In vitro partition of docetaxel and gemcitabine in human volunteer blood: the influence of concentration and gender

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We have performed *in vitro* incubations of blood from male and female volunteers with gemcitabine and docetaxel alone, and in combination, at different concentration gradients in order to investigate changes in partition between red blood cells (RBCs), total plasma and the free fraction. After extraction and sample pre-treatment, a validated high-performance liquid chromatography method followed by UV detection was used to determine the concentrations of both drugs in the different blood constituents. The partition ratio [the concentration in the erythrocytes divided by the concentration in plasma (E/P)] was calculated. The partition ratio of docetaxel varied from 0.02 to 1.44 (mean 0.35), reflecting its relatively low affinity for RBCs, probably because of its high plasma protein binding (more than 98%). For gemcitabine, the partition ratio varied from 1 to 5, reflecting a high affinity for RBCs (less than 10% plasma protein bound). The partition ratios of both drugs increased significantly with higher whole-blood concentrations, favoring uptake in the erythrocytes when plasma protein binding is saturated. Combination incubations showed a complex and unexplained interaction between gender and the influence of docetaxel on the partition of gemcitabine. We conclude that the incorporation of drugs into the RBC pool may be important for transportation to tumor tissue and efficacy. In combination, one anti-cancer agent can alter the partition ratios of other anti-cancer agents. *Anti-Cancer Drugs* 16:885–891 © 2005 Lippincott Williams & Wilkins.

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Introduction

Anti-cancer drugs and/or their metabolites are generally reactive substances, and are capable of inducing changes in cellular systems. In the case of blood cells, changes may even result in altered pharmacokinetics if the compartment affected is sufficiently large. Red blood cells (RBCs, i.e. erythrocytes) are most susceptible to temporary changes following exposure to anti-cancer drugs. As they have by far the largest cell volume and surface area when compared to other cellular components of the blood, they can induce alterations in pharmacokinetics [1].

In a previous phase I study we combined docetaxel with gemcitabine to investigate the pharmacokinetics of both agents [2]. The pharmacokinetics of docetaxel were consistent with single-agent data reported in the literature [3,4], indicating a lack of interference by gemcitabine. In contrast, gemcitabine pharmacokinetics changed significantly with the administration of docetaxel (Table 1). Since self-induction of gemcitabine clearance was excluded, it is likely that docetaxel significantly alters gemcitabine distribution. This change in distribution may be caused by altered partitioning of gemcitabine between the different blood constituents of cells, plasma and plasma water.

Docetaxel is extensively (more than 98%) bound to plasma proteins. It is generally accepted that only the unbound fraction of a drug in plasma partitions into RBCs [5,6]. Considering the protein data, one would expect relatively low amounts of docetaxel to be present in erythrocytes, as the protein binding has been reported to be non-saturable. Plasma protein binding of gemcitabine is negligible (less than 10%), so one would expect relatively high amounts of gemcitabine to be present in erythrocytes. Thus far, data of RBC versus plasma partitioning of docetaxel and gemcitabine are limited. We have performed *in vitro* incubations of blood from male and female volunteers with gemcitabine and docetaxel alone and in combination, at different concentration gradients, to investigate changes in partition between RBCs, total plasma and the free fraction. The new measurement of sediment (MESED) technology, which is not hindered by limitations generally encountered in the quantitative analysis of substances present in/at RBCs, was used to isolate 100-µl RBC pellets [7,8].

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Table 1 **Estimated pharmacokinetic parameters (means and SD) of gemcitabine [19]**

	C_{max}(ng/ml)	AUC (μg/ml·min)	$t_{1/2}$(min)	V_{ss}(l/m^2)	CL (ml/min/m^2)	MRT (min)
Group I + II						
day 1 (n=11)	40472	1431	29	36.6	827	48
	9794	282	14	12.3	292	28
day 8 (n=11)	53155	1434	48	29.8	650	61
	17503	608	25	10.1	261	40
day 15[a] (n=11))	38026	1253	138	76.4	806	71
	19711	668	69	65.8	411	23
Group IIIa						
day 1 (n=5)	52215	1153	112	58.7	747	48
	13431	414	134	43.3	187	5
day 8[a] (n=4)	28808	1485	561	328.7	546	190
	2002	187	467	213.4	75	69
Group IIIb						
day 1 (n=4)	42396	735	36	45.7	I I21	68
	10620	152	47	16.1	206	54
day 8 (n=4)	49824	439	36	56.9	1298	31
	11431	156	21	21.7	394	25
Group IV						
day 1 (n=3)	71823	1281	42	31.2	784	43
	3922	96	1	1.6	57	22
day 8[a] (n=3)	47689	812	29	34.2	1103	40
	13854	125	5	5.9	281	28

[a]Docetaxel administered before gemcitabine with a 1-h interval.Group I + II: 4-weekly schedule at two dose levels, gemcitabine 800 mg/m^2 on days 1, 8 and 15/docetaxel 85 or 100 mg/m^2 on day 15.Group III: 3-weekly schedule, gemcitabine 800 mg/m^2 on days 1 and 8/docetaxel 85 mg/m^2 on day 8.Group IV: 3-weekly schedule, gemcitabine 1000 mg/m^2 on days 1 and 8/docetaxel 85 mg/m^2 on day 8.

Methods

Docetaxel mono incubations

The clinical docetaxel formulation in Tween 80 (Taxotere 40 mg/ml; 2 mg/ml after reconstitution) was supplied by Aventis Pharma (Paris, France). Stock solutions of 100 μg/ml were made by dissolving 50 μl of the basic solution after reconstitution in 950 μl NaCl 0.9%. Working solutions of docetaxel were prepared by serial dilutions in NaCl 0.9% from the primary stock solutions to become 10 and 1 μg/ml docetaxel. Spiked plasma and RBC samples were prepared by the addition of calculated amounts of the working solutions to 100 μl of drug-free human plasma and RBCs, resulting in calibration standards of 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10 000 ng/ml docetaxel. Pools of quality control samples for docetaxel were prepared in human plasma in the concentrations of 40, 400 and 10 000 ng/ml, and in whole-blood in concentrations of 200 and 7500 ng/ml.

Blood (12 ml) was collected from 13 volunteers (seven females and six males) into EDTA tubes and divided into 10 Eppendorf cups (1 ml/cup). Calculated amounts of docetaxel were added to the cups to produce eight different concentrations: 10, 50, 100, 200, 500, 1000, 2000 and 5000 ng/ml. Subsequently these aliquots were transferred into MESED devices, and centrifuged twice at 3000 *g* and once at 500 *g* to separate plasma and RBCs, as described by Driessen *et al.* [7]. RBC and plasma samples were frozen at −20°C until further analysis.

Gemcitabine mono incubations

Gemcitabine was supplied by Eli Lilly (Brussels, Belgium) as a lyophilized powder in sterile vials containing 200 or 1000 mg of gemcitabine as the hydrochloride salt, mannitol and sodium acetate (Gemzar; 40 mg/ml basic solution after reconstitution). Stock solutions of 1 mg/ml gemcitabine were made by dissolving 25 μl of the basic solution after reconstitution in 975 μl NaCl 0.9% and stock solutions of 10 mg/ml were made by dissolving 25 μl of the basic solution in 75 μl NaCl 0.9%. Working solutions of gemcitabine were prepared by serial dilutions in NaCl 0.9% from the primary stock solutions to produce concentrations of 100 and 10 μg/ml. Spiked plasma and RBC samples were prepared by the addition of calculated amounts of the stock and working solutions to 100 μl of drug-free human plasma and RBCs, resulting in calibration standards of 200 and 500 ng/ml, and 1, 2, 5, 10, 20, 50, 100 and 200 μg/ml. Pools of quality control samples were prepared.

Gemcitabine is metabolized by plasma cytidine deaminase *ex vivo* and this can be inhibited by tetrahydrouridine (THU). We intentionally did not add THU to our volunteer samples since it is possible that THU itself can alter the partitioning of gemcitabine between RBCs and plasma. Furthermore, the metabolite dFdU cannot be eliminated during *in vitro* experiments and this possibly limits the conversion by a negative feedback mechanism.

Blood (12 ml) was collected from eight volunteers (two female smokers, two female non-smokers, two male smokers and two male non-smokers) into EDTA tubes and divided into 10 Eppendorf cups (1 ml/cup). Calculated amounts of gemcitabine were added to the cups to produce five different concentrations: 500 ng/ml, and 5, 10, 50 and 100 μg/ml. The duplicate concentration ranges allowed 1-h incubations at 37°C in both room air (0.04% CO_2) and concentrations of 20% CO_2. This is important,

as the physiology of the RBC is influenced *in vivo* by changes in CO_2 tension between the systemic and pulmonary circulations. Subsequently, these aliquots were transferred into MESED devices and centrifuged 3 times to separate plasma and RBCs as described by Driessen *et al.* [7]. The samples were frozen at –20°C until further analysis.

Combined docetaxel and gemcitabine incubations

Blood (12 ml) was collected from eight volunteers (two female smokers, two female non-smokers, two male smokers and two male non-smokers) into EDTA tubes and divided into 12 Eppendorf cups. Calculated amounts of docetaxel were added to the cups to produce three different concentrations: 50 ng/ml, and 1 and 5 μg/ml. After a 2-h incubation at 37°C, half of the samples at 0.04% CO_2 (room air) and the other half at 20% CO_2, the following concentrations of gemcitabine were added: 500 ng/ml, and 10 and 100 μg/ml. The samples were again incubated for 1 h at 37°C at different CO_2 concentrations, after which plasma and RBCs were separated by the MESED device and frozen at –20°C. This procedure was repeated with blood from another eight volunteers, in the reverse sequence of incubation: gemcitabine followed by docetaxel.

Solid-phase extraction and analysis of docetaxel

C_{18} columns (Alltech Associates, Deerfield, Illinois, USA) on a vacuum manifold were used for the reversed-phase extraction of docetaxel after conditioning with methanol and distilled water. An aliquot of 900 μl of distilled water was added to the 100-μl RBC samples to obtain lysis, after which the samples were centrifuged at 10 000 *g* for 10 min to remove cell debris. An aliquot of 100 μl of the plasma samples was also diluted with 900 μl of distilled water. A saline citrate buffer (3 M NaCl, 0.3 M Na citrate and 6 M urea, pH 7) was added to the samples to eliminate the proteins, to which docetaxel is strongly bound. Before centrifugation, 100 μl of pure paclitaxel (reference compound) 10 μg/ml in methanol was added to all the samples as an internal standard. Samples were introduced into the top of a C_{18} column, and extraction was performed by applying positive pressure with a syringe through the bed to produce a flow rate of 1–5 ml/min. Any weakly retained interfering compounds were washed off with 4 ml of distilled water, after which docetaxel and the internal standard paclitaxel were slowly eluted with 4 ml methanol. All the samples were evaporated in a vacuum centrifuge at 3000 g for 90 min. The residue of 100–150 μl was filtered over a 0.45-μm PVDF high-performance liquid chromatography (HPLC) filter (Acrodisc; Waters, Milford, Massachusetts, USA) and transferred to total recovery vials (Waters) for HPLC analysis.

A modification of the assay published by Loos *et al.* [9] was developed and validated to analyze docetaxel in RBCs, plasma and plasma water. The HPLC equipment comprised a constaMetric 3200 delivery system (LDC Analytical, a subsidiary of Thermo Instruments Systems, Riviera Beach, Florida, USA), a Waters 717plus autosampling device and a UV-2000 detector (Spectra Physics; Thermo Separation Products, Breda, The Netherlands). Separations were achieved on a stainless steel analytical column (250 mm × 1.0 mm internal diameter, 5 μm particle size) packed with Inertsil ODS-80A material (GL Science, Tokyo, Japan), protected by a Lichrospher 100RP-18 end-capped guard column (40 mm × 4.0 mm internal diameter, 5 μm particle size) obtained from Merck (Darmstadt, Germany). The mobile phase consisted of 680 ml methanol, 320 ml water and 3 ml phosphoric acid (85% orthophosphoric acid), with the pH adjusted to 6.0. The mobile phase was degassed by ultrasonication and delivered at a flow rate of 1.00 ml/min. The column was maintained at 60°C using a Model SpH99 column oven (Spark Holland, Meppel, The Netherlands) and the eluent was monitored at a wavelength of 230 nm. Peak recording and integration was performed with the Chrom-Card data analysis system (Fisons, Milan, Italy) connected to an ICW chromatographic workstation.

Ratios of docetaxel to the internal standard paclitaxel versus concentrations of the standard solutions were used for quantitative computations. Calibration curves were fitted by weighted linear regression analysis. These curves were linear in the range of 20–10 000 ng/ml of docetaxel, with regression correlation coefficients of above 0.999. The lower limit of quantitation for docetaxel was 100 pg (plasma) and 165 pg (RBC), and the limit of determination 10 ng/ml in plasma and 17 ng/ml in RBC. Partitioning ratios of the concentrations of docetaxel in RBCs and plasma were calculated for each set of samples (RBC/plasma).

Analysis of gemcitabine in plasma [10,11] and RBCs

To extract gemcitabine from the plasma samples, 100 μl of the internal standard 2′-deoxycytidine (10 μg/ml) was added to 200 μl of plasma. After vortexing, the sample was treated with 6 ml isopropanol (15%) in ethyl acetate and mixed thoroughly. After centrifugation, the organic phase was transferred to another polypropylene tube and evaporated until dry. The residue was redissolved in 1 ml of the mobile phase (see below, a 5 times dilution) and filtered over a 0.45-μm PVDF HPLC filter (Acrodisc) before HPLC injection (20 μl).

Distilled water (400 μl) of was added to 100 μl of RBCs. After vortexing and lysis of the RBCs, 100 μl of the internal standard was added and the sample treated in the same way as described for the plasma extraction.

An HPLC method has been used and validated for the determination of gemcitabine and its main circulating

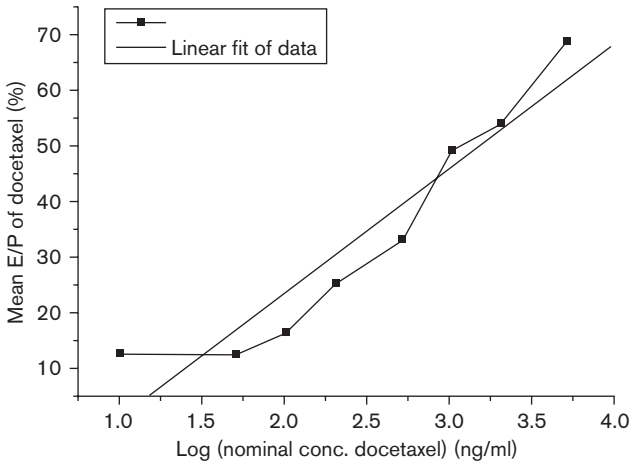
metabolite dFdU in human plasma and erythrocytes, with 2′-deoxycytidine as the internal standard. Separation was achieved on a Chrompack Spherisorb ODS-2 reversed-phase column (250 mm × 4.6 mm, 5 µm). The mobile phase was Pic B7 reagent (Waters) in 15% methanol (pH 3.1) with a flow rate of 1.0 ml/min. Gemcitabine and 2′-deoxycytidine were detected by UV detection at 270 nm. The limit of quantitation was about 100 ng/ml for gemcitabine. Within-run and between-run precisions were less than 10%, and average accuracies were 90–110%.

Results

The partition ratio [concentration of docetaxel in the erythrocytes divided by the concentration in the plasma (E/P)] was calculated for each sample in the mono incubations. The partition ratios varied from 0.02 to 1.44 (mean 0.35), indicating that the RBC has a relatively low affinity for docetaxel. Mean partition ratios per concentration are plotted against whole-blood concentrations in Figure 1. Above incubation concentrations of 50 ng/ml, the fraction of docetaxel in the erythrocytes increases significantly (on a semi-log scale, $p = 0.0003$).

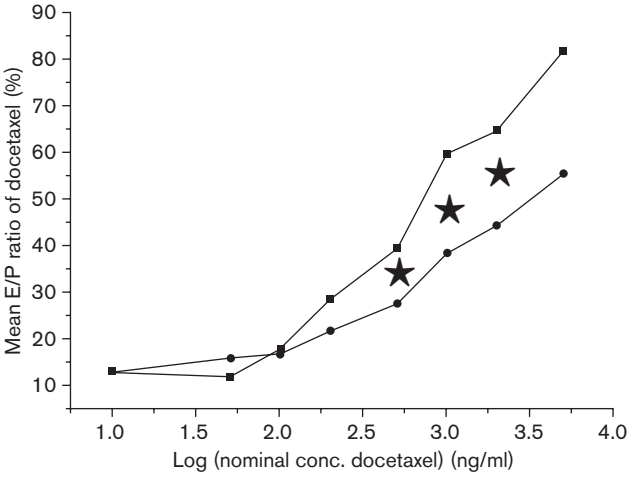
A comparison of males and females was performed, and the results are presented in Figure 2. At the lowest incubation concentrations there was no difference in partition ratios of docetaxel between men and women, but at whole-blood concentrations of 500, 1000 and 2000 ng/ml we detected a significant difference with higher partition ratios in women ($p < 0.02$). No difference was detected in the partition ratios of smokers and non-smokers.

Fig. 1



Mean E/P versus log total blood concentration of docetaxel. Linear regression analysis of the relationship is described as $y = -21.55 + 2.29\,x$ ($p = 0.0003$).

Fig. 2



Mean E/P versus log total blood concentration of docetaxel for male (circles) and female (squares) patients. Significant differences between male and female E/P are indicated by $*p < 0.05$ (Student's *t*-test, unpaired).

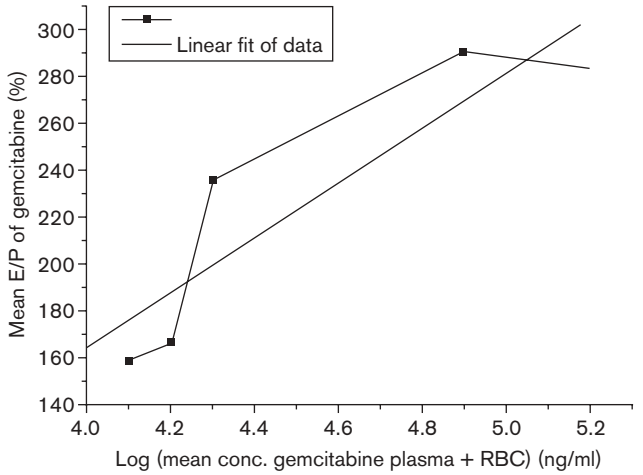
The partition ratio of gemcitabine was calculated for each sample. These ratios varied between 1 and 5 in the mono incubations. There was no difference in the partitioning of gemcitabine between the different blood constituents in smokers and non-smokers nor was there any influence of the CO_2 concentration during incubation. The partition ratio increased significantly with higher whole-blood concentrations (Fig. 3, $p = 0.038$).

A comparison of males and females was performed, and the results are presented in Figure 4. At the lowest incubation concentrations, there was no difference in the partition ratios of gemcitabine between men and women, but above 10 µg/ml we detected a major gender difference, with higher concentrations of gemcitabine in the erythrocytes of men versus women (unpaired *t*-test on the whole group: $p = 0.00009$).

The partition ratio of dFdU, the main circulating metabolite of gemcitabine, varied between 0.52 and 4.83, the same range as the parent drug. There was no significant change in these ratios with concentration, gender, CO_2 percentage during incubation or smoking habit. Nor was there any influence of docetaxel pre-incubation on the partition of dFdU when considering the whole group. Splitting gender and docetaxel pre-treatment, we detected more dFdU in the female erythrocyte with docetaxel pre-incubation, compared to the reversed sequence of incubation ($n = 8$, $p = 0.025$, data not shown).

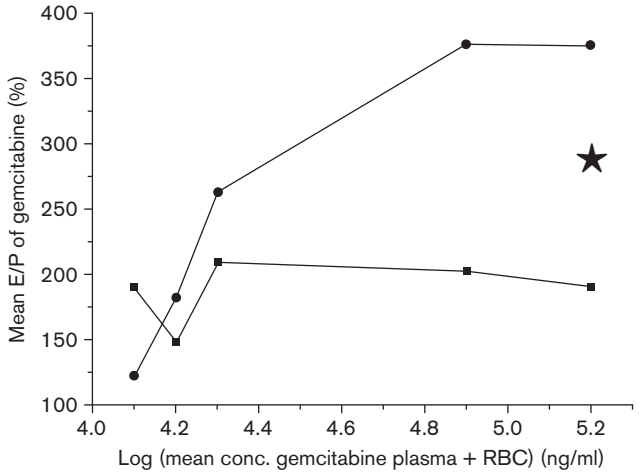
In the combination incubations, gemcitabine did not alter the partition ratios of docetaxel. The CO_2 concentrations

Fig. 3



Mean E/P versus log total blood concentration of gemcitabine. Linear regression of the relationship is described as $y = -299.98 + 11.61x$ ($p = 0.0380$).

Fig. 4



Mean E/P versus log total blood concentration of gemcitabine for male (circles) and female (squares) patients. Significant differences between male and female E/P are indicated by $*p < 0.05$ (Student's *t*-test, unpaired).

did not influence the partitioning of docetaxel, nor was there a significant difference between the partition ratios in smokers and non-smokers nor males and females. Comparing the lowest (50 ng/ml) to the highest (5 µg/ml) incubation concentrations of docetaxel, there was a trend to higher partition ratios at the higher concentrations ($p = 0.068$, data not shown), consistent with our findings in the mono incubations.

In the combination incubations with docetaxel, we were unable to detect any influence of docetaxel (including the vehicle polysorbate 80) on the partitioning of gemcitabine when comparing the whole sample pool (unpaired *t*-test: $p = 0.60$). By subanalysis of our combination incubations, however, we found significantly more gemcitabine in male erythrocytes after docetaxel exposure versus the inversed sequence of incubation ($n = 10$; $p = 0.02$). In women, the opposite was apparent; less gemcitabine in the erythrocytes after incubation with docetaxel ($n = 10$; $p = 0.001$).

Discussion

At incubation concentrations above 50 ng/ml, the fraction of docetaxel in the erythrocytes increases significantly (on a semi-log scale, $p = 0.0003$). This observation is consistent with the Hinderling equation [12], which states that the concentration of a drug in the RBCs is proportional to the free fraction of that drug. Assuming that the free fraction of docetaxel increases at the highest incubation concentrations, when proteins are saturated, we expected an increase in the RBC/plasma ratio at the higher concentrations; this did occur, but only gradually. The partition ratio increased almost linearly, with rising nominal concentrations, without a sudden jump after saturation of protein binding. A possible explanation could be that at our highest test concentration (5 µg/ml), which is still in the therapeutic range, the binding to plasma proteins is still not completely saturated. This would be in agreement with the observations of Urien *et al.* [13], who stated that the binding of docetaxel to α_1-acid glycoprotein and albumin was only saturated at very high docetaxel concentrations, above those encountered *in vivo*, and that binding to γ-globulins and lipoproteins was even unsaturable. They also reported substantial erythrocyte binding of up to 28% in a washed erythrocyte experiment and only 13% in total blood because of trapping of docetaxel by plasma proteins [13]. Surprisingly, binding of docetaxel to RBCs was found to be around 35% in our study, which is somewhat higher than expected, considering its high affinity for plasma proteins. Assuming that the MESED device preserves the physiological equilibrium between plasma proteins and erythrocytes, there is no obvious explanation for the difference with the 13% RBCs binding in whole blood described by Urien *et al.* Both our study and that of Urien *et al.* investigated the blood of healthy volunteers, but even so we cannot exclude variations in plasma protein composition, which may be responsible for the wide range in the extent of docetaxel binding to RBCs (from 2 to 144%).

The partition ratios of gemcitabine varied from 1 to 5 in the mono incubations, indicating a high affinity of gemcitabine for the RBCs, probably because of the low plasma protein binding of this drug, assuming that only the free fraction can be incorporated in the erythrocytes.

The partition ratio increased significantly with higher whole-blood concentrations (Fig. 3, $p = 0.038$), favoring uptake in the erythrocytes when plasma protein binding is saturated [12]. The free fraction will be studied in further experiments to support this hypothesis.

The influence of gender cannot be due to the difference in hematocrit between men and women, since the concentrations in the RBCs were measured after separation with the MESED device, providing exactly 100-µl RBC samples in every case. The higher affinity of docetaxel for the female erythrocyte and of gemcitabine for the male one should be attributed to intrinsic differences between the RBCs of both sexes.

In the combination incubations, gemcitabine did not alter the partition ratios of docetaxel, a finding consistent with the data of our phase I study [2].

We were unable to detect any influence of docetaxel (including the vehicle polysorbate 80) on the partitioning of gemcitabine when comparing the whole sample pool (unpaired *t*-test: $p = 0.60$). We therefore cannot explain the lower gemcitabine plasma concentrations following docetaxel pre-treatment in our phase I study (Table 1) by an altered partitioning of gemcitabine in the distribution phase. By subanalysis of our combination incubations, however, we found significantly more gemcitabine in male erythrocytes after docetaxel exposure versus the inverse sequence of incubation ($n = 10$; $p = 0.02$). In women, exactly the opposite was apparent; less gemcitabine in the erythrocytes after incubation with docetaxel ($n = 10$; $p = 0.001$).

In our phase I docetaxel and gemcitabine combination study, the decrease in plasma concentration of gemcitabine during the distribution phase only reached the level of significance when we compared the gemcitabine concentrations of the whole study population before and after docetaxel administration ($p = 0.012$) and when we compared both concentrations in women ($p = 0.010$). In men, the decrease in gemcitabine concentration following docetaxel administration was not significant compared to the concentration on day 1; it was significant when the gemcitabine mono administration on day 8 (in the first study cohort) was included in the analysis.

In order to further explore the apparent differences between males and females, we performed a subanalysis of two cohorts of our phase I study (IIIa + IV) with an equal ratio of females versus males. The mean gemcitabine plasma concentration in female patients before docetaxel administration was around 10% below the mean of male patients. However, pre-treatment with docetaxel increased this difference between male and female patient plasma concentrations to 33%. Both differences failed to reach the level of significance. Assuming a change of partition between RBCs and plasma, the *in vivo* situation in males can be explained by the present *in vitro* data; following docetaxel administration, relatively more gemcitabine is present in the male erythrocytes compared to plasma. In females, the opposite holds true. Nevertheless, the data from both studies strongly indicate an influence of docetaxel on the handling of gemcitabine by RBCs. It remains to be seen whether concomitant substances in the formulation of the taxane (polysorbate 80) are responsible, or docetaxel itself [14].

In conclusion, the RBC pool contains relatively more docetaxel and gemcitabine at higher incubation concentrations. Erythrocytes can play an important role in the transport of drugs because of their number and long lifetimes (120–140 days). The importance of the cellular uptake of cytotoxic drugs has also been recognized with respect to tumor kill. The incorporation of drugs into RBCs may occur by passive diffusion, active transport or linkage to RBC surface proteins. They may be transported to tumor tissue and mobilized from the erythrocytes by different active or passive transport mechanisms. The role of gender differences in the behavior of erythrocytes with respect to both drugs is not yet clear. However, the clinical relevance of these findings to treatment outcome remains uncertain and needs further investigation.

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