



Intracellular Pharmacokinetics of Pyrimidine Analogues used in Oncology and the Correlation with Drug Action

Ellen J. B. Derissen^{1,2,3} · Jos H. Beijnen^{1,4}

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Abstract

Pyrimidine analogues can be considered as prodrugs, like their natural counterparts, they have to be activated within the cell. The intracellular activation involves several metabolic steps including sequential phosphorylation to its monophosphate, diphosphate and triphosphate. The intracellularly formed nucleotides are responsible for the pharmacological effects. This review provides a comprehensive overview of the clinical studies that measured the intracellular nucleotide concentrations of pyrimidine analogues in patients with cancer. The objective was to gain more insight into the parallels between the different pyrimidine analogues considering their intracellular pharmacokinetics. For cytarabine and gemcitabine, the intracellular pharmacokinetics have been extensively studied over the years. However, for 5-fluorouracil, capecitabine, azacitidine and decitabine, the intracellular pharmacokinetics was only very minimally investigated. This is probably owing to the fact that there were no suitable bioanalytical assays for a long time. Since the advent of suitable assays, the first exploratory studies indicate that the intracellular 5-fluorouracil, azacitidine and decitabine nucleotide concentrations are very low compared with the intracellular nucleotide concentrations obtained during treatment with cytarabine or gemcitabine. Based on their pharmacology, the intracellular accumulation of nucleotides appears critical to the cytotoxicity of pyrimidine analogues. However, not many clinical studies have actually investigated the relationship between the intracellular nucleotide concentrations in patients with cancer and the anti-tumour effect. Only for cytarabine, a relationship was demonstrated between the intracellular triphosphate concentrations in leukaemic cells and the response rate in patients with AML. Future clinical studies should show, for the other pyrimidine analogues, whether there is a relationship between the intracellular nucleotide concentrations and the clinical outcome of patients. Research that examined the intracellular pharmacokinetics of cytarabine and gemcitabine focused primarily on the saturation aspect of the intracellular triphosphate formation. Attempts to improve the dosing regimen of gemcitabine were aimed at maximising the intracellular gemcitabine triphosphate concentrations. However, this strategy does not make sense, as efficient administration also means that less gemcitabine can be administered before dose-limiting toxicities are achieved. For all pyrimidine analogues, a linear relationship was found between the dose and the plasma concentration. However, no correlation was found between the plasma concentration and the intracellular nucleotide concentration. The concentration–time curves for the intracellular nucleotides showed considerable inter-individual variation. Therefore, the question arises whether pyrimidine analogue therapy should be more individualised. Future research should show which intracellular nucleotide concentrations are worth pursuing and whether dose individualisation is useful to achieve these concentrations.

✉ Ellen J. B. Derissen
e.derissen@etz.nl

Extended author information available on the last page of the article

Key Points

Pyrimidine analogues can be considered as prodrugs. Understanding the plasma pharmacokinetics is therefore not enough. It is important to investigate the extent to which these agents enter the cell, are activated intracellularly and actually reach their target in the activated form.

Intracellular pyrimidine nucleotide concentrations vary considerably between patients. This raises the question whether therapy with pyrimidine analogues should not be further individualised.

Although the intracellular accumulation of nucleotides appears critical for the pharmacological effect of pyrimidine analogues, only a few clinical studies have actually investigated the relationship between intracellular nucleotide concentrations and the clinical outcome of patients.

1 Introduction

Pyrimidine analogues form a classical group of cytostatic drugs that still have a very important place in the treatment of both solid and haematologic malignancies. Pyrimidine analogues are structural analogues of one of the physiological pyrimidine nucleobases: cytosine, uracil, or thymine or of their respective nucleosides: (deoxy)cytidine, uridine and thymidine (Fig. 1).

The first important pyrimidine analogue, 5-fluorouracil (5-FU), was synthesised in 1957 and still has an important place in the treatment of various solid tumours, including those originating into the gastrointestinal tract, breast, and head and neck region. After 5-FU, several other pyrimidine analogues followed. In 1969, cytarabine was introduced for the treatment of acute leukaemia. Today, cytarabine is still one of the cornerstones in the treatment of acute myeloid leukaemia (AML). Moreover, it has a place in the treatment of various other haematological malignancies. The success of cytarabine as an anti-leukaemic drug stimulated the research for other cytidine analogues that had anti-tumour activity and led to the first preclinical studies with azacitidine and decitabine in the 1960s [1]. Although azacitidine and decitabine initially seemed promising, the first clinical trials were not very impressive, limiting enthusiasm for these drugs as anti-tumour agents [2–4]. In 1995, gemcitabine was introduced. Although the structure of gemcitabine was similar to that of cytarabine, it had a different spectrum of anti-tumour activity [5]. Unlike cytarabine, gemcitabine

exhibited activity in solid tumours. Today, gemcitabine has an important place in the treatment of non-small cell lung cancer, pancreatic cancer, bladder cancer and ovarian cancer. In the 1990s, capecitabine was developed as a more convenient oral alternative to intravenous 5-FU therapy. As a prodrug of 5-FU, capecitabine was rationally designed to mimic continuous infusion of 5-FU with a certain degree of tumour selectivity [6]. In this decade, there was also renewed interest in azacitidine and decitabine. This time because of their DNA demethylating properties in patients with myelodysplastic syndrome (MDS) [7–10]. Today, azacitidine and decitabine have gained their place in the treatment of MDS and AML [11].

Pyrimidine analogues can be considered as prodrugs, like their natural counterparts, they have to be activated within the cell. The intracellular activation involves several metabolic steps including sequential phosphorylation of the pyrimidine analogue to its monophosphate, diphosphate and triphosphate (Fig. 2). Ultimately, these intracellularly phosphorylated anabolites, called nucleotides, are responsible for the pharmacological effects of the pyrimidine analogue. For instance, as the triphosphate form can compete with its natural counterpart for incorporation into the DNA or RNA, it thereby interferes with DNA or RNA synthesis and with their normal functions.

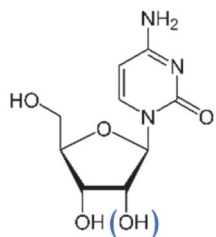
As the intracellularly formed nucleotides are ultimately responsible for the effect of the pyrimidine anticancer drugs, it would be useful to understand the intracellular pharmacokinetics (PK) of these nucleotides during treatment. In this review, we summarise the findings of clinical studies that measured the intracellular concentrations of pyrimidine nucleotides in patients with cancer. The objective of this review was to gain more insight into the parallels between the different pyrimidine analogues with regard to their intracellular PK. In this manner, we can learn the most from what is already known about the intracellular PK of the other drugs in this group. This information could ultimately be very useful to optimise treatment with these agents.

2 Literature Search

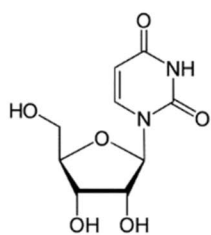
In this review, we focused on the six pyrimidine analogues that are currently considered the most important: 5-fluorouracil, capecitabine, cytarabine, gemcitabine, azacitidine and decitabine. All clinical studies were included in which intracellular nucleotide concentrations were measured in patients with cancer during or after treatment with one of these pyrimidine analogues.

Although much of our knowledge about the intracellular pharmacology of the pyrimidine analogues comes from *in vitro* and animal studies, these studies were not included in this review, as it remains difficult to extrapolate these

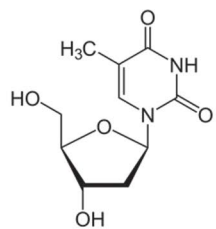
Physiological pyrimidines



Cytidine

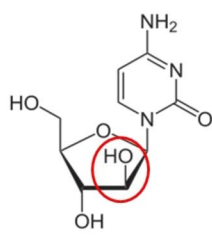
2'-Deoxycytidine

Uridine

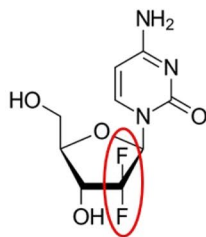


Thymidine

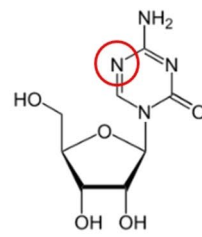
Pyrimidine analogues used in oncology



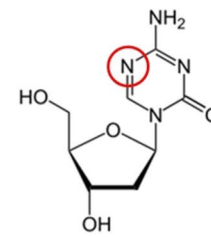
Cytarabine



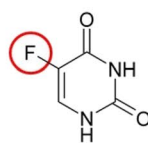
Gemcitabine



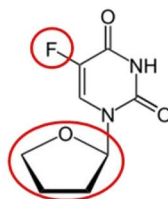
Azacitidine



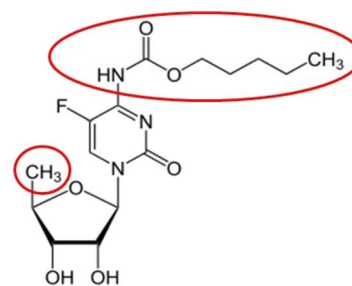
Decitabine



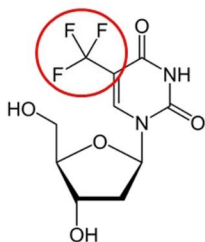
Fluorouracil



Tegafur



Capecitabine



Trifluridine

Fig. 1 Chemical structures of the physiological pyrimidines (left) and the pyrimidine analogues used as an anticancer drug (right)

results to the clinical situation. Knowledge derived from preclinical studies, which was considered relevant for the interpretation of the clinical studies, was briefly summarised at the beginning of each section.

More than 30 clinical studies were included in this review. The results of these studies are also summarised in Tables 1 and 2. The intracellular nucleotide concentrations measured in different studies are sometimes difficult to compare because different units were used. Concentrations were reported in μM , $\text{pmol}/10^6$ peripheral blood mononuclear cells (PBMCs) or ng/mg protein. In this review, data were presented as reported in the original publications. Units were not converted because not all required information,

for instance the measured cell volume, was published in the original articles.

3 5-Fluorouracil and Capecitabine

5-Fluorouracil is a uracil analogue with a fluorine atom at the C-5 position instead of a hydrogen atom (Fig. 1). Approximately 80% of 5-FU is inactivated in the liver by the enzyme dihydropyrimidine dehydrogenase [12]. This means that only a small fraction of the administered 5-FU is available for intracellular anabolism to the active nucleotides. Capecitabine was rationally designed to mimic continuous infusion of 5-FU [13, 14]. After oral administration,

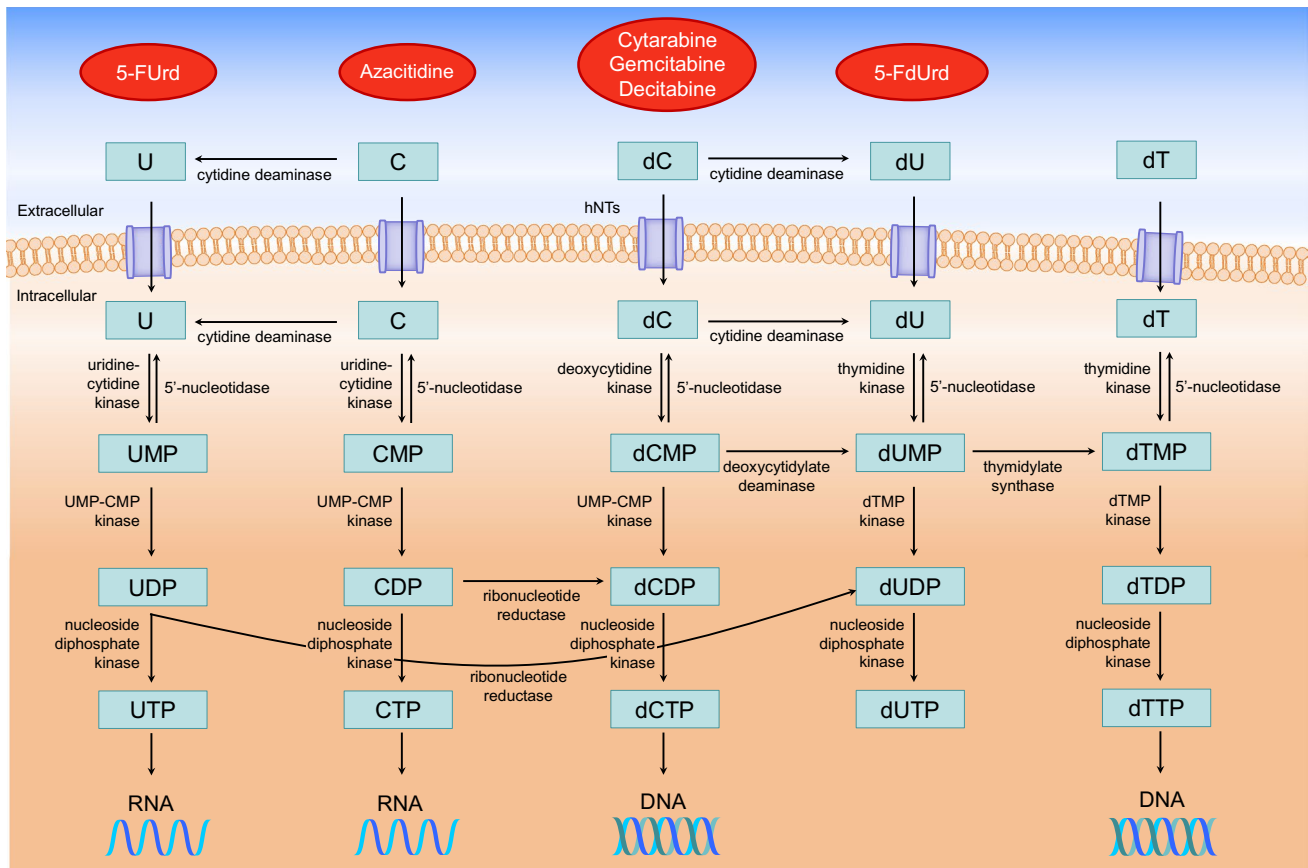


Fig. 2 Intracellular metabolism of pyrimidine anticancer drugs. Pyrimidine analogues and their natural counterparts share the same metabolic pathways. *5-FUrd* 5-fluorouridine, *5-FdUrd* 5-fluoro-2'-deoxyuridine, *C* cytidine, *CMP* cytidine monophosphate, *CDP* cytidine diphosphate, *CTP* cytidine triphosphate, *dC* 2'-deoxycytidine, *dCMP* 2'-deoxycytidine monophosphate, *dCDP* 2'-deoxycytidine diphosphate, *dCTP* 2'-deoxycytidine triphosphate, *DNA* deoxyri-

bonucleic acid, *dT* 2'-deoxythymidine, *dTMP* 2'-deoxythymidine monophosphate, *dTDP* 2'-deoxythymidine diphosphate, *dTTP* 2'-deoxythymidine triphosphate, *dU* 2'-deoxyuridine, *dUMP* 2'-deoxyuridine monophosphate, *dUDP* 2'-deoxyuridine diphosphate, *dUTP* 2'-deoxyuridine triphosphate, *hNTs* human nucleoside transporters, *RNA* ribonucleic acid, *U* uridine, *UMP* uridine monophosphate, *UDP* uridine diphosphate, *UTP* uridine triphosphate

capecitabine is rapidly and almost completely absorbed from the gastrointestinal tract. Subsequently, it is converted into 5-FU through a series of three metabolic steps [15, 16]. The third activation step preferentially takes place in tumour tissue rather than normal tissue, which confers a degree of tumour specificity to capecitabine [15–18].

3.1 Intracellular Metabolism and Mechanism of Action

5-Fluorouracil rapidly enters the cell using the same transport mechanism as uracil [12, 19]. Once in the cell, 5-FU has to be activated by ribosylation and sequential phosphorylation. The activation of 5-FU to its nucleotides can proceed via three pathways [20, 21]. First, 5-fluorouracil can be converted *directly* to 5-fluorouridine monophosphate by the enzyme orotate phosphoribosyl transferase. Second, 5-FU can be converted to 5-fluorouridine monophosphate *in two*

steps, via 5-fluorouridine, through the sequential actions of uridine phosphorylase and uridine kinase. 5-Fluorouridine monophosphate is further phosphorylated to 5-fluorouridine diphosphate and the active metabolite 5-fluorouridine triphosphate (FUTP) by uridine monophosphate kinase and uridine diphosphate kinase, respectively. 5-Fluorouridine diphosphate might also be converted to 5-fluoro-2'-deoxyuridine diphosphate by ribonucleotide reductase. The third activation pathway involves the conversion of 5-FU to 5-fluoro-2'-deoxyuridine by thymidine phosphorylase. 5-Fluoro-2'-deoxyuridine can then be phosphorylated to the active metabolite 5-fluoro-2'-deoxyuridine monophosphate (FdUMP) by thymidine kinase. 5-Fluoro-2'-deoxyuridine monophosphate can also be further phosphorylated to 5-fluoro-2'-deoxyuridine diphosphate and the third active metabolite 5-fluoro-2'-deoxyuridine triphosphate (FdUTP). These reactions are catalysed by uridine monophosphate kinase and uridine diphosphate kinase, respectively.

Table 1 Summary of the pharmacokinetic parameters in plasma (obtained in studies that also investigated the intracellular pharmacokinetics)

Reference	Drug	Treatment cycle	n	No. of samples per patient	Dose	Dose rate	T_{max}	C_{max}	Cl	Vd	ke	t1/2
5-Fluorouracil and capecitabine												
Derissen et al. [33]	Capecitabine	Cycle 1; day 1	13	10	1000 mg QD		Capecitabine: 0.7 ± 0.3 h	Capecitabine: 11 ± 4.6 µM	AUC ₀₋₄ : 8.8 ± 2.7 µM·h			t1/2β: 0.4 ± 0.08 h
							5-FU: 0.9 ± 0.3 h	5-FU: 2.6 ± 1.5 µM				
		Cycle 1; day 1	5	8	850 mg/m ² BID for 14 days, every 3 weeks		Capecitabine: 3.3 ± 2.9 h	Capecitabine: 7.3 ± 5.2 µM	AUC ₀₋₄ : 11 ± 5.1 µM·h			t1/2β: 0.7 ± 0.5 h
							5-FU: 3.4 ± 2.9 h	5-FU: 3.0 ± 2.1 µM				
Cytarabine												
Lillemark et al. [43]	Cytarabine	Cycle 1; day 1	11	8	3000 mg/m ² infused in 2 h	1500 mg/m ² /h	2 h	Css: 94 ± 32 µM	198 ± 67 µM ² ·h			t1/2α: 0.44 ± 0.10 h
Plunkett et al. [44]	Cytarabine	Cycle 1; day 1	2	8	3000 mg/m ² infused in 1 h	3000 mg/m ² /h		Css: 77 µM				
		Cycle 1; day 1	8	8	3000 mg/m ² infused in 4 h	750 mg/m ² /h						
		Cycle 1; day 1	11	8	1000 mg/m ² infused in 2 h	500 mg/m ² /h		Css: 21 µM				46 µM ² ·h
		Cycle 1; day 1	5	8	400 mg/m ² infused in 2 h	200 mg/m ² /h		Css: 5.3 µM				16 µM ² ·h
Gandhi et al. [37]	Cytarabine	Cycle 1; day 1	2	8	1000 mg/m ² infused in 2 h	500 mg/m ² /h		ara-C: 2 h	ara-C: 26.5 µM			
								ara-U: 3.3 h	ara-U: 294 µM			
		Cycle 1; day 1	3	9	2000 mg/m ² infused in 4 h	500 mg/m ² /h		ara-C: 3 h	ara-C: 29.3 µM			
								ara-U: 4.7 h	ara-U: 344 µM			
Gemcitabine												
Abbruzzese et al. [74]	Gemcitabine	NR	28	17	10–1000 mg/m ² weekly for 3 weeks followed by 12 days of observation	Escalating dose administered over 30 min	dFdC: A plateau was generally reached within 15 min after start of the infusion	dFdC: Css is proportional to the total dose administered	The AUC ₀₋₄ of plasma dFdC was proportional to the dose ($r=0.627$) as well as the AUC _{0-24h} of plasma dFdU ($r=0.758$) ($n=27$)	dFdC: 119 ± 279 [29–1239] L/m ² /h		dFdC: biphasic elimination observed for 13 patients t1/2α: 3.5 min; t1/2β: NR

Table 1 (continued)

Reference	Drug	Treat- ment cycle	n	No. of samples per patient	Dose	Dose rate	T _{max}	C _{max}	AUC	Cl	V _d	ke	t _{1/2}
Grunevald et al. [87]	Gemcit- abine	Cycle 1	19	≥ 16	1200–6400 mg/m ² weekly for 3 weeks	10 mg/m ² / min		C _{ss} : 26.5 ± 9 [13.0–53.5] µmol/L (n = 17)	AUC _{0–t} : The AUC for dF _{0C} increased with increased dose (r = 0.656)	149.3 [103–221] L/h	59 [18–257] L		dF _{0C} : biphasic elimi- nation observed for 6 patients t _{1/2α} : 6.7 [0.8–26] min; t _{1/2β} : 27 [6–80] min dF _{0U} : in general biphasic elimina- tion: t _{1/2α} : 23.5 [2.6–160] min; t _{1/2β} : 22.4 [6.9–124] h t _{1/2β} : dF _{0U} : 474 ± 43 min
Kroep et al. [131] ^a	Gemcit- abine	Cycle 1; day 1	9	5	1000 mg/m ² infused in 30 min + pacli- taxel 150 mg/m ²	33.3 mg/ m ² /min	30 min	dF _{0C} : 33.2 ± 7.1 µM dF _{0U} : 85.1 ± 6.3 µM	AUC _{0–inf} : dF _{0U} : 50.5 ± 7.1 mM*min				
van Moorsel et al. [132] ^b	Gemcit- abine	Cycle 1 + 2; day 1 + 2	33	5	1000 mg/m ² infused in 30 min + pacli- taxel 200 mg/m ² 800 mg/m ² infused in 30 min + cispl- atin 50 mg/m ²	33.3 mg/ m ² /min 26.7 mg/ m ² /min	30 min	dF _{0C} : 27.4 ± 1.5 µM dF _{0U} : 82.7 ± 5.7 µM	AUC _{0–inf} : dF _{0U} : 57.0 ± 4.3 mM*min				t _{1/2β} : dF _{0U} : 602 ± 54 min
					Gemcitabine 4 h before cisplatin			dF _{0C} : 21.9 ± 1.9 µM [7.6–36.2]	AUC _{0–inf} : dF _{0U} : 52.7 ± 17.2 mM* min [15.1–69.4]				
					Cisplatin 4 h before gemcitabine			dF _{0C} : 18.1 ± 1.6 µM [8.0–27.6]	AUC _{0–inf} : dF _{0U} : 34.4 ± 2.8 mM*min [15.6–48.9]				
					Gemcitabine 24 h before cisplatin			dF _{0C} : 32.0 ± 3.2 µM [11.0–64.1]	AUC _{0–inf} : dF _{0U} : 72.6 ± 15.5 mM* min [21.9–232.3]				
					Cisplatin 24 h before gemcitabine			dF _{0C} : 24.5 ± 3.1 µM [3.9–44.6]	AUC _{0–inf} : dF _{0U} : 49.5 ± 6.6 mM*min [8.2–97.4]				
Tempero et al. [92]	Gemcit- abine	Cycle 1	10	6	2200 mg/m ² infused in 30 min	73.3 mg/ m ² /min		99.5 ± 19 µM					
		Cycle 1	6	6	1500 mg/m ² infused in 150 min	10 mg/m ² / min		25.5 ± 5.5 µM					
			5	7	300 mg/m ² infused in 1 h	5 mg/m ² / min		3.10 mg/L	AUC _{0–t} : 3.55 mg*h/L	150.61 L/h	V _{ss} : 86.80 L	2.1 h ⁻¹	0.33 h
			5	7	300 mg/m ² infused in 2 h	2.5 mg/m ² / min		2.35 mg/L	AUC _{0–t} : 3.81 mg*h/L	124.39 L/h	V _{ss} : 64.98 L	1.87 h ⁻¹	0.37 h
Cattel et al. [90]	Gemcit- abine		5	7	300 mg/m ² infused in 3 h	1.67 mg/ m ² /min		1.33 mg/L	AUC _{0–t} : 3.12 mg*h/L	177.72 L/h	V _{ss} : 111.4 L	2.31 h ⁻¹	0.30 h

Table 1 (continued)

Reference	Drug	Treat-ment cycle	n	No. of samples per patient	Dose	Dose rate	T _{max}	C _{max}	AUC	Cl	Vd	ke	t1/2	
Soo et al. [95]	Gemcitabine	Cycle 1	5	7	1000 mg/m ² infused in 30 min	33.3 mg/m ² /min	51.3 ± 17.2 min	14.97 mg/L	AUC _{0-4h} : 10.75 mg* ^h /L	132.26 L/h	V _{ss} : 51.14 L	2.36 h ⁻¹	0.29 h	
			29	7	750 mg/m ² infused in 75 min	10 mg/m ² /min	20.8 ± 17.2 μM	AUC _{0-inf} : 1345.9 ± 1112.6 μM*min	261.8 ± 106.7 L/h or 164.0 ± 64.0 L/h/m ²	V _{ss} : 65.0 ± 37.2 L	18.2 ± 4.2 min (half-life of the terminal disposition phase)			
			29	7	1000 mg/m ² infused in 30 min	33.3 mg/m ² /min	41.2 ± 13.9 μM	AUC _{0-inf} : 1432.4 ± 528.9 μM*min	293.0 ± 128.1 L/h or 181.6 ± 74.5 L/h/m ²	V _{ss} : 74.5 ± 41.2 L	17.1 ± 3.1 min (half-life of the terminal disposition phase)			
Nieto et al. [133] ^c	Gemcitabine	Cycle 1	17	6	3000 mg/m ² infused in 5 h at 4 consecutive days	10 mg/m ² /min	Day 1: 8 ± 1.6 mg/L	Day 1: 145.6 ± 15.3 mg/L* ^h dFdU: 554 ± 90 mg/L* ^h	Day 1: dFdC: 147.1 ± 23 L/h dFdU: 24 ± 4 L/h	Day 1: 59 ± 13.5 L	Day 1: 15.5 ± 2.8 min			
Veltkamp et al. [106] ^d	Gemcitabine (per os)	Cycle 1; day 1	3	7	8 mg per os QD for 14 days of a 21-day cycle	-	Median: 2.3 [0.6-3.5] ng/mL dFdC: 127 [111-137] ng/mL	Median: 2.3 [0.6-3.5] ng/mL dFdC: 4.4 [2.7-6.2] ng/mL dFdU: 631 [620-645] ng/mL	AUC _{0-24h} : Median: 2379 [2338-2889] ng/mL* ^h	AUC _{0-24h} : Median: 13,747 [13,119-14,375] ng/mL* ^h	AUC _{0-24h} : Median: 6207 [5167-6797] ng/mL* ^h	Median: 17.1 [15-18] h dFdU: 101 [86-115] h		
			2	8	8 mg per os QD for 14 days of a 21-day cycle	-	Median: 2.3 [1.6-6.2] ng/mL dFdC: 825 [791-1134] ng/mL	Median: 2.3 [1.6-6.2] ng/mL dFdC: 23.8 ± 2.5 μM dFdU: 90.7 ± 10.3 μM	AUC _{0-24h} : Median: 32,552 [21,435-39,674] ng/mL* ^h	AUC _{0-24h} : Median: 39,674 [39,674-39,674] ng/mL* ^h	AUC _{0-24h} : Median: 39,674 [39,674-39,674] ng/mL* ^h	Median: 49 [39-79] h		
			3	7	20 mg per os every other day for 21 days of a 28-day cycle	-	Median: 2.3 [1.6-6.2] ng/mL dFdC: 825 [791-1134] ng/mL	Median: 2.3 [1.6-6.2] ng/mL dFdC: 23.8 ± 2.5 μM dFdU: 90.7 ± 10.3 μM	AUC _{0-24h} : Median: 32,552 [21,435-39,674] ng/mL* ^h	AUC _{0-24h} : Median: 39,674 [39,674-39,674] ng/mL* ^h	AUC _{0-24h} : Median: 39,674 [39,674-39,674] ng/mL* ^h	Median: 49 [39-79] h		
Poplin et al. [98]	Gemcitabine	Cycle 1	9	5	1000 mg/m ² infused in 30 min	33.3 mg/m ² /min	4678 ± 2472 ng/mL* ^h	9720 ± 2608 ng/mL* ^h	11,276 ± 8788 ng/mL* ^h					
			8	5	1500 mg/m ² infused in 150 min	10 mg/m ² /min								
			6	5	1000 mg/m ² infused in 100 min + oxaliplatin	10 mg/m ² /min								
Caffo et al. [134] ^e	Gemcitabine	Cycle 1; day 1	4	8	600 mg/m ² infused in 60 min	10 mg/m ² /min	Day 1: 23.8 ± 2.5 μM dFdC: 7.7 ± 1 μg* ^h /mL dFdU: 215.2 ± 31.8 μg* ^h /mL	AUC _{0-4h} : Day 1: 7.7 ± 1 μg* ^h /mL dFdC: 7.7 ± 1 μg* ^h /mL dFdU: 215.2 ± 31.8 μg* ^h /mL	AUC _{0-4h} : Day 1: 7.7 ± 1 μg* ^h /mL dFdC: 7.7 ± 1 μg* ^h /mL dFdU: 215.2 ± 31.8 μg* ^h /mL	AUC _{0-4h} : Day 1: 7.7 ± 1 μg* ^h /mL dFdC: 7.7 ± 1 μg* ^h /mL dFdU: 215.2 ± 31.8 μg* ^h /mL	AUC _{0-4h} : Day 1: 7.7 ± 1 μg* ^h /mL dFdC: 7.7 ± 1 μg* ^h /mL dFdU: 215.2 ± 31.8 μg* ^h /mL	AUC _{0-4h} : Day 1: 7.7 ± 1 μg* ^h /mL dFdC: 7.7 ± 1 μg* ^h /mL dFdU: 215.2 ± 31.8 μg* ^h /mL	AUC _{0-4h} : Day 1: 7.7 ± 1 μg* ^h /mL dFdC: 7.7 ± 1 μg* ^h /mL dFdU: 215.2 ± 31.8 μg* ^h /mL	

Table 1 (continued)

Reference	Drug	Treat- ment cycle	n	No. of samples per patient	Dose	Dose rate	T _{max}	C _{max}	AUC	Cl	Vd	ke	t1/2
		Cycle 1; day 8			600 mg/m ² infused in 60 min + cis- platin	10 mg/m ² / min		Day 8: dF ₀₋₁ : 22.5 ± 4.1 μM dF ₀₋₁ : 89.2 ± 17.2 μM	AUC ₀₋₁ : Day 8: dF ₀₋₁ : 6.4 ± 1.46 μg [*] /h/mL dF ₀₋₁ : 204 ± 33.7 μg [*] /h/mL				Day 8: dF ₀₋₁ : NR dF ₀₋₁ : 13.4 ± 5.8 min
		Cycle 2; day 1	4	8	1200 mg/m ² infused in 120 min	10 mg/m ² / min		Day 1: dF ₀₋₁ : 34.5 ± 20 μM dF ₀₋₁ : 138.5 ± 31.6 μM	AUC ₀₋₁ : Day 1: dF ₀₋₁ : 17.1 ± 5.8 μg [*] /h/mL dF ₀₋₁ : 346.7 ± 84.6 μg [*] /h/ mL				Day 1: dF ₀₋₁ : 27.8 ± 6.6 min dF ₀₋₁ : 10.2 ± 4.7 min
		Cycle 2; day 8			1200 mg/m ² infused in 120 min + cis- platin	10 mg/m ² / min		Day 8: dF ₀₋₁ : 27.5 ± 5 μM dF ₀₋₁ : 162.8 ± 55.2 μM	AUC ₀₋₁ : Day 8: dF ₀₋₁ : 14.4 ± 2.3 μg [*] /h/mL dF ₀₋₁ : 329.6 ± 96.2 μg [*] /h/ mL				Day 8: dF ₀₋₁ : 19.9 ± 6.6 min dF ₀₋₁ : 8.3 ± 1 min
		Cycle 1; day 1	4	8	1200 mg/m ² infused in 120 min	10 mg/m ² / min		Day 1: dF ₀₋₁ : 18.7 ± 1.9 μM dF ₀₋₁ : 94.8 ± 32.2 μM	AUC ₀₋₁ : Day 1: dF ₀₋₁ : 10.5 ± 0.8 μg [*] /h/mL dF ₀₋₁ : 220.3 ± 136.8 μg [*] /h/ mL				Day 1: dF ₀₋₁ : 19.9 ± 6.9 min dF ₀₋₁ : 7.1 ± 3.2 min
		Cycle 1; day 8			1200 mg/m ² infused in 120 min + cis- platin	10 mg/m ² / min		Day 8: dF ₀₋₁ : 21.2 ± 7.1 μM dF ₀₋₁ : 99.6 ± 35.5 μM	AUC ₀₋₁ : Day 8: dF ₀₋₁ : 10.4 ± 3.2 μg [*] /h/mL dF ₀₋₁ : 214.5 ± 81 μg [*] /h/mL				Day 8: dF ₀₋₁ : 21.2 ± 6.1 min dF ₀₋₁ : 10.3 ± 2.9 min
		Cycle 2; day 1	4	8	1200 mg/m ² infused in 120 min	10 mg/m ² / min		Day 1: dF ₀₋₁ : 17.8 ± 4.1 μM dF ₀₋₁ : 94.1 ± 19.4 μM	AUC ₀₋₁ : Day 1: dF ₀₋₁ : 9.2 ± 2.7 μg [*] /h/mL dF ₀₋₁ : 194.8 ± 43.9 μg [*] /h/ mL				Day 1: dF ₀₋₁ : 23.7 ± 13.3 min dF ₀₋₁ : 10 ± 2.3 min
		Cycle 2; day 8			1200 mg/m ² infused in 120 min + cis- platin	10 mg/m ² / min		Day 8: dF ₀₋₁ : 21.6 ± 12.5 μM dF ₀₋₁ : 88.8 ± 15.5 μM	AUC ₀₋₁ : Day 8: dF ₀₋₁ : 1.1 ± 5 μg [*] /h/mL dF ₀₋₁ : 215.2 ± 37.5 μg [*] /h/ mL				Day 8: dF ₀₋₁ : NR dF ₀₋₁ : 11.5 ± 3.3 min
Derissen et al. [105]	Gemcit- abine	Cycle 1; day 1	12	7	1000 mg/m ² infused in 30 min	33.3 mg/ m ² /min	dF ₀₋₁ : 0.5 [0.5-0.5] h dF ₀₋₁ : 0.8 [0.5-2] h	dF ₀₋₁ : 42.8 [16.7-81.7] μM dF ₀₋₁ : 147 [99.9-224] μM	AUC _{0-24h} : dF ₀₋₁ : 28.8 [11.1-56.9] μM ^{*h} dF ₀₋₁ : 999 [673-1595] μM ^{*h} AUC _{0-inf} : dF ₀₋₁ : 28.9 [11.1-57.0] μM ^{*h} dF ₀₋₁ : 1265 [869-2010] μM ^{*h}	dF ₀₋₁ : 153 L/h/m ² [66.7-341]		t1/2β: dF ₀₋₁ : 7.32 [3.43-19.5] h dF ₀₋₁ : 11.2 [8.65-13.2] h	

Table 1 (continued)

Reference	Drug	Treat-ment cycle	n	No. of samples per patient	Dose	Dose rate	T_{max}	C_{max}	AUC	Cl	Vd	ke	t1/2
	Gemcitabine	Cycle I; day 1	26	6	500 mg/m ² infused in 30 min	16.7 mg/m ² /min	dF ₀₋₁ : 0.5 [0.5-0.5] h dF ₀₋₂ : 0.52 [0.5-1] h	dF ₀₋₁ : 11.1 [0.802-29.3] μM dF ₀₋₂ : 75.8 [43.5-115] μM	AUC _{0-24h} : dF ₀₋₁ : 6.75 [0.535-17.3] μM* dF ₀₋₂ : 478 [289-748] μM* AUC _{0-inf} : dF ₀₋₁ : 7.89 [2.18-17.5] μM* dF ₀₋₂ : 662 [364-1060] μM*	dF ₀₋₁ : 324 L/h/m ² [109-873]			t1/2β: [4.16-42.8] h dF ₀₋₁ : 13.9 [8.78-24.5] h

Unless otherwise specified, the data are shown as mean values ± the standard deviation and between brackets the range

5-FU 5-fluorouracil, ara-C 1-β-D-arabinofuranosylcytosine (cytarabine), ara-U 1-β-D-arabinofuranosyluracil, AUC area under the concentration-time curve, AUC_{0-t} area under the concentration-time curve from time zero up to the last measured time point, AUC_{inf} area under the concentration-time curve extrapolated to infinity, BID twice daily, Cl plasma clearance, C_{max} maximum observed concentration, C_{ss} steady-state concentration, dF₀₋₁ 2',2'-difluoro-2'-deoxycytidine (gemcitabine), dF₀₋₂ 2',2'-difluoro-2'-deoxyuridine, FDR fixed-dose-rate, h hours, ke elimination rate constant, *mini* minutes, n number of patients, NR not reported, QD once daily, t1/2 elimination half-life, t1/2α initial elimination half-life, t1/2β terminal elimination half-life, T_{max} time to reach the maximum observed concentration, Vd volume of distribution, V_{ss} volume of distribution at steady state

^aPhase I/II study assessing pharmacokinetic and pharmacodynamic interactions between gemcitabine and paclitaxel. Concomitant treatment with paclitaxel was found to increase the intracellular accumulation of 2',2'-difluoro-2'-deoxycytidine triphosphate (gemcitabine triphosphate) in peripheral blood mononuclear cells, whereas no effects were found on the plasma pharmacokinetics of gemcitabine (Kroep, [131])

^bPhase I dose schedule finding study for the combination of gemcitabine and cisplatin (van Moorsel et al. [132])

^cPhase I study of gemcitabine at FDR combined with docetaxel/melphalan/carboplatin and autologous hematopoietic stem cell transplantation (Nioto et al. [133])

^dPhase I study investigating the feasibility of oral administration of gemcitabine. For both dosage regimens, only the highest dose tested was included in the table (Veltkamp et al. [106])

^ePhase II pharmacokinetic study of gemcitabine at FDR in combination with cisplatin. Administration of cisplatin before that of gemcitabine at day 8 of each cycle might have played a role in the induction of deoxycytidine kinase (Caffo et al. [134])

Table 2 (continued)

Reference	Drug	Treatment cycle	n	No. of Dose samples per patient	No. of Dose	Dose rate	Cell type	Metabolite	Triphosphate accumulation rate during infusion	T _{max}	C _{max}	Intracellular concentration after 24 h	AUC	t _{1/2}
Estey et al. [65]	Cytarabine	Cycle 1; day 1	147	7	3000 mg/m ² infused in 2 h	1500 mg/m ² /h	Circulating blasts	ara-CTP			Median: 303 [42–1264] μM		Median: 1731 [215–15,769] μM*h	Median: 2.8 [1.0–13.3] h
Gandhi et al. [37]	Cytarabine	Cycle 1; day 1	55	2	1500 mg/m ² infused in 24 h	62.5 mg/m ² /h	Circulating blasts	ara-CTP		ara-CTP: 2.8 h ara-UTP: 4 h	Css: median: 113 [16–470] μM ara-CTP: 467 μM ara-UTP: 28 μM			
	Cytarabine	Cycle 1; day 1	8	2	1000 mg/m ² infused in 2 h	500 mg/m ² /h	Circulating and AML blasts	ara-CTP and ara-UTP		ara-CTP: 4 h ara-UTP: 7.3 h	ara-CTP: 373 μM ara-UTP: 19.5 μM			
	Cytarabine	Cycle 1; day 1	4	9	2000 mg/m ² infused in 4 h	500 mg/m ² /h	Circulating and AML blasts	ara-CTP and ara-UTP						
Gemcitabine														
Abbruzzese et al. [74]	Gemcitabine	NR	26	NR	10–1000 mg/m ² weekly for 3 weeks followed by 12 days of observation	Escalating dose administered over 30 min	PBMCs	dFdCTP		<30 min of the end of the infusion				An early elimination phase was clearly observed for 4 patients: t _{1/2α} : 15 min
Grunewald et al. [87] ^a	Gemcitabine	Cycle 1	≥ 16	16	1200–6400 mg/m ² weekly for 3 weeks	10 mg/m ² /min	Leukemic cells	dFdCTP	163 [9.7–789] μmol/L/h	<15 min				Linear elimination (n=8): t _{1/2} : 275 min [42 min–9.5 h] Biphasic elimination (n=14): t _{1/2α} : 152 min [10 min–9.5 h] t _{1/2β} : 6.8 h [42 min–12 h] t _{1/2β} : 323±82 min
Kroep, et al. [131] ^b	Gemcitabine	Cycle 1; day 1	9	5	1000 mg/m ² infused in 30 min + paclitaxel 150 mg/m ²	33.3 mg/m ² /min	PBMCs	dFdCTP		2 h	55±10 pmol/10 ⁶ PBMCs		AUC _{0–4h} : 49.3±13.2 pmol/10 ⁶ PBMCs*min	
	Gemcitabine	Cycle 1; day 1	9	5	1000 mg/m ² infused in 30 min + paclitaxel 200 mg/m ²	33.3 mg/m ² /min	PBMCs	dFdCTP		4 h	106±16 pmol/10 ⁶ PBMCs		AUC _{0–4h} : 72.6±12.2 pmol/10 ⁶ PBMCs*min	
van Moorsele et al. [132] ^c	Gemcitabine	Cycle 1+2; day 1+2	33	5	800 mg/m ² infused in 30 min + cisplatin 50 mg/m ²	26.7 mg/m ² /min	PBMCs	dFdCTP						

Table 2 (continued)

Reference	Drug	Treatment cycle	n	No. of Dose sam- ples per patient	No. of Dose	Dose rate	Cell type	Metabolite	Triphos- phate accu- mulation rate during infusion	T _{max}	C _{max}	Intracellular concentration after 24 h	AUC	t1/2
					Gemcitabine 4 h before cisplatin								AUC _{0-24h} : 79.5 ± 18.6 [14.0–189.7] nmol/10 ⁶ PBMCs*min	
					Cisplatin 4 h before gemcitabine								AUC _{0-24h} : 93.5 ± 13.4 [41.7–219.6] nmol/10 ⁶ PBMCs*min	
					Gemcitabine 24 h before cisplatin								AUC _{0-24h} : 81.5 ± 6.3 [38.7–138.6] nmol/10 ⁶ PBMCs*min	
					Cisplatin 24 h before gemcitabine								AUC _{0-24h} : 124.8 ± 12.7 [47.5–222.0] nmol/10 ⁶ PBMCs*min	
Patel et al. [88]	Gemcitabine	Cycle 1	9	6	1000 mg/m ² infused in 30 min	33.3 mg/ m ² /min	PBMCs	dFdCTP		77 [30–120] min	144 [50–310] μM			
		Cycle 1	7	6	1000 mg/m ² infused in 150 min	6.66 mg/ m ² /min	PBMCs	dFdCTP		146 [120–150] min	201 [65–445] μM			
Gandhi et al. [89] [†]	Gemcitabine	Cycle 1	2	NR	480 mg/m ² infused in 8 h	10 mg/m ² / min	Circulat- ing AML blasts	dFdCTP		8 h	586 μM	193 μM		
			1		600 mg/m ² infused in 10 h	10 mg/m ² / min	Circulat- ing AML blasts	dFdCTP		4 h	409 μM	162 μM		
			1		750 mg/m ² infused in 12.5 h	10 mg/m ² / min	Circulat- ing AML blasts	dFdCTP		10 h	133 μM	33 μM		
			2		936 mg/m ² infused in 15.6 h	10 mg/m ² / min	Circulat- ing AML blasts	dFdCTP		15.6 h	704.5 μM	323 μM		
			2		1080 mg/m ² infused in 18 h	10 mg/m ² / min	Circulat- ing AML blasts	dFdCTP		18 h	905 μM	373 μM		
Tempero et al. [92]	Gemcitabine	Cycle 1	10	6	2200 mg/m ² infused in 30 min	73.3 mg/ m ² /min	PBMCs	dFdCTP		90 min after start infusion	Median: 188 [44–533] μM			
		Cycle 1	6	6	1500 mg/m ² infused in 150 min	10 mg/m ² / min	PBMCs	dFdCTP		150 min after start infusion	Median: 398 [111–682] μM			

Table 2 (continued)

Reference	Drug	Treatment cycle	n	No. of Dose sam- ples per patient	Dose	Dose rate	Cell type	Metabolite	Triphos- phate accu- mulation rate during infusion	T _{max}	C _{max}	Intracellular concentration after 24 h	AUC	t1/2
Cattell et al. [90]	Gemcit- abine		5	7	300 mg/m ² infused in 1 h	5 mg/m ² / min	Leuko- cytes	dFdCTP			35.78 μM		AUC ₀₋₁ : 226.67 μM ^h	
			5	7	300 mg/m ² infused in 2 h	2.5 mg/m ² / Leuko- cytes min		dFdCTP			50.09 μM		AUC ₀₋₁ : 408.41 μM ^h	
			5	7	300 mg/m ² infused in 3 h	1.67 mg/ m ² /min	Leuko- cytes	dFdCTP			68.60 μM		AUC ₀₋₁ : 535.71 μM ^h	
			5	7	1000 mg/m ² infused in 30 min	33.3 mg/ m ² /min	Leuko- cytes	dFdCTP			26.40 μM		AUC ₀₋₁ : 284.64 μM ^h	
Soo et al. [95]	Gemcit- abine	Cycle 1	15	7	750 mg/m ² infused in 75 min	10 mg/m ² / min	PBMCs	dFdCTP		1.6 ± 0.4 h	173.9 ± 77.3 μM		AUC _{inf} : 35,079 ± 18,216 μM ^h ·min	
			18	7	1000 mg/m ² infused in 30 min	33.3 mg/ m ² /min	PBMCs	dFdCTP		0.9 ± 0.2 h	224.8 ± 73.8 μM		AUC _{inf} : 32,249 ± 11,267 μM ^h ·min	
Nieto et al. [133] ^f	Gemcit- abine	Cycle 1	15	6	3000 mg/m ² infused in 5 h; at 4 consecutive days	10 mg/m ² / min	PBMCs	dFdCTP					Day 1: 1303 ± 1268 μmol ^h /10 ⁶ PBMCs Day 4: 6165 ± 5460 μmol ^h /10 ⁶ PBMCs	Day 1: 21 ± 26 h
Velkamp et al. [106] ^f (per os)	Gemcit- abine	Cycle 1; day 1	3	4	8 mg per os QD for 14 days of a 21-day cycle	–	PBMCs	dFdCTP and dFdUTP					Median: AUC _{0-24h} dFdCTP: 3.3 [1.4–4.8] ng/ mg protein dFdUTP: 4.6 [4.1–6.2] ng/ mg protein	
			2	4	8 mg per os QD for 14 days of a 21-day cycle	–	PBMCs	dFdCTP and dFdUTP					Median: AUC _{0-24h} dFdCTP: 40 [31–49] h*ng/ mg protein dFdUTP: 467 [240–694] h*ng/mg protein	
			3	4	20 mg per os every other day for 21 days of a 28-day cycle	–	PBMCs	dFdCTP					Median: AUC _{0-24h} dFdCTP: 15 [4.3–72] h*ng/ mg protein dFdUTP: NR	
			3	4	20 mg per os every other day for 21 days of a 28-day cycle	–	PBMCs	dFdCTP					Median: AUC _{0-24h} dFdCTP: 18 [7.5–29] h*ng/ mg protein dFdUTP: NR	
Poplin et al. [98]	Gemcit- abine	Cycle 1	8	5	1000 mg/m ² infused in 30 min	33.3 mg/ m ² /min	PBMCs	dFdCTP					1958.7 ± 794 μM ^h	
			8	5	1500 mg/m ² infused in 150 min	10 mg/m ² / min	PBMCs	dFdCTP					6804 ± 7763 μM ^h	

Table 2 (continued)

Reference	Drug	Treatment cycle	n	No. of samples per patient	Dose	Dose rate	Cell type	Metabolite	Triphosphate accumulation rate during infusion	T _{max}	C _{max}	Intracellular concentration after 24 h	AUC	t1/2	
			NR	5	1000 mg/m ² infused in 100 min + oxaliplatin	10 mg/m ² /min	PBMCs	dFdCTP					4501 ± 2113 μM*h		
Carfo et al. [134] ^g	Gemcitabine	Cycle 1; day 1	4	8	600 mg/m ² infused in 60 min	10 mg/m ² /min	PBMCs	dFdCTP		150 ± 117.5 min	92.6 ± 85.9 pmol*10 ⁶ PBMCs				
		Cycle 1; day 8			600 mg/m ² infused in 60 min + cisplatin	10 mg/m ² /min	PBMCs	dFdCTP		105 ± 130.8 min	173.0 ± 29.5 pmol*10 ⁶ PBMCs				
			Cycle 2; day 1	4	8	1200 mg/m ² infused in 120 min	10 mg/m ² /min	PBMCs	dFdCTP		101.3 ± 172.5 min	158.1 ± 134.7 pmol*10 ⁶ PBMCs			
			Cycle 2; day 8			1200 mg/m ² infused in 120 min + cisplatin	10 mg/m ² /min	PBMCs	dFdCTP		150 ± 60 min	140.1 ± 98.5 pmol*10 ⁶ PBMCs			
			Cycle 1; day 1	4	8	1200 mg/m ² infused in 120 min	10 mg/m ² /min	PBMCs	dFdCTP		191.3 ± 145.6 min	273.5 ± 139.5 pmol*10 ⁶ PBMCs			
			Cycle 1; day 8			1200 mg/m ² infused in 120 min + cisplatin	10 mg/m ² /min	PBMCs	dFdCTP		142.5 ± 155.6 min	316.9 ± 69.7 pmol*10 ⁶ PBMCs			
			Cycle 2; day 1	4	8	1200 mg/m ² infused in 120 min	10 mg/m ² /min	PBMCs	dFdCTP		243.8 ± 162.7 min	335.5 ± 117.5 pmol*10 ⁶ PBMCs			
			Cycle 2; day 8			1200 mg/m ² infused in 120 min + cisplatin	10 mg/m ² /min	PBMCs	dFdCTP		195 ± 139.6 min	445.3 ± 210.5 pmol*10 ⁶ PBMCs			
Derissen et al. [105]	Gemcitabine	Cycle 1; day 1	12	4	1000 mg/m ² infused in 30 min	33.3 mg/m ² /min	PBMCs	dFdCMP		1.4 [0.5–2] h	30.9 [5.12–67.7] μM		AUC _{0–24h} ⁱ 411 [68.5–873] μM*h		
								dFdCDP		1.5 [0.5–2] h	25.3 [75.0–461] μM		AUC _{0–24h} ⁱ 3480 [1080–6650] μM*h		
								dFdCTP		1.5 [0.5–2] h	497 [194–1060] μM		AUC _{0–24h} ⁱ 6580 [2830–15,700] μM*h		
								dFdUMP		1.8 [0.5–2] h	4.53 [1.39–7.99] μM		AUC _{0–24h} ⁱ 77.0 [31.7–171] μM*h		
								dFdUDP		5.4 [0.5–24] h	18.5 [5.78–32.0] μM		AUC _{0–24h} ⁱ 342 [117–674] μM*h		

Table 2 (continued)

Reference	Drug	Treatment cycle	n	No. of Dose sam- ples per patient	Dose rate	Cell type	Metabolite	Triphos- phate accu- mulation rate during infusion	T _{max}	C _{max}	Intracellular concentration after 24 h	AUC	t _{1/2}																														
	Gemcit- abine	Cycle 1; day 1	26	4	500 mg/m ² infused in 30 min	PBMCs	dFdUTP	5.4 [0.5–24] h	1.4 [0.5–2] h	38.5 [9.73–87.9] μM	695 [230–1620] μM* ^h	AUC _{0–24h} ⁱ	t _{1/2}																														
														16.7 mg/ m ² /min	dFdCMP	1.2 [0.5–2] h	128 [10.1–401] μM	1300 [138–3100] μM* ^h																									
																			dFdCDP	1.7 [0.5–22] h	96.1 [<LLOQ–440] μM	824 [54.9–3030] μM* ^h																					
																							dFdCTP	11 [0.5–22] h	10.7 [<LLOQ–48.5] μM	194 [23.5–938] μM* ^h																	
																											dFdUMP	16 [0.5–22] h	10.9 [<LLOQ–28.4] μM	193 [24.8–500] μM* ^h													
																															dFdUDP	12 [0.5–22] h	7.97 [<LLOQ–29.0] μM	135 [23.5–452] μM* ^h									
																																			dFdUTP	6.62 fmol/10 ⁶ PBMCs	6.62 fmol/10 ⁶ PBMCs						
																																						aza-dCTP	308 fmol/10 ⁶ PBMCs	308 fmol/10 ⁶ PBMCs			
																																									aza-dCTP	690 fmol/10 ⁶ PBMCs	690 fmol/10 ⁶ PBMCs
aza-dCTP	0.7 [0–2.7] pmol/10 ⁶ PBMCs	0.7 [0–2.7] pmol/10 ⁶ PBMCs																																									

Azacitidine and decitabine													
Jansen et al. [125]	Decit- abine	Cycle 1; day 3 after the 9th infu- sion	1	1	15 mg/m ² infused in 4 h repeated every 8 h for 3 days; every 6 weeks	PBMCs	aza-dCTP	6.62 fmol/10 ⁶ PBMCs	12 [0.5–22] h	6.62 fmol/10 ⁶ PBMCs	6.62 fmol/10 ⁶ PBMCs	6.62 fmol/10 ⁶ PBMCs	t _{1/2}
Cycle 5; day 1–3 after the 3rd–6th- 9th infusion	3	15 mg/m ² infused in 4 h repeated every 8 h for 3 days; every 6 weeks	PBMCs	aza-dCTP	690 fmol/10 ⁶ PBMCs	690 fmol/10 ⁶ PBMCs							
							Cycle 5; day 1–3 after the 3rd–6th- 9th infusion	3	15 mg/m ² infused in 4 h repeated every 8 h for 3 days; every 6 weeks	PBMCs	aza-dCTP	759 fmol/10 ⁶ PBMCs	759 fmol/10 ⁶ PBMCs
Wang et al. [126]	Decit- abine	Day 1	7	1	20 mg/m ² infused in 1 h for 10 days; every 4 weeks	Mononu- clear cells in bone marrow							

Conversely, the nucleotides may also be degraded to their inactive nucleosides by 5'-nucleotidases and phosphatases [20, 21].

Ultimately, three intracellularly formed metabolites (nucleotides) are held responsible for the antineoplastic effects of 5-FU. These are FUTP, FdUTP and FdUMP. **FUTP** is incorporated into RNA and interferes with normal RNA processing and function. **FdUTP** is incorporated into DNA, leading to DNA damage and ultimately cell death. **FdUMP** inhibits thymidylate synthase, the enzyme that catalyses the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTTP). Inhibition of thymidylate synthase by FdUMP leads to accumulation of deoxyuridine triphosphate (dUTP) and depletion of deoxythymidine triphosphate (dTTP). This imbalance has deleterious consequences for DNA synthesis and repair, ultimately leading to cell death [14, 22, 23].

3.2 Clinical Studies Investigating the Intracellular Concentrations of the 5-Fluorouracil Nucleotides

Most of our knowledge about the intracellular metabolism of 5-FU and its mechanisms of action was derived from in vitro experiments and animal studies [24–27]. Little is known about the formation of 5-FU nucleotides in patients who are treated with 5-FU or capecitabine.

In the 1980s and 1990s, several studies investigated the intracellular **FdUMP** concentrations in patients who were treated with an intravenous 5-FU bolus [28–30]. Spears et al. [28, 29] and Peters et al. [30] determined the intracellular FdUMP concentrations in healthy liver and colon biopsies as well as tumour biopsies obtained from patients who had previously received an intravenous 5-FU bolus of 500 mg/m² [28–30]. Surgical biopsies were obtained 20 min to 48 h after administration of 5-FU. For each patient, biopsies were obtained at a single time point after 5-FU administration. However, if the measured FdUMP concentrations for the total group of patients were expressed against the time interval between 5-FU administration and tissue biopsy, a graph was obtained that could be interpreted as a pharmacokinetic profile [28, 30].

The highest intracellular FdUMP concentrations were observed shortly after 5-FU administration, indicating that 5-FU is rapidly converted to FdUMP [28, 30]. At 1–5 h after 5-FU administration, the intracellular FdUMP concentrations measured in tumour tissue had a range of 10–1700 pmol/g [30]. The studies demonstrated that 5-FU plasma concentrations do not reflect intracellular FdUMP concentrations. Although 5-FU is rapidly eliminated from plasma, FdUMP is retained considerably longer in cells. At 19–27 h after 5-FU administration, FdUMP was still measurable in concentrations below 200 pmol/g [30]. After

48 h, FdUMP was measurable only in a few samples [30]. The concentrations of FdUMP measured in tissue samples obtained at the same time points after the 5-FU bolus showed large inter-individual variation [28–30]. Remarkably, the FdUMP concentrations in tumour tissue were higher than those observed in healthy tissue from the same patient [28, 30].

Spears et al. also measured, besides the FdUMP concentrations, the dUMP concentrations in the biopsy specimens and expressed these concentrations against the observed percentages of thymidylate synthase inhibition [29]. This suggested that a minimum FdUMP concentration of 75 pmol/g and a minimal FdUMP/dUMP ratio of 0.001 was needed to achieve maximal thymidylate synthase inhibition [29].

In the above studies, the intracellular FdUMP concentrations were determined using a competitive binding assay with bacterial thymidylate synthase as a binding protein. The quantification of FdUMP was based on the displacement of a known amount of radiolabelled [6-³H]-FdUMP, measured by scintillation counting [28–31]. This “isotope-dilution” assay for the quantification of FdUMP was available since 1979 [31]. However, a sufficiently sensitive assay for the quantification of the *other* two active nucleotides, **FUTP** and **FdUTP**, in cells of patients who were treated with 5-FU was lacking for a long time.

Therefore, in 2015, Derissen et al. developed an ultra-sensitive liquid chromatography-tandem mass spectrometry assay for simultaneous quantification of the three pharmacologically active 5-FU nucleotides FUTP, FdUTP and FdUMP in PBMCs [32]. As a start, the intracellular 5-FU nucleotide concentrations were measured in PBMC samples of five patients who were treated with an intravenous 5-FU bolus of 400 mg/m². Thirty minutes after the end of the infusion, FUTP concentrations between 4.7 and 11 μM were measured. 5-Fluoro-2'-deoxyuridine monophosphate concentrations were much lower with a range of 0.02–0.6 μM. The FdUTP concentrations were below the detection limits of the assay [32].

In a subsequent study, the intracellular PK of the three active 5-FU nucleotides were explored during capecitabine treatment [33]. As usual in these studies, PBMCs were selected as a surrogate matrix to measure the intracellular nucleotide concentrations. After treatment with capecitabine, only FUTP could be measured in PBMCs (Table 2). Apparently, capecitabine is mainly converted into FUTP and to a lesser extent into FdUTP and FdUMP. No significant correlation was found between the plasma 5-FU exposure and the intracellular FUTP exposure. The FUTP concentration–time profiles demonstrated considerable inter-individual variation. The study also showed that FUTP remained much longer present in PBMCs than 5-FU in plasma. Twenty-four hours after a single capecitabine dose of 1000 mg, intracellular FUTP concentrations were on

average still 41% of the maximum concentrations found. In accordance with this finding, after 14 days of treatment with capecitabine (850 mg/m²) twice daily, intracellular accumulation of FUTP was found in PBMCs. The area under the FUTP concentration–time curve was significantly increased on day 14 of the treatment compared with day 1. Remarkably, the intracellular FUTP concentrations measured on day 14 of capecitabine treatment were in the same range (0.6–14 µM) as the concentrations observed 30 min after an intravenous 5-FU bolus (400 mg/m² in 30 min) [33]. It is important to realise that, by measuring in PBMCs, the tumour-specific activation of capecitabine is not taken into account and that the concentrations in tumour cells might be different.

So far, to the best of our knowledge, it has not been possible to measure **FdUTP** in cells of patients who were treated with 5-FU or capecitabine. Perhaps an even more sensitive assay is needed to measure these FdUTP concentrations.

4 Cytarabine

Cytarabine (1-β-D-arabinofuranosylcytosine, ara-C) is a deoxycytidine analogue as it combines a cytosine base with an arabinose sugar. The difference with respect to cytidine is that the 2' hydroxyl group of cytarabine is oriented in the trans position, resulting in a metabolism similar to that of a deoxyribose nucleoside.

After administration, cytarabine is rapidly converted to 1-β-D-arabinofuranosyluracil (ara-U) by the enzyme cytidine deaminase. Cytidine deaminase is ubiquitous in the human body and catalyses the hydrolytic deamination of (deoxy)cytidine to (deoxy)uridine (Fig. 2). Cytidine deaminase is present in plasma, but also in the liver and kidneys, where high levels are found [34]. After cytarabine administration, plasma ara-U peak concentrations were generally 5–30 times higher than the peak concentrations of cytarabine [35–37].

4.1 Intracellular Metabolism and Mechanism of Action

Like the other pyrimidine analogues, cytarabine has to enter the tumour cell, where it has to be activated by phosphorylation (Fig. 2). Cytarabine is transported into the cell primarily by the human equilibrative nucleoside transporter (hENT) 1 [38]. Once in the cell, cytarabine is phosphorylated to its monophosphate (ara-CMP) by deoxycytidine kinase (dCK) and then to cytarabine diphosphate (ara-CDP) by pyrimidine nucleoside monophosphate kinase (UMP-CMP kinase). The final phosphorylation step to cytarabine triphosphate (ara-CTP) is catalysed by nucleoside diphosphate kinase. Ara-CTP competes with deoxycytidine triphosphate (dCTP) for incorporation into the DNA. Once incorporated into DNA,

further extension of the DNA strand by DNA polymerase is inhibited. Correlations have been found between cytarabine incorporation into DNA and cytotoxicity [39–41].

Within the cell, cytarabine is also subject to deamination by cytidine deaminase. Additionally, ara-CMP can be deaminated by deoxycytidylate deaminase [42].

4.2 Clinical Studies Investigating the Intracellular Concentrations of Cytarabine Nucleotides

4.2.1 Relationship Between Cytarabine Dose, Plasma Concentrations and Intracellular Ara-CTP Concentrations

Several clinical studies investigated the relationship between the cytarabine dose, the plasma PK and the intracellular PK of ara-CTP in leukaemic cells [43–46]. A linear relationship was found between the cytarabine dose and the steady-state concentrations and area under the concentration–time curves (AUCs) of cytarabine and ara-U in plasma. However, the intracellular PK of ara-CTP was much more heterogeneous [44–46]. No correlation was found between cytarabine plasma concentrations and the intracellular concentrations or AUC of ara-CTP [43]. Patients who had all received an identical cytarabine dose demonstrated substantial differences in the intracellular concentrations and AUCs of ara-CTP [44, 45]. However, within the same patient, measurements of the ara-CTP concentrations were reproducible with subsequent treatments [47]. The ability of leukaemic cells to phosphorylate cytarabine is characteristic to each individual [43]. Inter-individual differences in the ability of patients to phosphorylate cytarabine and retain ara-CTP are related to the ability to transport cytarabine into the cell and the activities of deoxycytidine kinase, cytidine deaminase and dephosphorylating enzymes.

When examining the PK of intracellular ara-CTP, there are also commonalities. The accumulation of ara-CTP in leukaemic cells was dependent on the dose regime; however, for an individual patient, the cellular elimination rate of ara-CTP remained constant under all infusion conditions. The elimination of ara-CTP from leukaemic cells was monophasic. The elimination rate differed substantially between patients. In the study of Liliemark et al., a median elimination half-life of 3.4 h (range 1.25–18.9 h) was found [43].

4.2.2 Saturation of the Intracellular Accumulation of Ara-CTP

The accumulation of ara-CTP in leukaemic cells was also dependent on the dose regime. In vitro studies in human leukaemic cells demonstrated that the intracellular accumulation of ara-CTP was saturated at cytarabine concentrations above 10 µmol/L [42, 48, 49]. Plunkett et al. 1987

studied the saturation of intracellular ara-CTP formation in patients and investigated which cytarabine infusion rate produced steady-state plasma concentrations that saturate the intracellular ara-CTP accumulation [44]. To this end, the intracellular ara-CTP accumulation in leukaemic cells of 11 patients with refractory leukaemia was studied after different cytarabine doses infused over 2 h. Varying the cytarabine dose between 500 and 3000 mg/m² infused over 2 h did not substantially change the accumulation rate and the AUC of ara-CTP in leukaemic cells. Only when the infusion rate was further reduced to 400 or 300 mg/m² over 2 h, resulting in steady-state cytarabine plasma concentrations of less than 7 µM, the accumulation rate of ara-CTP was substantially reduced as was the AUC of intracellular ara-CTP [44]. This study demonstrated that infusion rates of 200–250 mg/m²/h produce cytarabine plasma concentrations that saturate the intracellular ara-CTP accumulation in leukaemic cells.

4.2.3 Optimisation of Dosing Regimes in Relation to the Intracellular Accumulation of Ara-CTP

Remarkably, for more than two decades, not much was done with the finding that cytarabine infusion rates above 200–250 mg/m²/h led to saturation of the intracellular ara-CTP formation. Until 2011, high-dose cytarabine regimes remained common practice for the treatment of patients with AML [50–52]. High cytarabine doses were given with a high infusion rate, typically 3000 mg/m² over 1–3 h. These dosing regimes led to peak plasma concentrations that were about 5–15 times higher than the plasma concentrations that caused saturation of intracellular triphosphate formation in leukaemic cells [43, 53–55]. From a pharmacological perspective, this was not rational. Indeed, substantial toxicities were associated with these high-dose cytarabine regimes [56–60]. It is likely that plasma cytarabine in excess of the amount that can be phosphorylated by leukaemic cells will contribute to the observed toxicity, either directly or after deamination to ara-U [45, 61].

It was only in 2011 that clinical research by Löwenberg et al. showed that an intermediate-dose remission induction regime with an infusion rate just above the ‘saturation infusion rate’ was as effective as high-dose cytarabine, but was associated with less toxicity [62]. The idea is that these intermediate infusion rates still saturate the intracellular ara-CTP formation in leukaemic cells, but result in a substantial decrease of the peak plasma concentrations of cytarabine and ara-U leading to less toxicity [63].

In retrospect, you may wonder why it took so long before the findings from clinical pharmacological research were translated into clinical practice. Although many studies have been conducted over the last 30 years to find the most suitable cytarabine doses, little attention has been paid to the infusion rates. Yet, these infusion rates also determine

how much cytarabine is converted intracellularly into ara-CTP, particularly in view of the short plasma half-life of cytarabine.

4.2.4 Relationship Between Intracellular Ara-CTP Concentrations and Efficacy

Several studies investigated the relationship between the accumulation of ara-CTP in leukaemic cells and the response to cytarabine therapy. Kantarjian et al. and Estey et al. investigated the relationship between the intracellular PK of ara-CTP in circulating blasts and the response of patients with refractory AML to remission induction therapy [47, 64]. Patients were treated with ‘high-dose cytarabine’ and received 3000 mg/m² infused over 2 h, every 12 h for 4–12 doses. Intracellular ara-CTP concentrations were measured after the first dose and differed largely among patients. In the first study, a trend was observed and in the follow-up study a significant correlation was found between the AUC of intracellular ara-CTP and the complete remission rate. In addition to the AUC, a long half-life of ara-CTP and a high ara-CTP trough concentration correlated with a high complete remission rate. The number of patients achieving a complete remission was too small to investigate the association between the PK of ara-CTP and the remission *duration* [47, 64].

The subsequent study of Estey et al. did not confirm the correlation between the intracellular ara-CTP exposure and the remission rate [65]. However, this probably had to do with the fact that the PK of ara-CTP was investigated after a single gift of high-dose cytarabine for all patients, whereas these patients were subsequently treated according to three different treatment protocols probably resulting in different intracellular ara-CTP concentrations [65].

Rustum and Preisler [66] and Preisler et al. [67, 68] did not measure the intracellular ara-CTP concentrations during cytarabine therapy, but isolated leukaemic blasts from more than 80 patients with AML prior to therapy, incubated these blasts with [³H]-cytarabine and determined the percentage of ara-CTP that was retained after 4 h. Then, they studied the relationship between the *in vitro* retention of ara-CTP in the isolated blasts and patients’ response to subsequent therapy with cytarabine and an anthracycline. As the ara-CTP formation in leukaemic blasts was studied after incubation and not during treatment with cytarabine, this series of three studies did not meet the inclusion criteria of this review. Therefore, the results were not included in Tables 1 and 2. Nevertheless, we briefly mention this series here because it has contributed to a better understanding of the relationship between intracellular ara-CTP retention and the efficacy of cytarabine therapy. The design of the studies was not appropriate to examine the relationship between the *initial* ara-CTP formation and the probability of achieving a complete remission,

as patients received continuous cytarabine infusions during induction therapy. However, all three studies did find a significant correlation between the *in vitro* retention of ara-CTP in blasts and the *duration* of remission of patients receiving cytarabine as part of their remission induction and maintenance therapy [66–68].

4.2.5 Role of Arabinosyluracil Triphosphate (ara-UTP) During Cytarabine Therapy

Gandhi et al. demonstrated that, during cytarabine (1- β -D-arabinosylcytosine; ara-C) therapy, arabinosyluracil 5'-triphosphate (ara-UTP) was also formed in circulating leukaemic blasts [37]. The intracellular PK of ara-UTP was investigated in six patients with relapsed AML receiving cytarabine infusions of 500 mg/m²/h during 2–4 h. The intracellular peak concentrations of ara-UTP had a range of 6–50 μ M and were between 2 and 10% of the peak concentrations that were measured for ara-CTP. Ara-UTP was maintained for several hours after the end of the cytarabine infusion. The intracellular ara-UTP peak concentrations were not related to the peak concentrations of cytarabine and ara-U in plasma. However, a linear relationship was found between the intracellular ara-CTP and ara-UTP peak concentrations. These data indicate that the formation of ara-UTP may be dependent on ara-CTP and not on the free nucleoside [37]. This is in line with previous *in vitro* studies. Incubation of leukaemic blasts with up to 100 μ M [³H]-ara-U did not result in ara-UTP accumulation, indicating that ara-U is not phosphorylated directly in these cells [69]. *In vitro* studies, investigating the route of ara-UTP formation showed that ara-UTP was formed through the deamination of ara-CMP to ara-UMP by deoxycytidylate deaminase, followed by phosphorylation to ara-UTP [37].

The question remains as to whether ara-UTP also contributes to the cytotoxic effects of cytarabine. *In vitro* studies in leukaemic cells demonstrated that ara-UTP served as a good alternative substrate for deoxythymidine triphosphate for incorporation into DNA [70] and inhibited DNA polymerase [71, 72]. Hence, it could be postulated that ara-UTP may play a role in cytarabine-induced cytotoxicity [71, 73].

5 Gemcitabine

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC) is a deoxycytidine analogue in which the two hydrogen atoms at the 2'-position of the deoxyribose moiety are replaced by fluorine atoms. After administration, gemcitabine is rapidly cleared from plasma by deamination. More than 90% of the gemcitabine dose is converted into 2',2'-difluoro-2'-deoxyuridine (dFdU) by cytidine deaminase [74].

5.1 Intracellular Metabolism and Mechanism of Action

Like the other pyrimidine analogues, gemcitabine has to enter the tumour cell, where it has to be activated by phosphorylation (Fig. 2). Cellular uptake of gemcitabine is largely mediated by hENT 1 and, to a lesser extent, also by hENT2 and the human concentrative nucleoside transporters 1 and 3 [75, 76]. Once inside the cell, gemcitabine (dFdC) is phosphorylated in the cytoplasm by dCK to its monophosphate (dFdCMP) and then by pyrimidine nucleoside monophosphate kinase (UMP-CMP kinase) to gemcitabine diphosphate (dFdCDP). The enzyme responsible for the final phosphorylation step, the phosphorylation of dFdCDP to gemcitabine triphosphate (dFdCTP), is unclear, although nucleoside diphosphate kinase may play this role [77].

Intracellularly formed dFdCTP and dFdCDP are responsible for the cytotoxic effects of gemcitabine. **dFdCTP** competes with dCTP for incorporation into the DNA. Incorporation of dFdCTP into DNA leads to inhibition of the DNA synthesis and apoptosis of the cell [78, 79]. The mechanism of action of gemcitabine is self-potentiated by inhibition of two enzymes involved in the deoxynucleotide metabolism. **dFdCDP** inhibits the enzyme ribonucleotide reductase, which catalyses the reduction of ribonucleotides to deoxyribonucleotides [80]. By inhibiting this enzyme, the intracellular deoxyribonucleotide triphosphate pool is depleted. This increases the dFdCTP:dCTP ratio, which favours the incorporation of dFdCTP into the DNA. This effect is enhanced in several ways. dFdCTP directly inhibits the enzyme deoxycytidylate deaminase [81]. The same enzyme is also inhibited indirectly by the reduced intracellular deoxyribonucleotide triphosphate pool. As dCK activity is also regulated by dCTP, lowering the deoxyribonucleotide triphosphate pool also promotes gemcitabine phosphorylation [82, 83].

Like in plasma, also in the cell, a major form of elimination of gemcitabine is deamination to dFdU by cytidine deaminase. Likewise, dFdCMP is deaminated by deoxycytidylate deaminase [81, 84]. Gemcitabine can also become inactivated by dephosphorylation of the monophosphate form by 5'-nucleotidases, converting nucleotides back to nucleosides [82].

5.2 Clinical Studies Investigating the Intracellular Concentrations of Gemcitabine Nucleotides

5.2.1 Saturation of the Intracellular Accumulation of dFdCTP

The intracellular metabolism of gemcitabine follows the same pathways as that of cytarabine (Fig. 2). Therefore, in

the early 1990s, the intracellular accumulation of dFdCTP was expected to exhibit similar saturation kinetics as previously demonstrated for ara-CTP [85]. Grunewald et al. confirmed that the ability of PBMCs to convert gemcitabine into dFdCTP was saturable. In a phase I study, including 22 patients with solid tumours, gemcitabine doses between 53 and 1000 mg/m² (infused over 30 min) were compared. The rate of dFdCTP accumulation in PBMCs initially increased with higher doses, but was found to be maximal at a dose of 350 mg/m² infused over 30 min. At this dose rate, gemcitabine steady-state plasma concentrations of 15–20 µM were measured. Although the study demonstrated considerable inter-individual variation, above a gemcitabine steady-state concentration of 20 µM, no further increase of the dFdCTP AUC was found for the majority of patients [74, 85].

5.2.2 Increasing the Intracellular Accumulation of dFdCTP by Prolonging the Infusion Time

Subsequent studies attempted to maximise the intracellular formation of dFdCTP by giving more prolonged gemcitabine infusions at an infusion rate that would presumably saturate the intracellular dFdCTP formation. An infusion rate of 10 mg/m²/min was selected. This infusion rate was previously found to produce steady-state plasma concentrations above 15–20 µM [86].

Grunewald et al. conducted a phase I study with the administration of gemcitabine at this fixed dose rate and increased the dose intensity by prolonging the duration of the infusion (from 120 to 640 min in six steps) [87]. The mean steady-state plasma concentration of gemcitabine during infusion at 10 mg/m²/min was 26.5 µM, with a range of 13.0–53.5 µM. Only four of the 17 patients had steady-state plasma concentrations below 20 µM. For 12 of the 15 assessable patients, linear accumulation of intracellular dFdCTP was seen during the infusion of gemcitabine. In the remaining three patients, the intracellular dFdCTP accumulation reached a plateau during the infusion. The accumulation rate of dFdCTP in circulating leukaemic cells varied considerably among individuals, with a range of 9.7–789 µmol/L/h [87].

Since then, abundant evidence has been published that the intracellular accumulation of dFdCTP in PBMCs and leukaemic blasts indeed increased by prolonging the infusion time (Table 2) [88–90]. Patel et al. demonstrated that maximum intracellular dFdCTP concentrations were significantly higher if gemcitabine 1000 mg/m² was given as a 150-min infusion compared with a standard 30-min infusion (median increase 1.4-fold, range 1.0- to 2.6-fold) [88]. Cattel et al. compared gemcitabine 300 mg/m² infused over 1, 2 or 3 h with a conventional gemcitabine dose of 1000 mg/m² infused in 30 min [90]. The intracellular dFdCTP accumulation in leucocytes was highest in patients who received

gemcitabine 300 mg/m² infused over 3 h. Indicating that, to achieve the highest possible dFdCTP concentrations, a prolonged infusion time was more important than a high dose administered as a short infusion [90].

Grimison et al. performed a cross-over study in which patients received a gemcitabine dose of 1000 mg/m² over either 30 or 100 min [91]. At the second week, each patient received the alternate schedule. Intra-patient comparison showed that the intracellular dFdCTP exposure in leucocytes was significantly higher following the 100-min infusion. However, the effect was confounded by an increase in dFdCTP exposure in the second week compared to the first week, which was consistent with self-induction of the gemcitabine metabolism. Moreover, significant variations in intracellular dFdCTP exposure were observed both between and within individuals. We have to realise that self-induction of the gemcitabine metabolism (among others due to dCK activation) implicates that the optimal dose rate for saturation of the intracellular accumulation changes over time. Because of self-induction and inter-individual differences, a dose rate of 10 mg/m²/min will not be optimal for all patients all of the time [91].

5.2.3 Relationship between Intracellular dFdCTP Concentrations and Efficacy

Other than for cytarabine, for gemcitabine, there are no studies that investigated the relationship between the dFdCTP concentrations in cells of patients treated with a particular gemcitabine dose and treatment efficacy. Various clinical studies did compare the efficacy of prolonged administration of gemcitabine at a fixed-dose rate (FDR) (e.g. 10 mg/m²/min) with conventional 30-min infusion of gemcitabine (1000–1250 mg/m²) [92–98]. However, although these studies were all based on the principle that prolonged infusion would maintain gemcitabine plasma concentrations that result in saturation of the intracellular dFdCTP formation, only a few studies actually measured the intracellular concentrations of dFdCTP (Table 2) [91, 92, 95, 98].

One randomised phase II study showed a survival advantage for prolonged FDR infusion of gemcitabine in patients with advanced pancreatic cancer [92]. In this study, prolonged FDR infusion (1500 mg/m² in 150 min) was compared with standard gemcitabine infusion (2200 mg/m² in 30 min) in 92 patients [92]. The dose levels for both regimes were based on the maximum tolerated doses established in previous studies [99–101]. During the 150-min FDR infusion, the dFdCTP concentration in PBMCs increased linearly, whereas after the end of the 30-min infusion the accumulation rate of dFdCTP decreased markedly. Consistently, the maximum dFdCTP concentrations measured in PBMCs were significantly higher after the FDR infusion (median: 398 vs 188 µM). Median survival was longer for patients

in the FDR arm than for those in the standard arm (8.0 vs 5.0 months; $p=0.013$), and also the 1- and 2-year survival rates were significantly higher following the FDR infusion [92].

However, the confirmatory phase III trial in 832 patients with advanced pancreatic cancer failed to show a significant advantage with a prolonged infusion schedule [98]. In this study, three treatment arms were compared, namely standard gemcitabine 1000 mg/m² infused in 30 min (GEM), FDR gemcitabine 1500 mg/m² infused in 150 min (GEM-FDR) and gemcitabine 1000 mg/m² infused in 100 min in combination with oxaliplatin (GEMOX). It should be noted that patients received a 1.5-fold higher gemcitabine dose in the GEM-FDR arm. The AUC of intracellular dFdCTP measured in PBMCs was significantly increased for both GEM-FDR and GEMOX compared with GEM (Table 2). However, neither GEM-FDR nor GEMOX resulted in a significantly improved overall survival, progression-free survival or response rate compared to GEM [98].

In the phase III study of Gridelli et al., 440 patients with advanced non-small cell lung cancer were treated with a combination of cisplatin and gemcitabine and the effect of FDR gemcitabine (1200 mg/m²) infusion in 120 min was compared with standard 30-min infusion. Also, in this patient population, FDR infusion of gemcitabine did not improve overall survival, progression-free survival or the response rate compared to standard infusion. Unfortunately, this study did not examine the PK of gemcitabine or dFdCTP [97].

5.2.4 Interpretation and Future Perspectives

Overall, there is thus no evidence that a prolonged infusion of gemcitabine at a fixed dose rate results in better treatment efficacy than conventional infusion in 30 min. This is not unexpected. Prolonged gemcitabine infusions at a fixed dose rate aim at a more efficient accumulation of the intracellular anabolites dFdCDP and dFdCTP. If both the antineoplastic effects and the toxic effects of gemcitabine are equally and exclusively mediated by dFdCDP and dFdCTP, more efficient administration of gemcitabine also means that less gemcitabine can be administered before dose-limiting toxicities are achieved. Looking at the phase I dose-escalation studies performed, the maximum tolerated dose for a prolonged FDR infusion of gemcitabine was indeed lower than the maximum tolerated dose for a conventional 30-min infusion due to myelotoxicity (1500–1800 vs 2200 mg/m²/week; for 3 weeks every 4 weeks) [99–101]. If a prolonged FDR infusion is adjusted to this lower maximum tolerated dose, and the total amount of gemcitabine administered is reduced, no increase in efficacy is to be expected.

If we really want to improve the dosing regimen of gemcitabine, we need to find out if there are places where the

mechanisms underlying toxicity do not completely parallel the mechanisms that are responsible for the antineoplastic effects of gemcitabine. One could think of differences in the accumulation rate between target tissues and healthy tissues due to different membrane transporter capacities or enzyme capacities, differences in cell division rate, but also of other gemcitabine metabolites that might contribute to the dose-limiting toxicities. In this light, the role of the deaminated metabolite dFdU could be interesting.

5.2.5 Role of dFdU Nucleotides During Gemcitabine Therapy

Although dFdU was traditionally considered as an inactive metabolite, *in vitro* studies demonstrated that dFdU has cytotoxic activity in tumour cell lines [102–104]. The half-maximal inhibitory concentration (IC₅₀) for dFdU in gemcitabine-sensitive tumour cell lines was at least 1000- to 2000-fold higher than for gemcitabine [102–104]. However, during gemcitabine treatment, the exposure to dFdU highly exceeds the exposure to gemcitabine [74, 105]. The question is therefore what the contribution of dFdU is to the cytotoxic effect of gemcitabine.

In vitro studies demonstrated that dFdU is taken up into cells, for instance by human concentrative nucleoside transporter 1 [103]. In the cell, dFdU can be phosphorylated to its monophosphate (dFdUMP), diphosphate (dFdUDP) and triphosphate (dFdUTP) [103]. Alternatively, dFdCMP can be deaminated to dFdUMP, by deoxycytidylate deaminase, and then further phosphorylated [81, 84]. *In vitro* studies also demonstrated that dFdUTP is incorporated into both DNA and RNA and that the extent of incorporation correlated with the cytotoxicity of dFdU [103].

Veltkamp et al. [106] performed a feasibility study with orally administered gemcitabine and demonstrated for the first time that, besides dFdCTP, dFdUTP was also formed in PBMCs of patients. After oral administration, the systemic gemcitabine exposure proved to be very low as a result of extensive first-pass metabolism to dFdU. Consistently, the intracellular dFdUTP exposure in PBMCs was significantly higher than the dFdCTP exposure. One patient died during the study experiencing grade 4 hepatic failure. It was noted that the exposure to dFdUTP was excessively high in this patient. This suggested that dFdUTP could have played a role in the toxicity experienced by this patient [106].

Derissen et al. [105] explored the intracellular PK of both the dFdC nucleotides and the dFdU nucleotides (i.e. dFdUMP, dFdUDP and dFdUTP) during intravenous gemcitabine treatment. Both in plasma and in PBMCs, dFdU was present in higher concentrations than gemcitabine. However, dFdU was only phosphorylated to a very limited extent in PBMCs. For all patients, intracellular dFdU nucleotide concentrations were much lower than the gemcitabine

nucleotide concentrations. This suggests that the contribution of dFdU nucleotides to the cytotoxic effect of gemcitabine is limited [105].

6 Azacitidine and Decitabine

Azacitidine (aza-C) and decitabine (5-aza-2'-deoxycytidine; aza-dC) are analogues of cytidine and deoxycytidine, respectively, in which the carbon atom at position 5 of the pyrimidine ring has been replaced by a nitrogen atom. Azacitidine and decitabine are rapidly cleared from plasma. Azacitidine undergoes spontaneous hydrolysis or is deaminated to 5-aza-uridine (aza-U) by cytidine deaminase [109, 110]. Decitabine is primarily eliminated through deamination to 5-aza-2'-deoxyuridine (aza-dU) by cytidine deaminase [109, 111, 112].

6.1 Intracellular Metabolism and Mechanism of Action

Cellular uptake of azacitidine is mediated by all seven human nucleoside transporters, with human concentrative nucleoside transporter 3 showing the highest transport rates [107]. Decitabine is transported into cells by hENT1 and 2 [107]. As azacitidine is an analogue of cytidine and decitabine an analogue of deoxycytidine, different enzymes are involved in their intracellular metabolism (Fig. 2). Once in the cell, azacitidine is phosphorylated to 5-azacytidine monophosphate (aza-CMP) by uridine-cytidine kinase and then to 5-azacytidine diphosphate (aza-CDP) and 5-azacytidine triphosphate (aza-CTP) by UMP-CMP kinase and nucleoside diphosphate kinase, respectively. A small fraction, about 10–20%, of the aza-CDP is instead converted to 5-aza-2'-deoxycytidine diphosphate (aza-dCDP) via the enzyme ribonucleotide reductase, followed by further phosphorylation to 5-aza-2'-deoxycytidine triphosphate (aza-dCTP) [108–110]. Decitabine is, after uptake in the cell, converted to its monophosphate (aza-dCMP) by deoxycytidine kinase. dCMP kinase and nucleoside diphosphate kinase convert the monophosphate to its diphosphate form (aza-dCDP) and triphosphate form (aza-dCTP), respectively [111]. Like in plasma, azacitidine and decitabine are also in the cell primarily eliminated by deamination by cytidine deaminase. Likewise, aza-dCMP is deaminated to 5-aza-2'-deoxyuridine monophosphate (aza-dUMP) by deoxycytidylate deaminase [111].

Intracellularly formed aza-CTP and aza-dCTP are responsible for the pharmacological effects of azacitidine and decitabine. **Aza-CTP** is incorporated into RNA. This ultimately disrupts messenger RNA metabolism and protein synthesis, leading to apoptosis [109, 110]. Furthermore, incorporation of aza-CTP into RNA inhibits ribonucleotide

reductase, leading to a reduced deoxyribonucleotide pool, which has deleterious consequences for DNA synthesis and repair [109]. **Aza-dCTP** competes with endogenous dCTP for DNA incorporation. At high doses, aza-dCTP incorporation into DNA leads to inhibition of DNA synthesis and cell death [111, 112]. However, at lower doses, a smaller amount of aza-dCTP is incorporated into the DNA, which primarily causes inhibition of DNA methylation [111]. DNA methylation has an important role in the regulation of gene expression. Aberrant DNA methylation has a place in the pathogenesis of several malignancies including MDS and AML [113, 114]. The most studied change of DNA methylation is the silencing of tumour suppressor genes by hypermethylation of the CpG islands within their promoter regions [115]. The enzymes responsible for DNA methylation are DNA methyltransferases (DNMTs). When aza-dCTP is incorporated into the DNA, covalent bonds are formed between the aza-dC in the DNA and these DNMTs. At high doses, the DNA is not able to recover and cell death occurs. However, at lower doses, the formed DNA-DNMT adducts are degraded by the proteasome, after which the DNA is restored. DNA synthesis is then resumed in the absence of DNMTs. As a consequence, the aberrant DNA hypermethylation patterns, which play an important role in the pathogenesis of the mentioned hematologic abnormalities, can no longer be reproduced toward the daughter strands [116]. In this manner, a low dose of azacitidine or decitabine is able to induce re-expression of previously silenced genes. Reactivation of cell cycle-regulating genes (i.e. tumour suppressor genes), which were initially silenced due to hypermethylation, may restore cell differentiation and normal growth control in the daughter cells [110, 117, 118].

6.2 Clinical Studies Investigating the Intracellular Concentrations of Azacitidine and Decitabine Nucleotides

In the 1970s and 1980s, the anti-leukaemic effects of azacitidine and decitabine were studied in high doses based on the maximum tolerated dose. The mechanism of action at such high doses is based on cytotoxicity. From the 1990s, researchers also started to study low azacitidine and decitabine doses, based on the idea of their demethylating effect [119–122]. It was only at these low doses that the therapeutic benefits of azacitidine and decitabine were demonstrated [123, 124]. Both drugs were therefore registered in low doses.

Few clinical studies have examined the intracellular PK of the active nucleotides aza-CTP and aza-dCTP. Other than for the other pyrimidine analogues discussed in this review, for low-dose azacitidine and decitabine therapy, we are not interested in maximising intracellular triphosphate concentrations to maximise the effect. The optimal dose is more

nuanced and comes down to the dose that maximises the demethylating effect without the cytotoxic effect gaining the upper hand.

Only at the beginning of this decade, three studies investigated the intracellular PK of the active metabolites aza-CTP and aza-dCTP in patients who were treated with azacitidine or decitabine [125–127]. The main objective of these studies was to gain insight into inter-individual differences in the intracellular PK, especially as some patients experience no effect from treatment with azacitidine or decitabine or become resistant to the treatment at some point [128].

Jansen et al. investigated the intracellular aza-dCMP, aza-dCDP and aza-dCTP concentrations in PBMCs obtained from three patients diagnosed with MDS who were treated with decitabine 15 mg/m² administered as a 4-h infusion, repeated every 8 h for 3 days with a cycle duration of 6 weeks [125]. In contrast to plasma decitabine, intracellular aza-dCTP was found to accumulate during the treatment cycle of nine infusions. Two patients responded well to decitabine therapy. Remarkably, considerable aza-dCTP concentrations were measured in the PBMCs from these two patients, whereas only low concentrations were present in the third patient who did not respond. The authors also searched for the possible presence of deaminated decitabine nucleotides, i.e. 5-aza-2'-deoxyuridine nucleotides, but these were not observed [125].

Wang et al. investigated the intracellular aza-dCTP concentrations in bone marrow cells and PBMCs from seven patients with AML who were treated with decitabine 20 mg/m² infused in 1 h for 10 days [126]. Samples were collected on days 1 and 5 within 3 h after completion of the decitabine infusion. Other than in the study by Jansen et al., repeated dosing did not result in accumulation of intracellular aza-dCTP during the treatment cycle. Aza-dCTP concentrations were found to be relatively stable from day 1 to 5. In line with the study by Jansen et al., higher intracellular aza-dCTP concentrations also appeared to be associated with clinical response in this study. Patients who eventually achieved a complete response had higher aza-dCTP concentrations in both bone marrow and PBMCs than those who did not respond to the treatment. However, also in this explorative study, the number of patients was too small to draw definitive conclusions [126].

Following these two studies with decitabine triphosphate (aza-dCTP), Derissen et al. developed an assay to quantify azacitidine triphosphate (aza-CTP) in PBMCs [127]. This proved to be more complex than it had been for aza-dCTP. High-resolution mass spectrometry had to be used, because regular liquid chromatography-tandem mass spectrometry could not distinguish between aza-CTP and some of the endogenous nucleotides. The developed assay was applied to quantify aza-CTP in PBMCs of two patients diagnosed with MDS who were treated with azacitidine 75 mg/m²

subcutaneously for 7 days, every 4 weeks. The aza-CTP concentrations measured were about 10–25 times higher than the aza-dCTP concentrations found by Jansen et al. and Wang et al. (Table 2) [125–127].

We have not found any other studies that determined the intracellular nucleotide concentrations after azacitidine therapy. Nevertheless, the recent study by Unnikrishnan et al. is very interesting to mention in this light [128]. This study examined the successive steps in the pharmacology of azacitidine for eight patients diagnosed with MDS or chronic myelomonocytic leukaemia, and showed that responders to azacitidine therapy demonstrated more aza-dC incorporation into DNA and more DNA demethylation than non-responders. Unfortunately, the intracellular aza-CTP and aza-dCTP concentrations were not measured in this study. Instead, all intracellular nucleotides were enzymatically dephosphorylated during sample preparation, after which the total intracellular aza-C and aza-dC concentrations were measured. Interestingly, the non-responders, showing minimal DNA incorporation and minimal DNA demethylation, showed high intracellular aza-C and aza-dC concentrations (after dephosphorylation) as well as high aza-C incorporation into RNA. This means that the low aza-dC DNA incorporation could not be explained by limited cellular uptake and that the intracellular metabolism (at least the RNA pathway) was also intact. The authors suggest that aza-dC incorporation into DNA might be low in patients if an increased proportion of the bone marrow cells is quiescent and not undergoing DNA replication [128]. It would be interesting to measure the aza-CTP and aza-dCTP concentrations in these patients and examine if they correlate with DNA incorporation and response.

6.2.1 Interpretation and Future Perspectives

Only three small studies were found that examined the intracellular PK of azacitidine and decitabine nucleotides. Nevertheless, the two exploratory studies conducted so far for decitabine both suggest a relationship between intracellular aza-dCTP concentrations and clinical response to decitabine therapy. This relationship should be further investigated in larger numbers of patients. In addition, this relationship should also be investigated for azacitidine. If a relationship between intracellular triphosphate concentrations and response is confirmed, the intracellular concentrations of aza-CTP or aza-dCTP can serve as a biomarker for early identification of individuals who are unlikely to respond to treatment with azacitidine or decitabine. Further studies should then show whether these patients would benefit from a higher azacitidine or decitabine dose or whether they would be better off switching to another therapy.

7 Conclusions and Discussion

7.1 Differences Between Pyrimidine Analogues

For cytarabine and gemcitabine, the intracellular PK of the active nucleotides has been extensively studied over the years. However, for 5-FU, capecitabine, azacitidine and decitabine, the intracellular PK of the active nucleotides has only been studied to a very limited extent. This is remarkable. Of course, also for the latter agents, it is useful to gain insight into the intracellular PK of their active metabolites. For a long time, however, there were no suitable bioanalytical assays that made it possible to quantify the nucleotides of 5-FU, capecitabine, azacitidine and decitabine in cells.

In the past decade, using modern analytical techniques, we have managed to develop suitable bioanalytical assays to quantify also the nucleotides of these pyrimidine analogues [32, 125, 127]. Indeed, quantification of the active nucleotides of 5-FU, capecitabine, azacitidine and decitabine proved more difficult than quantifying cytarabine triphosphate and gemcitabine triphosphate [32, 125, 127]. Azacitidine and decitabine are very similar to endogenous nucleotides and also have the same molecular mass, which makes quantification by liquid chromatography-tandem mass spectrometry difficult [125, 127]. In addition, the intracellular nucleotide concentrations during treatments with 5-FU, capecitabine, azacitidine and decitabine were found to be very low compared with the intracellular nucleotide concentrations during treatment with cytarabine or gemcitabine (Table 2). Now that suitable bioanalytical assays are finally also available for quantifying the active nucleotides of 5-FU, capecitabine, azacitidine and decitabine, it is time that the intracellular PK of these pyrimidine analogues is also better studied.

7.2 Relationship Between the Intracellular Nucleotide Concentrations and the Antineoplastic Effect

Based on pharmacology, a relationship is expected between the intracellular concentrations of the active nucleotides and the cytotoxic effects of pyrimidine analogues. For cytarabine and gemcitabine, this relationship has also been demonstrated in *in vitro* studies and animal studies [41, 129]. However, not many clinical studies have investigated the relationship between intracellular nucleotide concentrations and the clinical outcome in patients. Only for cytarabine, a relationship was demonstrated between the intracellular triphosphate concentrations in leukaemic cells and the response rate in patients with AML [64]. Future clinical studies should show, for the other pyrimidine analogues, whether there is a relationship between the intracellular

nucleotide concentrations and the therapeutic effect in patients.

7.3 Optimisation of Dosing Regimes

Research that examined the intracellular PK of cytarabine and gemcitabine focused primarily on the saturation aspect of the intracellular triphosphate formation [44, 45, 74, 85]. Attempts to improve the dosing regimen of gemcitabine were aimed at maximising the intracellular gemcitabine triphosphate concentrations [88–92, 95, 98]. However, if the dose is based on the maximum tolerated dose and the mechanisms underlying the therapeutic effect and toxicity are exactly the same, this strategy does not make sense. Efficient administration of gemcitabine, aimed at maximum triphosphate formation, also means that less gemcitabine can be administered before dose-limiting toxicities are achieved [99–101]. In all cases, the dosage is limited by the ‘maximum tolerated intracellular triphosphate exposure’.

If we really want to improve the dosing regimens of pyrimidine analogues, we will have to examine if there are areas where the mechanism of action and the mechanism of toxicity are not completely parallel. One could think of differences in cellular uptake or phosphorylation capacity between healthy cells and tumour cells. It is also useful to investigate the effects of high plasma peaks, which far exceed plasma concentrations that saturate the intracellular triphosphate formation. What happens to the excess of pyrimidine analogue that is not phosphorylated intracellularly? What is, for instance, the effect of deamination products such as ara-U and dFdU?

A recent study investigating the intracellular PK of gemcitabine showed that dFdU was only phosphorylated to a very limited extent in PBMCs [105]. Future studies should show whether dFdU is also hardly phosphorylated in tumour cells. If this is the case, dFdU is unlikely to contribute substantially to the cytotoxic effects of gemcitabine. In this light, it would be rational to pay more attention to inter-individual differences in the deamination rate of gemcitabine to dFdU. Some clinical studies have shown that gemcitabine therapy was more effective and more toxic in patients with low cytidine deaminase activity, i.e. a decreased deamination of gemcitabine to dFdU [50–52]. Future studies should show whether individualisation of the gemcitabine dose, based on the measured cytidine deaminase activity, leads to better treatment results.

7.4 Inter-Individual Differences

For all pyrimidine analogues, a linear relationship was found between the dose and the plasma concentration [18, 43–46, 74, 130]. However, no correlation was found between the plasma concentration and the intracellular nucleotide

concentration [33, 43, 85]. The concentration–time curves for the intracellular nucleotides showed considerable inter-individual variation. This is not unexpected as many different proteins are involved in cellular uptake and the extensive intracellular metabolism of the pyrimidine analogues. The activity of all these membrane transporters and enzymes might vary from patient to patient.

Given the considerable inter-individual differences observed for the intracellular nucleotide concentrations, the question arises whether pyrimidine analogue therapy should be more individualised. Future research should show which intracellular nucleotide concentrations are worth pursuing and whether dose individualisation is useful to achieve these concentrations. In addition, the possibility of using the intracellular nucleotide concentration as a predictive marker for the therapeutic effect might be investigated. In this way, potential non-responders, owing to inadequate intracellular metabolism, could be recognised early.

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Declarations Not applicable.

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Affiliations

Ellen J. B. Derissen^{1,2,3} · Jos H. Beijnen^{1,4}

¹ Department of Pharmacy and Pharmacology, Antoni van Leeuwenhoek Hospital-The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands

² Department of Clinical Pharmacology and Pharmacy, Amsterdam UMC, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands

³ Department of Pharmacy, Elisabeth-TweeSteden Hospital, Dr. Deelenlaan 5, 5042 AD Tilburg, The Netherlands

⁴ Science Faculty, Division of Pharmaco-epidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands