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Inhibition of Catabolic Pathway of 5-Fluorouracil by 3-Cyano-2,6-dihydroxypyridine in Human Lung Cancer Tissues

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Our studies of the degradation and the phosphorylation of 5-fluorouracil (5-FU) in normal and tumor lung tissues from 10 cases of lung cancer have shown that the phosphorylation of 5-FU in the tumor tissues was about 2- to 3-fold higher than that in normal tissues, and that the degradation of 5-FU in tumor tissues was nearly 6-fold higher than that in normal tissues. BOF-A2 is an anti-neoplastic agent newly synthesized from 1-ethoxymethyl-5-FU and 3-cyano-2,6-dihydroxypyridine (CNDP). The inhibitory effect of CNDP on the degradation of 5-FU in the tumor tissues was potent (IC_{50} 3.9×10^{-9} *M*). Thus, BOF-A2 exerts its anti-neoplastic effect on tumors by potentiating the action of 5-FU through inhibition of 5-FU degradation by the CNDP moiety.

Key words: 5-Fluorouracil degradation — 5-Fluorouracil phosphorylation — 3-Cyano-2,6-dihydroxypyridine — Human lung cancer — BOF-A2

It has been reported that 5-fluorouracil (5-FU) is directly phosphorylated by pyrimidine phosphoribosyltransferase, or indirectly via 5-fluorouridine (FUrd), to 5-fluorouridine 5′-monophosphate (FUMP), which causes disturbance of RNA metabolism. Another route is via 2′-deoxy-5-fluorouridine (FdUrd) to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which inhibits thymidylate synthase, the rate-limiting enzyme of DNA synthesis.[1-6] 5-FU is also degraded to dihydro-5-fluorouracil by dihydrouracil (DHU) dehydrogenase, a rate-limiting enzyme, and thence rapidly to 2-fluoro-3-ureidopropionic acid (FUPA) and 2-fluoro-β-alanine (F-β-Ala) by catabolic enzymes present mainly in normal liver tissues[7-10] (Fig. 1). It was expected, therefore, that a potent inhibitor of 5-FU degradation would enhance the anti-neoplastic effect of 5-FU. In fact, Fujii *et al.*[11] have already found that the anti-neoplastic effect of Tegafur, a masked compound of 5-FU, was enhanced by combination with uracil, an inhibitor of 5-FU degradation. Accordingly, they developed UFT (Tegafur combined with uracil in a molar ratio of 1 to 4), which is now widely used as an effective anti-neoplastic agent for many cancer patients.

BOF-A2 has recently been synthesized as a bifunctional compound composed of 3-cyano-2,6-dihydroxypyridine (CNDP), a new inhibitor[12] of 5-FU degradation 2000 times more potent than uracil, and 1-ethoxymethyl-5-fluorouracil (EM-FU), a masked compound of 5-FU. BOF-A2 has exhibited a potent anti-neoplastic effect on various experimental and human tumors xenografted in nude mice.[13] It has also been proved clinically that BOF-A2 is particularly effective for human lung cancer, against which administration of conventional 5-fluorinated pyrimidines has not been successful.[14] To elucidate the anti-neoplastic mechanisms of BOF-A2, we examined the degradation of 5-FU and the inhibitory effect of CNDP on 5-FU catabolism in human lung cancer tissues.

MATERIALS AND METHODS

Chemicals [6-^{3}H]5-FU was obtained from Japan Radioisotope Association, Tokyo. 5-FU was purchased from Daikin Kogyo Co., Ltd., Tokyo. Nicotinamide adenine dinucleotide phosphate in reduced form (NADPH) and adenosine 5′-triphosphate (ATP-2Na) were purchased from Oriental Kobo Co., Ltd., Tokyo. F-β-Ala and *p*-dimethylaminobenzaldehyde were purchased from Tokyo Kasei Co., Ltd., Tokyo. Ribose 1-phosphate (Rib1P), deoxyribose 1-phosphate (dRib1P), phosphoribosyl 1-pyrophosphate (PRibPP) and FdUMP were purchased from Sigma Chemical Co., USA. FUMP was purchased from Hoechst Chemical Co., USA. Nicotinamide, NaF, and ninhydrin were purchased from Wako Chemical Co., Ltd., Osaka. CNDP, FUPA, FUrd and FdUrd were synthesized by Otsuka Pharmaceutical Co., Ltd., Tokyo. Other chemicals were all commercial products.

Preparation of human lung tissues Freshly prepared normal and tumor lung tissues from 10 patients with lung cancer were used (Table I). The tissues were resected from the same pulmonary lobe of each patient during

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surgical operations, and immediately frozen at −80°C. Histological examinations of the specimens followed the General Rules for Clinical and Pathological Classification of Lung Tumors (UICC 1987). For the examination of 5-FU degradation, 5 cases of well-differentiated adenocarcinomas, 2 cases of poorly differentiated adenocarcinomas, 3 cases of squamous cell carcinomas, and their respective normal lung tissues were used. For the examination of 5-FU phosphorylation, 3 cases of well-differentiated adenocarcinomas, 1 case of poorly differentiated adenocarcinoma, 3 cases of squamous cell carcinomas, and their respective normal lung tissues were used.

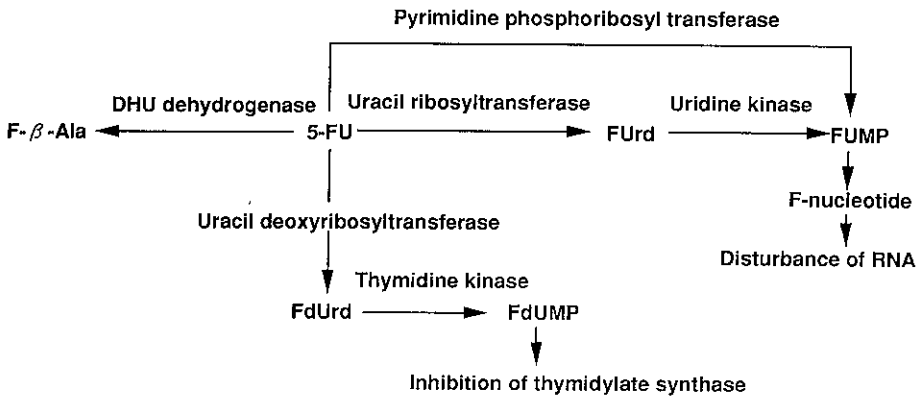

Fig. 1. Possible metabolic pathways of 5-FU.

Table I. Details of Human Lung Tumor Tissues Used

Patient	Age	Sex	Stage	Clinical and histological findings	Combination therapy	Concomitant disease	Resection site	Treatment
T.K.	45	♂	IIIB	Cancer of pulmodexter well-differentiated adenocarcinoma	None	None	Pulmodexter Lobus superior	Curative
R.T.	71	♂	I	Cancer of pulmodexter well-differentiated adenocarcinoma	None	Low respiratory function	Pulmodexter Lobus superior-medius	Curative
Y.M.	54	♂	I	Cancer of pulmosinister poorly-differentiated adenocarcinoma	None	None	Pulmosinister Lobus inferior	Curative
H.W.	69	♂	IIIB	Cancer of pulmodexter poorly-differentiated adenocarcinoma	None	None	Pulmodexter Lobus superior-medius	Non-curative
I.N.	73	♂	IV	Cancer of pulmosinister Squamous cell carcinoma	None	Pneumoconiosis Low respiratory function	Pulmosinister Lobus inferior	Non-curative
T.J.	72	♂	I	Cancer of pulmodexter Squamous cell carcinoma	None	None	Pulmodexter Lobus inferior	Curative
T.N.	68	♂	IIIA	Cancer of pulmodexter Squamous cell carcinoma	CDDP VP-16 MMC	None	Pulmodexter	Curative
S.T.	65	♂	IIIB	Cancer of pulmosinister well-differentiated adenocarcinoma	Non	None	Pulmosinister Lobus superior	Palliative
I.T.	72	♀	I	Cancer of Pulmodexter well-differentiated adenocarcinoma	None	None	Pulmodexter Lobus superior	Curative
N.A.	72	♀	I	Cancer of Pulmodexter well-differentiated adenocarcinoma	None	None	Pulmodexter Lobus superior	Curative

For the examination of inhibitory effect of CNDP on 5-FU degradation, 1 case of squamous cell carcinoma and 2 cases of well-differentiated adenocarcinomas were used.

Preparation of enzyme solutions All procedures were done in an ice-water bath. Tissue samples weighing 0.3–2.0 g were thawed, minced with scissors and homogenized with 4 volumes of 0.25 *M* sucrose solution containing 5 m*M* β-mercaptoethanol and 0.5 m*M* ethylenediaminetetraacetic acid (EDTA). The homogenates were centrifuged at 105,000*g* for 60 min. A part of the supernatant was dialyzed overnight against 10 m*M* potassium phosphate buffer (pH 7.5) for the assay of 5-FU degradation, and the remainder was stored at −80°C until use in the assay of the inhibitory effect of CNDP on 5-FU degradation and 5-FU phosphorylation.

Assay of 5-FU degradation The assay of 5-FU degradation essentially followed the methods of Ikenaka *et al.*[8] The total volume of 0.5 ml of incubation mixture contained 45 m*M* potassium phosphate buffer (pH 7.5), 50 m*M* nicotinamide, 5 m*M* ATP, 25 m*M* NaF, 1 m*M* NADPH, 10 μM [^{3}H]5-FU (1.02 μCi/ml) and 0.2 ml of enzyme solution after serial dilution. After incubation for 60 min at 37°C, the mixture was cooled in an ice water bath, mixed with 2 *M* perchloric acid (0.125 ml) and centrifuged at 3,000 rpm for 10 min. A 0.25 ml aliquot of the supernatant was added to 0.09 ml of 2 *M* KOH and the mixture was centrifuged at 3,000 rpm for 10 min. Then, 10 μl of the supernatant was spotted onto thin layer chromatography (TLC) plates (Merck silica gel 60 F_{254}-precoated plate). The standard solution of 5-FU, F-β-Ala and FUPA was similarly applied to the plates before spotting the test sample. The plates were developed with a mixture of chloroform, methanol and acetic acid (17:3:1, v/v/v). 5-FU was detected by its UV absorption at 254 nm, while F-β-Ala or FUPA was detected by spraying ninhydrin reagent (50 mg ninhydrin/30 ml acetone) or Ehrlich reagent (200 mg *p*-dimethylaminobenzaldehyde/1 ml HCl plus 9 ml ethanol), respectively. The spots of 5-FU, F-β-Ala and FUPA were located at *Rf* values of 0.6, 0 and 0, respectively. Each spot was scraped into vials, extracted with 0.1 ml of 4 *N* HCl and mixed with 10 ml of scintillation fluid (ACSII, Amersham). The radioactivity was measured in an Aloka LSC liquid scintillation spectrometer. The assay was done in duplicate, and mean values were taken. From the percentage degradation of 5-FU [counts of origin/(counts of origin plus counts of 5-FU)] × 100, the degradation activity of 5-FU (nmol/g tissue/min) in the enzyme solution was calculated as 5 nmol × degradation % × [1/(0.04 g × 1/dilution rate)] × 1/60 min. Statistical analysis was carried out by using Wilcoxon's sign rank test.

Assay of the inhibition of 5-FU degradation by CNDP The 105,000*g* supernatant frozen at −80°C was thawed and dialyzed against 10 m*M* potassium phosphate buffer (pH 7.5). The assay was carried out by the same method as the 5-FU degradation assay. The total volume of 0.25 ml of reaction mixture contained the same components as in the assay of the 5-FU degradation, plus CNDP (10^{-5}–10^{-10} *M*). The enzyme concentration was limited to within a range giving 30 to 40% 5-FU degradation in the absence of CNDP. CNDP was added at various concentrations, and 5-FU degradation (%) was measured. Degradation inhibitory rate (%) was calculated as 100 − (5-FU degradation (%) in the presence of CNDP/5-FU degradation (%) in the absence of CNDP) × 100. The IC_{50} (*M*) was calculated by probit analysis from the inhibition (%) by CNDP.

Assay of 5-FU phosphorylation The 105,000*g* supernatant frozen at −80°C was thawed and dialyzed against 10 m*M* Tris-HCl buffer (pH 8.0). The assay of 5-FU phosphorylation essentially followed the method of Ikenaka *et al.*[8] For the assay of phosphorylation activity with uracil ribosyltransferase and uridine kinase or uracil deoxyribosyltransferase and thymidine kinase, a total volume of 0.5 ml of the incubation mixture (sometimes 0.25 ml) was used, which contained 25 m*M* Tris-HCl buffer (pH 8.0), 5 m*M* $MgCl_2$, 10 m*M* NaF, 12.5 μM [^{3}H]5-FU (1.28 μCi/ml), 5 m*M* ATP, 2 m*M* Rib1P or 2 m*M* dRib1p, and 0.2 ml (0.1 ml) of serially diluted enzyme solution. For the assay direct phosphorylation activity with pyrimidine phosphoribosyltransferase, 0.5 ml (0.25 ml) of the incubation mixture was used, which contained 25 m*M* Tris-HCl buffer (pH 8.0), 5 m*M* $MgCl_2$, 10 m*M* NaF, 12.5 μM [^{3}H]5-FU (1.28 μCi/ml), 2 m*M* PRibPP and 0.2 ml (0.1 ml) of serially diluted enzyme solution. The reaction was stopped by cooling in an ice-water bath after incubation of the mixture for 30 min at 37°C. Then 2 *M* perchloric acid (0.125 ml) was added, and the mixture centrifuged at 3,000 rpm for 10 min. A 0.25 ml aliquot of the supernatant was added to 0.09 ml of 2 *M* KOH and centrifuged at 3,000 rpm for 10 min. Ten μl of the supernatant was then spotted onto TLC plates (Merck silica gel 60 F_{254}-precoated plates). The standard solution of 5-FU, FUrd, FdUrd, FUMP and FdUMP was similarly applied to the plates before spotting the test sample. The plates were developed with a mixture of chloroform, methanol and acetic acid (17:3:1, v/v/v). The spots were detected by measuring UV absorption at 254 nm. 5-FU, FdUrd, FUrd, FUMP and FdUMP were located at *Rf* values of 0.6, 0.4, 0.3, 0 and 0, respectively. Each spot was scraped into vials and extracted with 0.1 ml of 4 *N* HCl and mixed with 10 ml of scintillation fluid (ACSII, Amersham), and the radioactivity was measured in an Aloka LSC liquid scintillation spectrometer. From the percentage phosphorylation of 5-FU, i.e., from the value of [FUMP/(5-FU+FUMP+FUrd)]×100 or [FdUMP/(5-FU+FdUMP+

FdUrd)] × 100 or [FUMP / (5-FU + FUMP)] × 100, hosphorylation activity (nmol/g tissue/min) was calculated as 6.25 nmol × % phosphorylation × [1/(0.04 g × 1/dilution rate)] × 1/30 min. Statistical analysis was carried out by using Wilcoxon's sign rank test.

RESULTS

Phosphorylation of 5-FU in human lung tumors As shown in Table II, the phosphorylation activity of 5-FU in human lung tumors (7 cases) was 2- to 3-fold higher than that in normal tissues (7 cases), either with uracilribosyltransferase plus uridine kinase (1.12 nmol/g tissues/min), or with uracildeoxyribosyltransferase plus thymidine kinase (1.16 nmol/g tissue/min). The phosphorylation activity (0.1 nmol/g tissue/min) of pyrimidine phosphoribosyltransferase in tumor as well as in normal tissues was lower than the activities of the other two phosphorylation pathways. A significant difference in the latter activity was observed between the tumor tissues and the normal tissues.

Degradation of 5-FU in human lung tumors As shown in Fig. 2, the mean values of degradation activity of 5-FU were 13.9 (nmol/g tissue/min) in the 10 tumor tissues and 2.2 (nmol/g tissue/min) in 10 normal tissues, i.e., the degradation activity of 5-FU in tumor tissues was about 6-fold higher than that in normal tissues ($P<0.005$). However, there was no significant difference in the activity among the different histological types of the tumors.

Inhibition of 5-FU degradation by CNDP in human lung tumors As shown in Fig. 3, CNDP strongly inhibited the degradation activity of 5-FU in all the three types of tumor tissues. The IC_{50} values (M) of two cases of well differentiated adenocarcinomas were 3.2×10^{-9} M (95% confidence interval: $2.3–4.5\times10^{-9}$ M) and 3.8×10^{-9} M (95% confidence interval: $2.8–5.3\times10^{-9}$ M), respectively. The IC_{50} values (M) of squamous cell carcinoma was 4.6×10^{-9} M) (95% confidence interval: $3.3–6.5\times10^{-9}$ M). The mean IC_{50} (M) was remarkably low (3.9×10^{-9} M).

Table II. Phosphorylation Activity of 5-FU in Normal and Tumor Lung Tissues[a)]

Enzyme	Phosphorylation activity (nmol/g tissue/min)	
	Normal tissues	Tumor tissues
Phosphoribosyltransferase	0.02±0.01	0.10±0.09[b)]
Uracil ribosyltransferase + Urd kinase	0.39±0.19	1.12±0.26[b)]
Uracil deoxyribosyltransferase + dThd kinase	0.66±0.25	1.16±0.33[b)]

a) The reaction mixture contained [^{3}H]5-FU (12.5 μM, 1.28 μCi/ml), 25 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 10 mM NaF, 5 mM ATP, 2 mM Rib1P, 2 mM PRib1P, and 2 mM dRib1P for phosphorylation.
b) Significant difference from the control group, $P<0.05$. n=7, mean±SD.

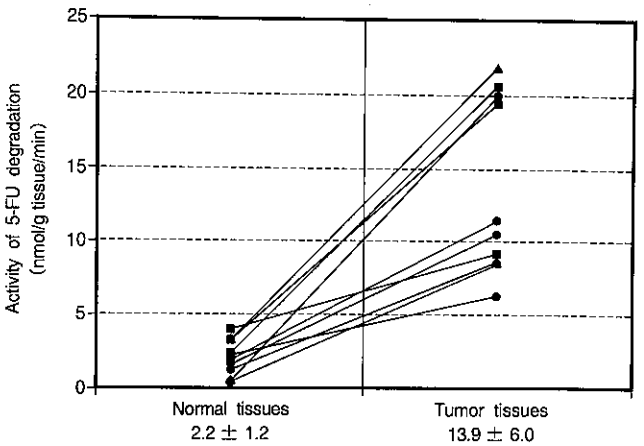

Fig. 2. Comparison of degradative activity of 5-FU in tumor tissues and normal tissues of the 10 lung cancer patients. The reaction mixture contained [^{3}H]5-FU (10 μM, 1.02 μCi/ml), 1 mM NADPH, 45 mM potassium phosphate buffer (pH 7.5), 50 mM nicotinamide, 5 mM ATP, 25 mM NaF and enzyme solution obtained from tumor tissues and normal tissues of the patients. Statistical analysis was carried out by using Wilcoxon's sign rank test. Values by the abscissa are means±SD for 5-FU degradation activity. A significant difference was observed between normal and tumor lung tissues ($P<0.005$). ●, well differentiated adenocarcinoma; ▲, poorly differentiated adenocarcinoma; ■, squamous cell carcinoma.

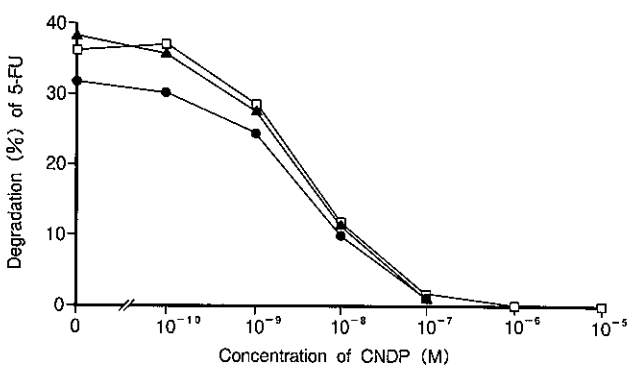

Fig. 3. Inhibitory effect of CNDP of 5-FU degradation in human lung tumor tissues *in vitro*. The reaction mixture contained [^{3}H]5-FU (10 μM, 1.02 μCi/ml), CNDP, 1 mM NADPH, 45 mM potassium phosphate buffer (pH 7.5), 50 mM nicotinamide, 5 mM ATP, 25 mM NaF and enzyme solution obtained from tumors (3 cases). ▲, ●, well differentiated adenocarcinoma; □, squamous cell carcinoma.

DISCUSSION

It has already been reported[8] that 5-FU is metabolized via four pathways: a catabolic pathway from 5-FU to FUPA and F-β-Ala; a phosphorylating pathway from 5-FU to FUMP through FUrd; a phosphorylating pathway from 5-FU to FdUMP through FdUrd; and directly phosphorylation from 5-FU to FUMP. Ikenaka *et al.*[8] and Maehara *et al.*[15] have reported that the phosphorylation activity of 5-FU by the second or the third pathway is higher in tumor tissues than in normal tissues. Ikenaka *et al.*,[8] Ho *et al.*[10] and Maehara *et al.*[15] have also reported that the degradation of 5-FU by the catabolic pathway is carried out mainly in normal tissues of liver (in rat and human) but scarcely in those of other organs, and they confirmed that the degradation activity of 5-FU in rat tumor[8] or in human tumor[15] is low compared with that in the normal liver. Naguib *et al.*[9] have reported the presence of a potent activity of 5-FU degradation in tumor cell lines of human pancreas and lung. In the present study, our results on 5-FU phosphorylation activity were the same as those of Maehara *et al.*[15] However, the degradation activity of 5-FU in the 10 cases of human lung tumor tissues was about 6-fold higher than that in normal lung tissues. The high activity of 5-FU degradation in tumor tissues is in agreement with the report by Naguib *et al.*[9] From these results, we suggest that BOF-A2 shows its enhanced anti-neoplastic effect on lung cancer as a result of inhibition of the degradation of 5-FU by its CNDP moiety.

Our results showed that the inhibitory effect of CNDP was remarkably high, with an IC_{50} value of 3.9×10^{-9} *M*. It thus seems likely that the anti-neoplastic effect of BOF-A2 against human lung cancer can be attributed to the inhibition by CNDP of the degradation of 5-FU incorporated into the lung tumor tissues after BOF-A2 administration.[16]

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