FLUOROPYRIMIDINE METABOLISM IN HUMAN HEAD AND NECK
CANCER XENOGRAPHS AND MURINE COLON TUMORS

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INTRODUCTION

Fluoropyrimidines are widely used for the treatment of solid tumors, such as breast-, colorectal- and head & neck cancer [1-3]. A better therapeutic efficacy than for 5-fluorouracil (5FU) has been claimed for the analog 5'-deoxy-5-fluorouridine [1-6] (5'dFUR, doxifluridine) which cannot be converted directly to the nucleotide level due to the presence of a 5-deoxy-ribose moiety and requires conversion to 5FU (Fig. 1) for its activation [5,7,8].

The human xenograft model has a potential unique value in screening and selecting new drugs for clinical trials [9]. We have developed a panel of head and neck cancer xenograft (HNX) tumor lines in order to select compounds for Phase II trials [10,11]. Table 1 summarizes the sensitivities of several HNX lines and 2 murine colon tumor lines in mice for 5FU and 5'dFUR. Up to now no biochemical evaluation in relation to sensitivity to fluoropyrimidines of HNX lines has been performed.

Fig. 1. Metabolism of 5FU and 5'dFUR; The enzymes catalyzing these reactions are: 1, orotate phosphoribosyltransferase (OPRT); 2, pyrimidine nucleoside phosphorylases; 3, 5'nucleotidase and phosphatases; 4, uridine kinase. PRPP, 5-phosphoribosyl-1-pyrophosphate; 5dRib-1-P, 5-deoxy-Rib-1-P; FUR, fluorouridine.

Table 1. SENSITIVITY OF 6 TUMOR LINES TO 5FU AND 5'dFUR

Tumor	Origin	Drug (mg/kg)	Dose	Sensitivity
HNX-DU	Hypopharynx	5FU	50	-
		5'dFUR	1000	-
HNX-KE	Larynx	5FU	50	-
		5'dFUR	1000	++
HNX-G	Oral cavity	5FU	50	-/+
		5'dFUR	400	-
HNX-E	Skin	5FU	50	-
		5'dFUR	1000	+
Colon 26	Murine colon	5FU	100	-/+
		5'dFUR	2000	+++++
Colon 38	Murine colon	5FU	100	+++++
		5'dFUR	1000	+++++

Drugs were administered i.p. weekly.

The conversion of 5'dFUR to 5FU is catalyzed by a pyrimidine nucleoside phosphorylase. It has been suggested that activation of 5'dFUR to 5FU occurred selectively in tumor cells compared to normal cells [12]. The activity of pyrimidine nucleoside phosphorylase appears to be related to the toxicity of 5'dFUR [5,8,12,13], however, a strict correlation has not been demonstrated [5,14]. We postulated that although sufficient conversion of 5'dFUR to 5FU is essential for a cell to be sensitive to 5'dFUR, this is not the only critical factor which determines the activity of 5'dFUR [14]. Further activation of the 5FU formed from 5'dFUR appeared to be essential. A cell line in which the direct conversion of 5FU to FUMP plays an important role, appeared to be very sensitive to 5'dFUR [14]. In this cell line 5'dFUR was also able to decrease the levels of PRPP [15]. 5FU can also be converted to FUMP via FUR. In the presence of sufficient dRib-1-P as co-substrate, 5FU can be directly converted to 5-fluoro-deoxy-uridine (FUDR). However, the concentration of dRib-1-P is neglectable under physiological concentrations [16,17], but enhanced at administration of e.g. deoxyinosine.

In order to know whether 5FU resistance could be overcome by administration of 5'dFUR we attempted to correlate the sensitivity of several tumor lines to fluoropyrimidines with the biochemical pharmacology of the drugs in each line; four human tumor xenografts and two murine colon carcinomas. In these tumors we measured the activities of enzymes involved in the activation of 5'dFUR and 5FU. The results demonstrated that anti-tumor activity of 5'dFUR not only depends on the rate of conversion to 5FU but also on the further metabolism of 5FU.

MATERIALS AND METHODS

5FU and 5'dFUR were obtained from Hoffman-La Roche, Mijdrecht, the Netherlands. All other chemicals were of the highest quality commercially available. The head and neck tumor lines were established from tumors from untreated patients (Table 1) and maintained in female B10.LP/Cpb nude mice as described previously [10]. The murine colon tumors Colon 26 and Colon 38 were maintained in female Balb/c mice and C57B1/6 mice, respectively, as described previously [18]. Tumors (ranging in size between 200-2000 mm³) were obtained from non-treated mice. Tumors were removed immediately, and stored directly either in liquid nitrogen or at -70°C. Frozen tissues were pulverized using a micro-dismembrator as described [19]. The powder was suspended in assay buffer (50 mM Tris-1 mM EDTA, pH

7.4), (one gram tissue per 4 ml buffer). After centrifugation the supernatant was immediately used for determination of enzyme activities. Enzyme assays were performed at 37°C. Assays with 5FU as substrate were performed as described previously [14,19]. Products were separated from the substrate 5FU using thin-layer chromatography [20]. The reaction mixture for the pyrimidine nucleoside phosphorylase assay contained 0.3-60 µg protein, 5 mM MgCl₂ and the cofactors Rib-1-P or dRib-1-P at 2.5 mM final concentration. For the measurement of FUMP and FdUMP synthesis (via FUR or FdUR, respectively), more protein (200-650 µg) was present in the assay. In order to prevent breakdown of nucleotides by phosphatases we added 15 mM 2-glycerol-phosphate to these assays. ATP and (d)Rib-1-P were present at 2.5 mM final concentration. For the measurement of the direct conversion of 5FU to FUMP catalyzed by OPRT the pentose phosphates were substituted by 2 mM PRPP; 0.6 mM α,β-methylene-ADP was present to inhibit 5'-nucleotidases; 200-650 µg protein was present. The reactions were initiated by addition of radiolabeled 5FU (final concentration 0.27 mM [6-¹⁴C]-5FU). The phosphorolysis of 5'dFUR to 5FU was measured using a recently developed HPLC assay [21]. The assay was performed with the 11,000 g supernatant. The assay mixture contained 5-100 µg protein, 40 mM KH₂PO₄ and was initiated by addition of 5'dFUR (final concentrations 0.25-2 mM).

RESULTS

Pyrimidine nucleoside phosphorylase was assayed using a recently described HPLC assay [21] with 5'dFUR as substrate. A high K_m was observed in all tumors varying from 0.6-2 mM. The maximal activity with 5'dFUR varied considerably between the several tumor lines (Table 2). The activity in the Colon 26 was about 50 times higher than in HNX-DU and HNX-KE. The activity in the other two HNX tumor was intermediate.

Pyrimidine nucleoside phosphorylases also catalyzes the further anabolism of the product 5FU with Rib-1-P or dRib-1-P as co-substrates (Fig. 2). The activity with dRib-1-P was higher than with Rib-1-P in all tumors, in the HNX tumors at least 10-fold, in both colon tumors 3-fold. With Rib-1-P the highest activity was found in the Colon 26 and the lowest activity in the HNX-KE and Colon 38. With dRib-1-P the pattern was different, the highest activity was present in Colon 26 and the lowest in Colon 38. However, the activities in the HNX tumors were all higher than in Colon 38 and the difference of HNX-E with Colon 26 was less than with Rib-1-P as co-substrate.

TABLE 2. ACTIVITY OF PYRIMIDINE NUCLEOSIDE PHOSPHORYLASE WITH 5'dFUR AS SUBSTRATE.

Tumor line	V _{max} (nmol/hr per mg protein)	K _m (mM)
HNX-E	2300 ± 460	1.16 ± 0.30
HNX-DU	273 ± 28	0.80 ± 0.11
HNX-KE	366 ± 54	1.16 ± 0.09
HNX-G	2565 ± 293	1.02 ± 0.34
Colon 26	9892 ± 2087	1.89 ± 0.54
Colon 38	671 ± 238	2.19 ± 1.56

Values are means ± SE of 3-5 different tumors.

Fig. 2. Activity of pyrimidine nucleoside phosphorylase with 5FU as substrate and Rib-1-P or dRib-1-P as co-substrate. Values represent means + SE of 3-5 different tumors.

Conversion of 5FU to either FUMP or FdUMP via FUR or FUDR, respectively, was measured by supplying for the ATP at a physiological concentration in the assay for pyrimidine nucleoside phosphorylase. Under these conditions an estimate of the conversion of 5FU to the nucleotide will be obtained [14]. This conversion was termed "channelling". The rate of channelling was measured in the presence of a phosphatase inhibitor to prevent the degradation of newly formed nucleotides. The rate of channelling of 5FU to FUMP was highest in the Colon 26 and very low in Colon 38 (Fig. 3). The activity in all HNX tumor was at least 10 times higher than in Colon 38, but also less than 5% of that in Colon 26. The rate of channelling of 5FU to FdUMP showed a completely different pattern. The activity was highest in Colon 26 but the difference with the other tumors was less, the activity in HNX-DU and KE was even 50% of that in Colon 26. The rate of channelling in the other 3 tumor lines was much lower. The direct conversion of 5FU catalyzed by the pyrimidine de novo enzyme OPRT was highest in Colon 26 and Colon 38 (Fig. 4); for the HNX tumors the activity was highest in the HNX-KE.

Fig. 3. Synthesis of FUMP and FdUMP from 5FU via FUR or FdR, respectively. The "channeling" reaction was measured with Rib-1-P or dRib-1-P as co-substrates in the presence of ATP. Values represent means + SE of 3-5 different tumors.

Fig. 4. Activity of OPRT with 5FU as substrate and PRPP as co-substrate. Values represent means + SE of 3-5 different tumors.

DISCUSSION

An attractive way to select active anticancer agents is the use of human xenograft tumor lines [9,10]. The present study tries to give a biochemical explanation for the fact that in human xenografts resistant to 5FU the therapeutic efficacy might be increased by treatment with the 5FU prodrug 5'dFUR. The improved therapeutic efficacy might be related to the rate of activation of 5'dFUR to 5FU which is very high in the sensitive Colon 26, but the HNX-KE tumor and Colon 38, which are also sensitive to 5'dFUR, have rather low activities. All sensitive tumors have a relatively high activity of OPRT, which might play a key role in the further activation of 5U.

It has been postulated that the sensitivity of tumors to 5'dFUR is correlated with the activity of pyrimidine nucleoside phosphorylase [5,8,12,13] although no strict correlation has been demonstrated [5,14]. This discrepancy might be related to the role of uridine phosphorylase in the activation of 5'dFUR. Uridine phosphorylase might be responsible for the conversion of 5'dFUR to 5FU [8] but 5'dFUR is also a substrate for thymidine phosphorylase [22]. The pattern of activity of pyrimidine nucleoside phosphorylase with 5'dFUR as a substrate correlated with that of 5FU and dRib-1-P as substrates. This conversion of 5FU to FudR and the cleavage of FudR are mainly catalyzed by thymidine phosphorylase and/or a uridine-deoxyuridine phosphorylase [14,23]. Enzyme kinetics of pyrimidine nucleoside phosphorylase with 5'dFUR as a substrate were comparable to other studies. The K_m for 5'dFUR observed by others was about 1 mM [7,24,25]. The K_m for 5'dFUR is much higher than that for uridine [26] or thymidine [27] it may be concluded that 5'dFUR has a low affinity for pyrimidine phosphorylases. It might be substrate for the various phosphorylases which have been reported thusfar [23,24].

From the data presented it can be concluded that for this panel of tumors the activity of uridine phosphorylase is not related to the sensitivity of the tumor to 5'dFUR. The Colon 38 is most sensitive to 5'dFUR but the rate of 5'dFUR phosphorolysis is very low. Colon 26, which is also sensitive to 5'dFUR has a very high rate of 5'dFUR phosphorolysis, but the HNX-KE which is also sensitive has low activity, lower than HNX-E and HNX-G. This means that other factors than the rate of phosphorolysis of 5'dFUR play an important role in the sensitivity to 5'dFUR. Activation to FdUMP via FudR can be excluded because of the low endogenous levels of the co-substrate dRib-1-P. The levels of Rib-1-P are usually high enough for activation of 5FU to FUMP via FUR, but the rate of this pathway was very low in the 5'dFUR sensitive Colon 38, high in the 5'dFUR resistant HNX-DU, and very high in Colon 26. So, this pathway might only be important for Colon 26. However, previously we demonstrated for cell lines that this way of phosphorylation of 5FU formed from 5'dFUR is unlikely [14]. This could be partially due to; a) the presence of the modified pentose phosphate 5-deoxy-ribose-1-phosphate which might interact with the substrate Rib-1-P and b) an interference of 5'dFUR itself with pyrimidine nucleoside phosphorylase. The only alternative for phosphorylation of 5FU will be direct conversion to FUMP. WiDr cells, appeared to be most sensitive to 5'dFUR of the panel of cell lines which were tested [14,15] and had a relatively high activity of OPRT both with the analog substrate 5FU [14] and the natural substrate orotic acid [15]. With the present tumor lines it appears that the most sensitive lines Colon 26, Colon 38 and HNX-KE have a relatively high activity of OPRT with 5FU as a substrate. This means that only those human tumors with a sufficient capacity to convert 5'dFUR to 5FU and subsequently 5FU to nucleotides may be sensitive to 5'dFUR.

It may be that 5FU resistant tumors do not use efficiently the pathway catalyzed by OPRT, for the activation of 5FU, possibly due to a low availability of PRPP or a high activity of pyrimidine nucleoside phosphorylase. Thus inhibition of pyrimidine nucleoside phosphorylase may lead to a more efficient use of the OPRT pathway, and therapy of 5FU resistant tumors might be improved by an efficient use of the OPRT pathway.

Recently we demonstrated that several human colon tumor have a relatively high activity of OPRT compared to adjacent normal mucosal tissue [19]. This might lead to the conclusion that colorectal cancer is an attractive tumor type to be treated with 5'dFUR, although results up to now only showed a limited therapeutic effect. Tumors with a high OPRT activity might also be treated better with a combination of 5FU and a modulator which selectively employs the activation of 5FU via the direct phosphorylation pathway [28].

In conclusion, the enhanced sensitivity to 5'dFUR might not strictly be related to the rate of conversion of 5'dFUR to 5FU. Although a certain amount of activity is essential to convert 5'dFUR to 5FU, further metabolism to nucleotides might be limiting. This might lead to an improved therapeutic efficacy of 5'dFUR compared to 5FU in tumors with a high OPRT activity such as the HNX-KE and the Colon 26. These findings indicate that further studies attempting at reducing the severe clinical toxicity observed with 5'dFUR are warranted.

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REFERENCES

1. Abele, R., Alberto, P., Kaplan, S., & 6 others (1983). *J. Clin. Oncol.* 1, 750.
2. Abele R., Kaplan E., Grossenbacher, R., Schmidt, H.J. & Cavalli, F. (1984). *Eur. J. Cancer Clin Oncol.*, 20, 333.
3. Alberto, P., Rozencweig, M., Clavel, M. & 6 others (1986). *Cancer Chemother. Pharmacol.*, 16, 78.
4. Bollag W. & Hartmann, H.R. (1980). *Eur. J. Cancer*, 16, 427.
5. Hartmann, H.R. & Matter, A. (1982). *Cancer Res.*, 42, 2412.
6. Hurlteloup, P., Armand, J.P., Cappelaere, P., & 16 others (1986). *Cancer Treatm. Rep.*, 70, 731.
7. Armstrong, R.D. & Diasio, R.B. (1980). *Cancer Res.*, 40, 3333.
8. Ishitsuka, H., Miwa, M., Takemoto, K., Fukuoka, K., Itoga, A., & Maruyama, H.B. (1980). *Gann* 71, 112.
9. Winograd, B., Boven, E., Lobbezoo, M.W. & Pinedo, H.M. (1987). *In Vivo*, 1, 1.
10. Braakhuis, B.J.M., Schoevers, E.J., Heijerman, E.C.M., Sneeuwloper, G. & Snow G.B. (1983). *Br. J. Cancer*, 48, 711.
11. Braakhuis, B.J.M. & Snow, G.B. (1988). *ESO monographs, Human xeno grafts in anticancer drug development.* (Eds.: Winograd, B., Peckham, M.J. & Pinedo, H.M.). Springer Verlag, Heidelberg, pp. 37-39.
12. Armstrong, R.D. & Diasio, R.B. (1981). *Cancer Res.*, 41, 4891.

13. Armstrong, R.D., Gesmonde, J., Wu, T. & Cadman, E. (1983). *Cancer Treatm. Rep.*, 67, 541.
14. Peters, G.J., Laurensse, E., Leyva, A., Lankelma, J. & Pinedo, H.M. (1986). *Cancer Res.*, 46, 20.
15. Peters, G.J., Laurensse, E., Leyva, A. & Pinedo, H.M. (1985). *Int. J. Biochem.*, 17, 95.
16. Barankiewicz, J. & Henderson, J.F. (1977). *Biochem. Med.*, 17, 45.
17. Peters, G.J., Laurensse, E., Leyva, A. & Pinedo, H.M. (1987). *Eur. J. Cancer Clin. Oncol.*, 23, 1869.
18. Peters, G.J., Van Dijk, J., Nadal, J.C., Van Groeningen, C.J. Lankelma, J. & Pinedo, H.M. (1987). *In Vivo*, 1, 113.
19. Peters, G.J., Laurensse, E., Leyva, A. & Pinedo, H.M. (1986). *Clin. Chim. Acta.*, 158, 193.
20. Peters, G.J., Laurensse, E., Lankelma, J., Leyva, A. & Pinedo, H.M. (1984). *Eur. J. Cancer Clin. Oncol.*, 20, 1425.
21. Peters, G.J., Laurensse, E., Leyva, A. & Pinedo H.M. (1987). *Klin. Wochenschr.*, 65 (Suppl X), 18.
22. Siegel, S.A. & Lin, T.-S. (1986). *Drugs Future*, 11, 961.
23. Woodman, P.W., Saril, A.M. & Heidelberger, C. (1980). *Cancer Res.*, 40, 507.
24. Choong, Y.S. & Lee, S.P. (1985). *Clin. Chim. Acta*, 149, 175.
25. Miwa, M., Nakamura, J. & Ishitsuka, H. (1981). *Gann* 72, 965.
26. Leyva, A., Kraal, I., Lankelma, J., Delemarre, J.F.M. & Pinedo, H.M. (1983). *AntiCancer Res.*, 3, 227.
27. Wataya, Y., Santi, D.V. (1981). *Anal. Biochem* 112, 96.
28. Pinedo, H.M. & Peters, G.J. (1988). *J. Clin. Oncol.*, in press.