

# Measurement of in vitro cellular pharmacokinetics of 5-fluorouracil in human and rat cancer cell lines and rat hepatocytes using a flow-through system\*

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Received 4 January 1990/Accepted 21 July 1990

**Summary.** A flow-through system was used to study the cellular pharmacokinetics of 5-fluorouracil (5-FU) in four human cell lines (squamous-cell carcinoma HEp-2, colon carcinoma WiDr, hepatoma Hep G2, and breast carcinoma MCF-7) as well as in the rat hepatoma H35 cell line and in freshly isolated rat hepatocytes. The system made it possible to restrict the decrease in the concentration of 5-FU in the medium, to keep the volume in which the metabolites accumulated relatively small, and to study the dynamics of a response during and after a change in the composition of the eluent. Clearance of 5-FU from the eluent was achieved predominantly (>95%) by its catabolism to dihydrofluorouracil in the tumor cell lines and to 2-fluoro- $\beta$ -alanine in the hepatocytes. Not only rat hepatocytes but also HEp-2 cells showed relatively high clearance values. A concentration-dependent 5-FU elimination was observed, indicating saturation of 5-FU elimination according to Michaelis-Menten kinetics ( $K_m$  14–22  $\mu M$ ). The maximal velocity ( $V_{max}$ ) values ranged from 0.025 to 0.13 nmol 5-FU/ $10^6$  cells per minute. For HEp-2 cells, high-concentration pulse injections of 5-FU, thymine, uridine, or uracil immediately led to a reduction in 5-FU conversion, followed by recovery within 5 min. The flow-through system proved to be adequate for the study of the non-linear pharmacokinetics of 5-FU in different intact cells and for the comparison of various manipulations of these pharmacokinetics.

## Introduction

5-Fluorouracil (5-FU) is widely used either as a single agent or in combination with other drugs in the treatment of gastrointestinal, breast, head and neck, and ovarian cancers [11, 15, 21, 23]. Metabolism plays an important role in the cytotoxic effects and elimination of 5-FU. The anabolic and catabolic pathways have been mapped [5, 12, 22, 28]. 5-FU anabolism is complex and leads to the formation of several potentially active fluoronucleotides. 5-FU catabolism leads to the formation of dihydrofluorouracil (F-DHU), which can be further hydrolyzed to  $\alpha$ -fluoroureidopropionic acid (F-UPA), 2-fluoro- $\beta$ -alanine (F- $\beta$ -ala), or carbon dioxide and ammonia. In patients, >90% of the delivered dose is cleared by catabolism [4]; the liver is thought to be the major site of 5-FU catabolism [4, 23]. The catabolites were originally assumed to lack antitumor activity [22], but it has been shown that F-DHU can be cytotoxic to some cancer cells in vitro [9].

The pharmacokinetics of 5-FU on bolus and continuous intravenous infusion have been compared by Myers [22]. The clearance values after constant intravenous infusion were significantly higher than those after bolus administration, and some values even approached the cardiac output [6, 30, 37]. These observations indicate (a) non-linear or saturable pharmacokinetics for 5-FU in man, and (b) additional clearance of 5-FU in extrahepatic tissue, such as lung tissue or blood [6]. The cellular mechanisms responsible for the observed saturable elimination of 5-FU or its relatively high clearance values are not known.

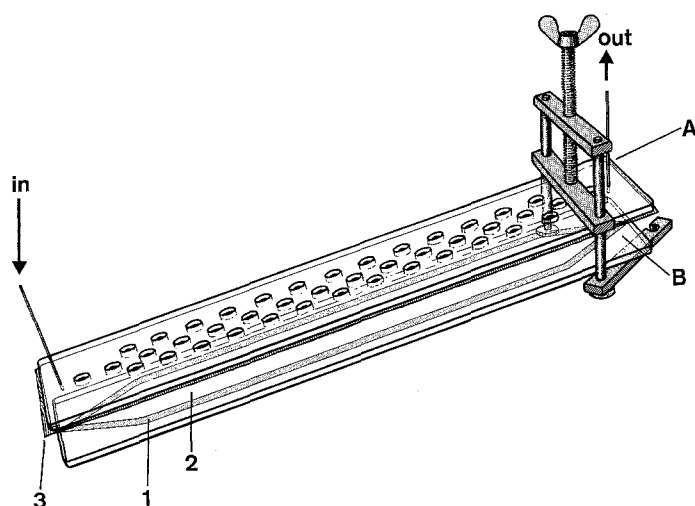
The aim of the present study was a systematic investigation of the cellular elimination of 5-FU in vitro and of its relation to the drug's metabolic pathway. Therefore, we compared the cellular metabolism with the cellular clearance of 5-FU at different concentrations using rat hepatocytes as well as various cancer cell lines. Because 5-FU plasma concentrations in patients can change rapidly (over more than five decades [37]), we also designed an experiment to record the cellular dynamics of changes in 5-FU elimination.

We made use of a flow-through system [18] to study the elimination of 5-FU at the cellular level. In this system,

\*Supported by grant IKA 87-16 from the Netherlands Cancer Foundation. One author (G. J. P.) is the recipient of a senior research fellowship from the Royal Netherlands Academy of Arts and Sciences (KNAW)

**Abbreviations:** 5-FU, 5-fluorouracil; FUR, 5-fluorouridine; F-DHU, dihydrofluorouracil; F- $\beta$ -ala, 2-fluoro- $\beta$ -alanine; F-UPA,  $\alpha$ -fluoroureidopropionic acid; HEPES, 4-(2-hydroethyl)-1-piperazine-ethane sulfonic acid; MEM, modified minimal essential medium; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography

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**Fig. 1.** Schematic representation of the flow-through tissue-culture system. A, Top plate; B, bottom plate; 1, silicone glue forming the walls of the chamber when the plates are pressed together; 2, bottom of the chamber on which the cells are seeded; 3, polypropylene sheet (30  $\mu$ m thick), which in combination with the upper plate forms the ceiling of the chamber, enabling oxygen supply to the cells

extracellular concentrations of 5-FU and its metabolites could be maintained at steady state and the fate of 5-FU could be relatively easily investigated. 5-FU and its metabolites were measured in the effluent of the system after passage over the cells. The response of uracil, uridine, and thymine, three potent modulators of the catabolic route of 5-FU [13, 33], on 5-FU processing was studied as well. We selected cancer cell lines derived from colon, head and neck, and breast cancer, three sources with relevance for 5-FU therapy in the clinic. Because of the high catabolic activity in the liver, we also studied human-derived hepatoma Hep G2 cells, rat-derived hepatoma H35 cells, and freshly isolated rat hepatocytes. In this way, more insight into the cellular mechanisms of dose-dependent 5-FU elimination could be gained. We also obtained indications for the involvement of extrahepatic tissues in the catabolic elimination of 5-FU.

## Materials and methods

### Cells

The sources of both the human cell lines HEP-2 squamous-cell carcinoma [20], HEP G2 hepatoma [1, 16], MCF-7 breast carcinoma [3], and WiDr colon carcinoma [26] and the H35 rat hepatoma cell line [29] have been described elsewhere. The monolayers were cultured in Dulbecco's MEM (Grand Island Biological Co.; Paisley, UK) supplemented with 10% fetal bovine serum (undialyzed, heat-inactivated; Flow Laboratories, Irvine, UK). The cells were checked for mycoplasma at 3-month intervals by the Hoechst stain test. Suspended trypsinized cells ( $5 \times 10^6$ ) were allowed to attach to the bottom glass plate of the flow-through system overnight at 37°C before the plates were mounted to form the chamber. After the experiments, the cells were removed from the system by trypsinization and counted in a hemocytometer, and their viability (routinely >90%) was determined by the trypan blue exclusion test.

Freshly isolated rat hepatocytes were kindly provided by Dr. E. Roos of the Netherlands Cancer Institute, Amsterdam. Immediately after their isolation, rat hepatocytes were resuspended in incubation medium

composed of 20 mM HEPES, 5 mg/l insulin (bovine pancreas; Sigma, St. Louis, Mo.), and 4 mM glutamine (Merck; Darmstadt, FRG) in Dulbecco's MEM ( $10^6$  cells/ml). The cells were then allowed to attach to a glass plate that had been pretreated with 5% newborn calf serum (undialyzed, heat-inactivated; Gibco, Paisley, UK) in HBSS (Gibco). After 2 h, the plate was washed twice with incubation medium supplemented with 10% newborn calf serum and then held at 37°C for 24 h. The rat hepatocytes attached to the plate had a viability of >90%, which persisted during the experiments as determined by the trypan blue exclusion test.

### Flow-through system experiments

**Flow-through system.** This system, which has been described elsewhere [18], is represented schematically in Fig. 1. A sheet of polypropylene (30  $\mu$ m thick) was attached to the perforated upper plate (70 holes with a diameter of 4 mm), which enabled oxygen transfer to the cells in the system. Before its use, the bottom plate was washed with laboratory soap, thoroughly rinsed with de-ionized water, and sterilized for 3 h at 150°C. The perfusion medium (medium A) was Dulbecco's MEM without bicarbonate but containing 20 mM HEPES (Serva; Heidelberg, FRG), 5% fetal bovine serum, glucose (5.6 mM), and glutamine (4 mM). After installation of the flow-through system, the medium was flushed through rapidly (flow rate, 200  $\mu$ l/min) to remove cells that had not attached to the bottom plate. The stock of perfusion medium and the flow-through system were held at 37°C. An HPLC pump (equipped for micro liquid chromatography; Gilson model 302, 5S pump head) was used to pump the medium over the cells, after which the cells were allowed to equilibrate for 30 min at a flow rate of 50  $\mu$ l/min. During the experiments, 60%–70% of the surface was covered with a layer of contiguous cells, and cell densities were 100,000–130,000 cells/cm<sup>2</sup>. Cell exposure to the drug routinely lasted <8 h.

**Determination of eliminated 5-FU.** The chosen 5-FU input concentrations were achieved by stepwise addition of 5-FU (Sigma) to the perfusion medium, starting at the lowest concentration. We used [ $6\text{-}^3\text{H}$ ]-5-FU (1 mCi/mmol; final concentration, 0.01 or 0.1  $\mu$ M; Radiochemical Center, Amersham, UK) to assess metabolites and added nonradioactive 5-FU at higher input concentrations. Samples of input and output effluent were collected 15 min after a change in the input concentration, when steady-state concentrations had been reached. Cellular clearance values were calculated for HEP-2, MCF-7, WiDr, Hep G2, and H35 cells, as well as for freshly isolated rat hepatocytes according to Eq. 1:

$$\ln C_e/C_o = -k \times p/W, \quad (1)$$

where  $C_e$ ,  $C_o$ ,  $k$ ,  $p$ , and  $W$  represent the output concentration, input concentration, clearance constant, number of cells, and flow rate, respectively [18].

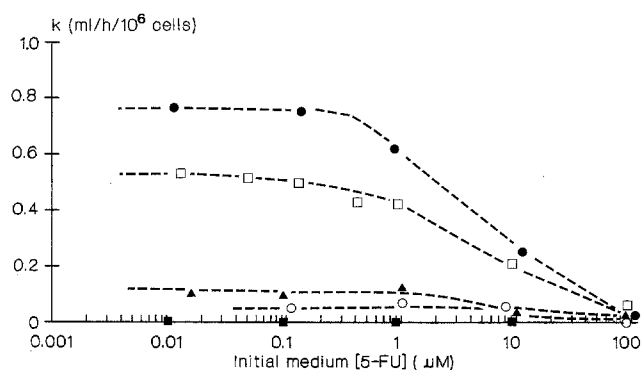
**Cellular pharmacokinetics.** The Michaelis-Menten parameters were estimated according to procedure P3R of the statistical software program BMDP [10], in which the Michaelis-Menten equation (Eq. 2) was fitted. For this equation, the initial velocity ( $V_o$ ) was estimated according to equation 3:

$$V_o = V_{\max} \times C_o/(K_m + C_o) \quad (2)$$

$$V_o = (C_o - C_e) \times W/p. \quad (3)$$

No dramatic overestimation of the 5-FU concentrations could have been made by using the 5-FU input concentrations for the calculation of the Michaelis-Menten parameters, since the decrease in the initial 5-FU concentrations at those inducing saturation phenomena appeared to be <30%. Because small differences between the input and the output concentrations of 5-FU at high drug concentrations led to relatively large errors in the calculation of the  $V_o$  values, we used a weight code equal to  $1/\log V_o$  in the curve-fitting process for the determination of  $K_m$ .

**Dynamic response after pulse injections.** In the flow-through system we used  $7\text{--}8 \times 10^6$  HEP-2 cells, perfusion medium A (see above) supplemented with [ $6\text{-}^3\text{H}$ ]-5-FU (1 mCi/mmol; final concentration, 0.1  $\mu$ M), and a flow rate of 50  $\mu$ l/min for measurement of the dynamic response. When steady-state concentrations of radioactive 5-FU and its catabolites



**Fig. 2.** Cellular clearance of 5-FU by the indicated cell lines, plotted as a function of the initial concentration of the drug in medium. Cellular clearance was calculated according to Eq. 1. A representative example is given for 2–5 experiments. Conditions: number of cells,  $5\text{--}8 \times 10^6$ ; cell-chamber volume, 500  $\mu\text{L}$ ; flow rate, 50  $\mu\text{L}/\text{min}$ ; perfusion medium, medium A; pH 7.4; temperature, 37°C; initial input concentrations of 5-FU varied stepwise from 0.01 to 100  $\mu\text{M}$ , starting with the lowest concentration.  $\blacktriangle$ , WiDr;  $\bullet$ , HEp-2;  $\circ$ , H35;  $\blacksquare$ , MCF-7 and Hep G2;  $\square$ , rat hepatocytes

were reached, pulse injections (15  $\mu\text{L}$ ) of 5 mM nonradioactive 5-FU, thymine, uridine, or uracil were introduced into the flowing perfusion medium via an HPLC injection valve. Samples (50  $\mu\text{L}$ ) of each fraction were then collected. In all experiments, blanks and effluent samples were immediately cooled to a low temperature and the pH was adjusted to a low value (0–5°C; pH 3), and all material was stored at –20°C until analyzed.

### Incubation of HEp-2 cells

HEp-2 cells collected from a suspension of trypsinized cells were seeded in culture flasks (25  $\text{cm}^2$ ) at a density of  $0.5\text{--}5 \times 10^6$  cells/flask and then cultured overnight. The monolayers were washed with HBSS (pH 7.4) and then incubated at 37°C with 1 ml medium A supplemented with 5% fetal calf serum and 0.1  $\mu\text{M}$  [6- $^3\text{H}$ ]-5-FU (1 mCi/mmol). After 30 min, the incubation medium was removed from the monolayer, immediately put on ice, and then centrifuged at 0–5°C in a Runne Heidelberg centrifuge (model 85-2, Stram-Instrumenten; Eerbeek, The Netherlands) for 5 s at 3,000  $g$  to remove unattached cells. The supernatant was kept at –20°C until analysis. Clearance was calculated according to equation 4:

$$\ln C_t/C_0 = -k \times p \times t/V, \quad (4)$$

where  $C_t$ ,  $C_0$ ,  $t$ , and  $V$  are the concentration of 5-FU after incubation in medium, the initial 5-FU concentration, the incubation time, and the volume of incubation medium, respectively.

### Analysis

Determination of 5-FU concentrations between 0.01 and 1  $\mu\text{M}$  was performed by capillary gas chromatography and negative-ion chemical ionization mass spectrometry as described elsewhere [17]. Samples with higher 5-FU concentrations were measured by HPLC (Perkin Elmer liquid chromatography pump, series 2; Norwalk, Conn.) using a UV detector (Uvikon 740 LC; Kontron Analytical, Basel, Switzerland) as described elsewhere [2]. We used a  $\mu\text{Bondapak C-18}$  column (30  $\text{cm} \times 3.9$  mm; average particle diameter, 10  $\mu\text{m}$ ; Waters Associates, Milford, Mass.) and an acetate buffer (10 mM, pH 5.4) as the eluent. The flow rate was 1 ml/min (HPLC method I). When radioactive 5-FU was used, the total amount of radioactivity was measured by liquid scintillation counting in 25- $\mu\text{L}$  aliquots of the input and output samples.

F- $\beta$ -ala, F-UPA, 5-FU, F-DHU, and 5-fluorouridine (FUR) were separated according to an HPLC method described elsewhere [32]. We used a  $\mu\text{Bondapak C-18}$  column as described above, with 5 mM tet-

rabutylammonium hydrogen sulfate and 1.5 mM potassium phosphate (pH 8) as the mobile phase. Elution was performed at a rate of 1 ml/min (HPLC method II). We analyzed 10  $\mu\text{L}$  of each sample and found the following capacity factors (difference between the retention times of retarded and unretarded fractions divided by the retention time of the unretarded fraction): 0.2 for F- $\beta$ -ala, 0.6 for F-DHU, 1.4 for F-UPA, 2.0 for 5-FU, and 4.1 for FUR. Samples were spiked with 5-FU, FUR, F-DHU, and F- $\beta$ -ala (F-DHU and F- $\beta$ -ala were gifts from Hoffmann-La Roche B. V., Mijdrecht, The Netherlands). Because radiolabeled compound was not available, the capacity factor of F-UPA was determined by comparing the only unknown radioactive fraction with published data [32]. Detection occurred at a wavelength of 254 nm except for F-DHU, which was detected at 215 nm. Fractions (500  $\mu\text{L}$ ) were collected and radioactivity was measured by liquid scintillation counting.

Separation of [6- $^3\text{H}$ ]-5-FU and its metabolites was routinely performed on PEI-cellulose thin layers using distilled water as the eluent as described elsewhere [25]. F- $\beta$ -ala and F-UPA remained at the origin; for 5-FU, F-DHU, and FUR the  $R_f$  values were 0.67, 0.75, and 0.80, respectively. The samples were spiked with 5-FU and FUR (Sigma), which could be detected under a UV lamp (254 nm). Because the other metabolites could not be detected by UV absorption at a wavelength of 254 nm, these  $R_f$  values were determined by counting the radioactivity on PEI cellulose. Values obtained by different separation methods were similar (deviations, <20%).

## Results

### Concentration dependence of 5-FU elimination

At a fixed input concentration, the 5-FU output concentration remained constant for several hours. The cellular clearance values were calculated according to Eq. 1 for various cell lines and freshly isolated rat hepatocytes at various 5-FU concentrations. Representative experiments are shown in Fig. 2. At 5-FU concentrations of between 0.01 and 1  $\mu\text{M}$ , cellular clearance was constant. This maximal clearance (expressed in milliliters per hour per  $10^6$  cells) was  $0.7 \pm 0.3$  ( $n = 8$ ), 0.7 or 0.5 ( $n = 2$ ),  $0.05 \pm 0.02$  ( $n = 4$ ), and 0.04 or 0.1 ( $n = 2$ ) for HEp-2 cells, rat hepatocytes, WiDr cells, and H35 cells, respectively. Values represent the means  $\pm$  SD from ( $n$ ) separate experiments. No clearance was detected in either Hep G2 or MCF-7 cells.

We used the HEp-2 cell line to examine whether the cellular clearance was dependent on the flow rate or the cell density in our system. At an input concentration of 0.1  $\mu\text{M}$  5-FU, clearance as calculated using Eq. 1 was constant between 25 and 100  $\mu\text{L}/\text{min}$  (data not shown). The relationship between decreases in concentration and cell density at a fixed time point was tested for cell densities between 50,000 and 200,000 cells/ $\text{cm}^2$ . Calculation of the clearance values for the data from these incubation experiments (Eq. 4; data not shown) gave the same clearance constants that were found with the flow-through system (differences, <10%).

Cellular clearance showed saturation kinetics, with  $K_m$  values of 16.4 and 17.3  $\mu\text{M}$  and  $V_{\text{max}}$  values of 0.095 and 0.091 nmol/min/ $10^6$  cells being calculated for HEp-2 cells. For rat hepatocytes, we obtained  $K_m$  values of 14.3 and 21.8  $\mu\text{M}$  and  $V_{\text{max}}$  values of 0.12 and 0.13 nmol/min/ $10^6$  cells. The values were calculated from separate experiments, fitting at least four points per series. On the basis of these values, the differences between experimental and predicted values for the initial velocity were usually <10%.

**Table 1.** Metabolism of 5-FU by cells of various types

Cell type	Metabolite formed		
	F-DHU (%)	F-UPA (%)	F- $\beta$ -ala (%)
Hepatocytes	11 $\pm$ 1	6 $\pm$ 1	83 $\pm$ 5
HEp-2	91 $\pm$ 4	4 $\pm$ 1	5 $\pm$ 1
H35	64 $\pm$ 4	18 $\pm$ 1	18 $\pm$ 1
WiDr	76 $\pm$ 1	<sup>a</sup>	<sup>a</sup>

<sup>a</sup> F-UPA plus F- $\beta$ -ala accounted for 24% of the eliminated radioactivity/ml in the 5-FU fraction

Metabolism of 5-FU by various cell lines at a constant initial concentration of 5-FU is expressed as the percentage of metabolite formed from eliminated 5-FU. All data represent the means  $\pm$  SD of 2–5 experiments. For experimental conditions, see Fig. 2; the initial input concentration of 5-FU was 0.1  $\mu$ M

**Table 2.** Concentration-dependent catabolism of 5-FU in rat hepatocytes and HEp-2 cells

5-FU ( $\mu$ M)	F-DHU (%)	F-UPA (%)	F- $\beta$ -ala (%)
Hepatocytes:			
100	40 $\pm$ 4	13 $\pm$ 2	47 $\pm$ 4
10	27 $\pm$ 3	7 $\pm$ 1	66 $\pm$ 5
1	21 $\pm$ 3	6 $\pm$ 1	73 $\pm$ 4
0.1	11 $\pm$ 1	6 $\pm$ 1	83 $\pm$ 5
HEp-2:			
100	89 $\pm$ 6	<sup>a</sup>	<sup>a</sup>
10	83 $\pm$ 3		
1	98 $\pm$ 5		
0.1	91 $\pm$ 4		
0.01	90 $\pm$ 3		

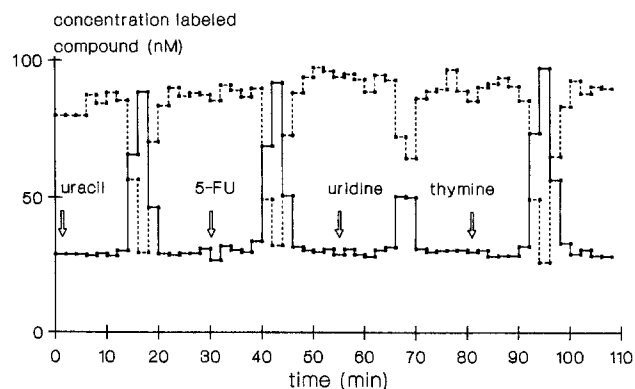
<sup>a</sup> F- $\beta$ -ala plus F-UPA accounted for 10% of the eliminated radioactivity/ml in the 5-FU fraction

Metabolism of 5-FU by hepatocytes and HEp-2 cells was compared at various initial 5-FU concentrations. Metabolism is expressed as in Table 1. All data represent the means  $\pm$  SD of 2–5 experiments. For experimental conditions, see Fig. 2

The low elimination capacity of WiDr and H35 cells led to marked deviation of the estimated initial velocity values from the predicted curve. The  $K_m$  and  $V_{max}$  values could be estimated only roughly for WiDr cells ( $K_m$ , 15  $\mu$ M;  $V_{max}$ , 0.025 nmol/min/10<sup>6</sup> cells).

### Cellular metabolism

For the analysis of 5-FU metabolites present in the medium, radioactive 5-FU and catabolites were separated. Table 1 shows the relative amounts of 5-FU metabolites found in cell lines and hepatocytes. Depending on the flow rate, initial 5-FU concentration, and cell line used, the ratio of 5-FU output concentration to input concentration ranged from 0.12 to 1.0. Metabolism was evaluated only if this ratio was  $\leq$  0.9. In all tumor cell lines, the main catabolite of 5-FU was F-DHU (60%–90%); in the rat hepatocytes, the main catabolite was F- $\beta$ -ala (>80%). Table 2 shows the relative amounts of 5-FU metabolites occurring at various drug input concentrations. No influence of flow rate (not shown) or dose was found in the cell line HEp-2. In rat



**Fig. 3.** Dynamic response of steady-state concentrations of radioactive 5-FU and catabolites after a high concentration pulse of uracil, 5-FU, uridine, or thymine. We plotted the concentrations of radioactive F-DHU and 5-FU. Concentrations were calculated with the specific activity of the steady-state radiolabeled 5-FU. Time of pulse injections are indicated by arrows. Results are representative of 2 experiments; for experimental conditions, see Fig. 2. Initial input concentration of [6-<sup>3</sup>H]-5-FU in perfusion medium A, 0.1  $\mu$ M; pulse injections: concentration of non-radioactive 5-FU, uracil, uridine or thymine, 5 mM; injection volume, 15  $\mu$ L. ●—●, [6-<sup>3</sup>H]-5-FU; ●---●, [6-<sup>3</sup>H]-F-DHU

hepatocytes we found a significant increase in the ratio of F- $\beta$ -ala to eliminated 5-FU at lower drug input concentrations. We also used HPLC method II to determine whether 5-FUR was present in the medium. No 5-FUR was detected in the output of either the tumor cell lines or the hepatocytes used.

### Dynamic response after pulse injections

The dynamic response after pulse injections of 5-FU as well as of compounds capable of modulating 5-FU metabolism was measured. In HEp-2 cells, we assessed the effect on 5-FU clearance of a high pulse injection of nonradioactive 5-FU, uracil, uridine, or thymine, given in conjunction with a steady-state concentration of tritium-labeled 5-FU. The results showed disturbed 5-FU clearance after bolus injections of the compounds used (see Fig. 3). There was no significant difference between the dynamic responses to a series of distinct pulse injections given in a different time order (not shown). The sum of radioactivity in the 5-FU and F-DHU fractions was >95% of the total radioactivity in the medium. The response was rapid and the initial situation according to our standards was restored within 5 min. Of the three compounds, thymine had the strongest effect on the catabolism of 5-FU.

### Discussion

This study presents a novel method for the examination of cellular events following the administration of 5-FU. To a certain extent, the setup simulates 5-FU perfusion of tumor tissue. Measurement of extracellular products of 5-FU metabolism by intact tumor is hampered because blood samples collected before and after passage through tumor tissue are usually not available. The 5-FU concentrations in the perfusion medium of the flow-through system were

chosen for their correspondence with the plasma concentrations previously found in patients [37].

We found a concentration-dependent 5-FU elimination for rat hepatocytes as well as some cancer cell lines. Dose-dependent 5-FU pharmacokinetics in man have previously been reported [6, 30]. However, the initial disappearance curves after intravenous bolus administration do not show the convex log plasma concentration-time curve one would expect from a saturable process. Collins et al. [6] have developed a two-compartment physiologic pharmacokinetic model that satisfactorily predicts the initial disappearance kinetics of 5-FU in patients. This model postulates two compartments, one with linear and the other with non-linear disappearance. The best-fit  $K_m$  value for saturation of 5-FU elimination in this model is 15  $\mu\text{M}$ .

Hepatic dose-dependent metabolism and elimination have been studied in isolated perfused rat liver, isolated rat hepatocytes, and cytosolic fractions of hepatocytes [19, 32, 38], and  $K_m$  values from these *in vitro* studies have been reported. However, fitting these data to the model for the whole body was difficult; Mentre et al. [19] found wide variation among these values (8–58  $\mu\text{M}$ ); Warren et al. [38] found  $K_m$  values in the range of 33–84  $\mu\text{M}$ . These latter values were obtained using perfused rat livers. In these experiments, a relatively sharp decrease in the initial 5-FU concentration per perfusion cycle could have led to overestimation of the initial 5-FU concentrations in the calculation of  $K_m$  values, especially at low flow rates. This might explain the higher  $K_m$  values found by Warren and his colleagues. The  $K_m$  values we obtained for saturation of 5-FU removal (14–22  $\mu\text{M}$ ) are in agreement with the best-fit  $K_m$  value of 15  $\mu\text{M}$  according to the proposed model for the whole body. In conclusion, for the first time, clear confirmation of the two-compartmental model of Collins et al. [6] has been obtained at the cellular level.

We found a significant increase in the ratio of F- $\beta$ -ala to eliminated 5-FU at lower drug input concentrations in rat hepatocytes (Table 2), indicating concentration-dependent F-DHU elimination. An explanation for these observations might be found at the biochemical level by the study of relative enzymatic conversion rates. Increasing attention has been paid to the pharmacokinetics of F-DHU, since it was suggested that this compound might contribute to the toxicity of 5-FU *in vivo*. Nonlinear disappearance of F-DHU *in vivo* has also been reported [7]. In *in vitro* experiments, F-DHU was found to be cytotoxic for Ehrlich ascites tumor cells [9], and the authors suggested a reverse conversion of F-DHU to 5-FU. Although F-DHU has been found to be unstable in human plasma [8, 35], a reverse reaction converting F-DHU to 5-FU *in vivo* has not been reported. The formation of F-DHU in tumor cells may contribute to 5-FU cytotoxicity to such cells. However, we observed fast cellular excretion of F-DHU (Fig. 3), which may prevent accumulation of this metabolite in these cells.

Several studies have been performed on biochemical pathways for 5-FU in isolated rat hepatocytes and perfused rat livers, and saturation of the catabolic pathway was found to be responsible for dose-dependent 5-FU elimination [19, 22, 31, 38]. Reports on dihydrouracil dehydrogenase activity in extrahepatic tissues are contradictory. Some investigators have found only small amounts of this

enzyme in tumor tissue [13]. Comparing dihydrouracil dehydrogenase activity in extracts of various human tissues, including neoplastic tissue, others have reported the lack of a pattern [24, 34]. 5-FUR is known to be the main anabolic compound leaked by human melanoma cell lines [25]. This compound, however, was absent in all of the medium samples tested in the present study. With respect to the metabolism of 5-FU in intact cells, our results show that in rat hepatocytes as well as in three tumor cell lines – WiDr, H35 and HEp-2 – most of the 5-FU (95%–100%; see Tables 2 and 3) is eliminated via the catabolic pathway. We found differences in 5-FU metabolism by the tumor cell lines and hepatocytes, the main catabolites being F-DHU and F- $\beta$ -ala, respectively. According to previous reports, liver and kidney differ from other tissues in uracil breakdown, as cytosolic extracts of liver and kidney were found to catabolize uracil to  $\beta$ -alanine, whereas only dihydrouracil was formed in intestinal mucosa, lung, pancreas, and lymphocytes [24]. Our results show that high clearance values were achieved not only when 5-FU was totally metabolized into F- $\beta$ -ala, as has previously been suggested [38], but also when F-DHU was the main catabolite. These findings support the expectation of Collins et al. [6] of high clearance values in lung tissue.

No association was found between the cellular 5-FU clearance values and the type of cell. The cellular clearance values we obtained (range, 0.03–1.4 l/h/g) are much higher than the average blood flow previously reported in normal liver tissue or tumor tissues *in vivo* (range, 0.001–0.010 l/h/g) [14, 27, 39]. Thus, 5-FU clearance values *in vivo* are likely determined by the blood flow in the tissue involved, and one can speculate that tissues with high blood-flow rates may have high values for extrahepatic clearance of 5-FU. Another important feature of blood flow is the delivery of drug to tissues. At low blood-flow rates and 5-FU concentrations below the  $K_m$  values, depletion of 5-FU by catabolism could occur. In this case, the rate of anabolic metabolism may also become lower. Since average blood flow in tumor tissue has been reported to be lower than that in normal tissue [14, 27], one can assume that 5-FU depletion and the associated changes in anabolic metabolism take place primarily in tumor tissue.

Comparison of the dynamic responses of uracil, uridine, and thymine was limited, since we selected only one high concentration for each modulator. Nevertheless, the observed low effect of uridine is in agreement with the absence of effect of a bolus uridine injection on 5-FU pharmacokinetics previously reported in patients [36]. The total dynamic response to different pulse injections (see Fig. 3) showed a rapid alteration in 5-FU conversion, with a return to original values occurring within 5 min. The enzymes responsible for catabolism are located in the cytosol of the cells [24]. We conclude that underlying processes such as drug uptake, intracellular transport, and metabolism in these cells are fast. These results indicate that changes in plasma composition in patients are immediately followed at the cellular level.

In summary, we found cellular catabolism to be responsible for concentration-dependent 5-FU elimination *in vitro*. Not only cells derived from hepatic tissue but also some cancer cell lines showed strong clearance of 5-FU

from the medium. The flow-through system proved to be adequate for the assessment of the cellular pharmacokinetics of 5-FU, especially the rapid dynamic processes. The system is also useful for comparison of the effects of compounds that may potentially affect these cellular pharmacokinetics. Such information may be valuable for the design of chemotherapy protocols.

**Acknowledgements.** The authors thank Dr. H. J. Broxterman for his valuable comments during the preparation of the manuscript. We are also indebted to Olga Tol and Dr. E. Roos of the Netherlands Cancer Institute, who kindly supplied HepG2 cells and freshly isolated rat hepatocytes, and to Dr. J. J. P. Nauta for his statistical advice.

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