Zasshi 94: 193
3. Scanlon KJ, Newman EM, Lu Y, Priest DG (1986) Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. Proc Natl Acad Sci USA 83: 8923
4. Rustum YM, Trave F, Zakrzewski SF, Petrelli N, Herrera L, Mittelman A, Arbuck SG, Creaven PJ (1987) Biochemical and pharmacologic basis for potentiation of 5-fluorouracil action by leucovorin. NCI Monogr 5: 165
5. Mini E, Mazzei T, Coronnello M, Criscuoli L, Gualtieri M, Periti P, Bertino JR (1987) Effect of 5-methyltetrahydrofolate on the activity of fluoropyrimidines against human leukemia (CCRF-CEM) cells. Biochem Pharmacol 36: 2905
6. Dohden K, Ohmura K, Watanabe Y (1993) Ternery complex formation and reduced folate in surgical specimens of human adenocarcinoma tissues. Cancer 71: 471
7. Kemeny N, Lokich JJ, Anderson N, Ahlgrem JD (1993) Recent advances in the treatment of advanced colorectal cancer. Cancer 71: 9
8. Martin DS (1987) Biochemical modulation: perspective and objectives In: Harrap K, Connors T (ed) New avenues in developmental chemotherapy. Academic Press, London, p 113
9. Omura K, Doden K, Munemoto Y, Kawakami K, Miyanaga T, Hashimoto T, Ishida F, Watanabe Y (1992) Treatment of advanced cancer of the alimentary tract with cisplatin and 5-fluorouracil. Jpn J Cancer Chemother 19: 723
10. Fujii S, Ikenaka K, Shirasaka T (1978) Effect of uracil and its derivatives on antitumor activity of 5-fluorouracil and 1-(2-tetrahydrofuryl)-5-fluorouracil. Gann 69: 763
11. Panasci L, Ford J, Margolese R (1985) A phase II study of sequential methotrexate and fluorouracil in advanced colorectal cancer. Cancer Chemother Pharmacol 15: 164

12. Ajani JA, Kanojia MD, Bedikian AY, Korinek JK, Stein SH, Espinosa EG, Bodey GP (1985) Sequential methotrexate and 5-fluorouracil in advanced colorectal carcinoma. Am J Clin Oncol 8: 69
13. Walder S, Schwartz EL, Goldman M, Lyver A, Rader M, Zimmerman M, Itri L, Weinberg V, Wiernik PH (1989) Fluorouracil and recombinant alpha-2a-interferon: an active regimen against advanced colorectal carcinoma. J Clin Oncol 7: 1769
14. Kemeny N, Younes A, Seiter K, Kelsen D, Sammarco P, Adams L, Derby S, Murray P, Houston C (1990) Interferon alpha-2a and 5-fluorouracil for advanced colorectal carcinoma; assessment of activity and toxicity. Cancer 66: 2470
15. Pazdur R, Ajani JA, Winn R, Bearden J, Belt RJ, Pilat S, Hallinan R, Levin B (1992) A phase II trial of 5-fluorouracil and recombinant alpha-2a-interferon in previously untreated metastatic gastric carcinoma. Cancer 69: 878
16. O'Connell MJ (1989) A phase III trial of 5-fluorouracil and leucovorin in the treatment of advanced colorectal cancer. Cancer 63: 1026
17. Laufman LR, Brenckman WD Jr, Stydnicki KA, Morgan ED, Collier M, Knick VB, Duch DS, Mullin R, Ferone R (1989) Clinical experience with leucovorin and 5-fluorouracil. Cancer 63: 1031
18. Doroshow JH, Multhauf P, Leong L, Margolin K, Litchfield T, Akman S, Carr B, Bertrand M, Goldberg D, Blayney D, Odujinrin O, Delap R, Shuster J, Newman E (1990) Prospective randomized comparison of fluorouracil versus fluorouracil and high-dose continuous infusion leucovorin calcium for the treatment of advanced measurable colorectal cancer in patients previously unexposed to chemotherapy. J Clin Oncol 8: 491
19. Petrelli N, Douglass HO, Herrera L, Russell D, Stablein DM, Bruckner HW, Mayer RJ, Schinella R, Green MD, Muggia FM, Megibow A, Greenwald ES, Bukowski RM, Harris J, Levin B, Gaynor E, Loutfi A, Kalser MH, Barkin JS, Benedetto P, Woolley PV, Nauta R, Weaver DW, Leichman LP (1989) The modulation of fluorouracil with leucovorin in metastatic colorectal carcinoma: a prospective randomized phase III trial. J Clin Oncol 7: 1419
20. Scanlon KJ, Newman EM, Lu Y, Priest DG (1986) Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. Proc Natl Acad Sci USA 83: 8923
21. Shirasaka T, Shimamoto Y, Ohshimo H, Saito H, Fukushima M (1993) Metabolic basis of the synergistic antitumor activities of 5-fluorouracil and cisplatin in rodent tumor models in vivo. Cancer Chemother Pharmacol 32: 167
22. Scanlon KJ, Safirstein R, Guttenplan JB, Thies HL, Waxman S (1982) Inhibition of the ACS amino acid transport system by cisplatin. Proc AACR 23: 167
23. Jeske J, Hansen RM, Libnoch JA, Anderson T (1990) 5-fluorouracil infusion and low-dose weekly cisplatin: an analysis of increased toxicity. Am J Clin Oncol 13: 485