In vitro pharmacokinetic study of the novel anticancer agent E7070: red blood cell and plasma protein binding in human blood

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E7070 is a novel sulfonamide anticancer agent that arrests the G_1/S phase of the cell cycle. Preclinical and phase I studies have demonstrated non-linear pharmacokinetics (PK) of the drug. A population PK analysis revealed that the human plasma concentration–time data were best described by a three-compartment model with non-linear distribution. We have studied the *in vitro* interaction of ^{14}C-radiolabeled E7070 with red blood cells (RBC) and its binding to plasma proteins in the concentration range where non-linearity in disposition was observed in humans to get more insight into the behavior of the drug. After the addition of E7070 to whole blood at 37°C, the drug is taken up or binds to RBC in a concentration-dependent manner. The addition of sodium azide, however, did not result in a decrease of drug uptake by RBC, indicating passive diffusion processes. A non-linear increase in drug uptake was observed at incubation concentrations above 4 μg/ml E7070 in whole blood. This non-linearity was confirmed by lower partition coefficients between RBC and plasma at higher incubation concentrations (from 2.37 at 4 μg/ml to 0.31 at 200 μg/ml). The plasma protein binding of E7070 was high (98–99%) and linear in the concentration range studied (20–200 μg/ml). In conclusion, E7070 in whole blood is preferentially bound to RBC and exhibits high plasma protein binding. The non-linear distribution of E7070 in humans can be caused, in part at least, by saturable binding of E7070 to RBC. *Anti-Cancer Drugs* 14:405–410 © 2003 Lippincott Williams & Wilkins.

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Introduction

Several sulfonamide derivatives have been synthesized to develop novel cytotoxic agents against solid tumors [1,2]. Sulfonamides are well known to have a variety of pharmacologic activities including antibacterial, carbonic anhydrase inhibitory, antidiabetic, diuretic and antithyroid [3]. The novel sulfonamide derivative E7070 [*N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide] arrests the transition of the G_1/S phase of the cell cycle by inhibiting the phosphorylation of cyclin E and activation of cyclin-dependent kinase 2 [1,2,4,5]. E7070 exhibited a potent antitumor activity in murine and human tumor cell lines, and in human xenograft studies. The highest antitumor activity was observed in colorectal and lung cancer xenografts (HCT116 colorectal cancer and LX-1 lung cancer models) [6].

Phase I trials of E7070 in patients with solid tumors using four different infusion schedules revealed non-linear pharmacokinetics (PK) of the drug. There was a pronounced disproportional increase of the area under the concentration–time curve with dose [7,8]. A population PK analysis of E7070 in phase I studies ($n = 143$) revealed that the concentration–time data could best be fitted to a three-compartment model with saturable distribution to one compartment, and both linear and Michaelis–Menten elimination from the central compartment [9].

In general, non-linear drug distribution can be caused by saturable plasma protein binding, saturable binding to blood cells, or saturation of tissue uptake or binding sites [10]. Sulfonamide agents are well known to bind to carbonic anhydrase, an enzyme abundant in red blood cells (RBC) [11–13]. We hypothesized that non-linear binding of E7070 either to RBC or to plasma proteins may explain its PK behavior. Therefore, we studied the *in vitro* distribution of E7070 in human whole blood and human plasma protein binding within the concentration range observed during the phase I studies.

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Methods

Materials

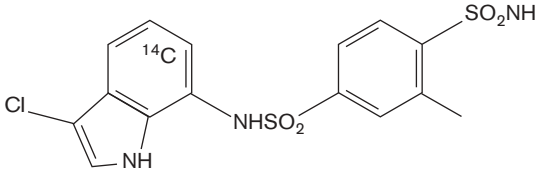
E7070 was supplied by Eisai (London, UK). The ^{14}C-radiolabeled E7070 compound ([indole ring-U-^{14}C]ER-35744, Fig. 1) was manufactured by Amersham International (Little Chalfont, UK). The ^{14}C-radiolabeled compound was dissolved in saline to a final concentration of 1 mg/ml. The radiochemical purity and the chemical purity were at least 98%. The radiolabeled drug was stored at –20°C until used. Isotonic phosphate-buffered saline (PBS, pH 7.4) was prepared. 'Solvable' was purchased from Packard Bioscience (Groningen, The Netherlands). EDTA (Titriplex) and hydrogen peroxide (Perhydrol) were purchased from Merck (Darmstadt, Germany).

Fresh whole blood samples from seven drug-free healthy volunteers were collected from an antecubital vein into heparinized tubes. Additional blood samples were taken from these volunteers to determine the hematological and chemical parameters of the collected blood.

Uptake of ^{14}C-radiolabeled E7070 in RBC

The uptake of E7070 in RBC was studied at various drug concentrations. Uptake studies were performed *in vitro* by incubation of whole blood with ^{14}C-radiolabeled E7070 in 50 ml Falcon tubes in a water bath at 37°C ($n = 6$) and at approximately 0°C (melting ice water, $n = 6$) on a plate with gentle shaking. After pre-incubation of whole blood for 10 min, radiolabeled compound was added to yield a final concentration of 200 μg/ml and incubated for 24 h to determine the time when distribution equilibrium was achieved between plasma and RBC. To determine whether the E7070 uptake in RBC was an adenosine triphosphate (ATP)-dependent transport, the effect of sodium azide on uptake was determined at 37°C (in duplicate). Sodium azide produces a decrease in the intracellular ATP concentration, and was added to give a final concentration of 20 mM, which is sufficient to inhibit ATP-dependent systems [14,15]. Whole blood samples (2.5 ml) were taken immediately after the addition of ^{14}C-radiolabeled E7070, at 30 and 60 min, and at 3, 7 and 24 h after the addition of ^{14}C-radiolabeled E7070. After the whole blood samples were taken, they were immediately centrifuged at approximately 1610 *g* for 5 min (at 4°C). Then, 50 μl of plasma was transferred to a plastic vial. The rest of the plasma layers and the buffy coats with the leukocytes were carefully removed with sufficient margins, followed by transfer of 200 μl of RBC in 15-ml Falcon tubes. After one wash step of the RBC with ice-cold isotonic PBS wash-solvent (1800 μl), the RBC were centrifuged at approximately 1610 *g* for 15 min (at 0°C), and the supernatant was discarded. RBC samples (200 μl) were dissolved and decolorized using Solvable (1 ml), 0.1 M EDTA (100 μl) and hydrogen peroxide (500 μl).

Fig. 1



Chemical structure of ^{14}C-radiolabeled E7070 ([indole ring-U-^{14}C]ER-35744).

This experiment was followed by the incubation of 2 ml of whole blood without sodium azide (in triplicate) with ^{14}C-radiolabeled E7070 at concentrations in the range of 0 (blank), 4, 20, 50, 100, 150 and 200 μg/ml in 15-ml Falcon tubes in a water bath at 37°C or at approximately 0°C (melting ice water) on a plate with gentle shaking. All whole blood samples were centrifuged (at 1610 *g* for 5 min at 4°C) after the equilibrium between plasma and RBC was attained as determined in the previous experiment. After the centrifugation, 100 μl of plasma was transferred from each Falcon tube to a plastic vial, and the rest of the plasma layers and buffy coats were carefully removed with sufficient margins. This was followed by transfer of 500 μl of the RBC samples in 15-ml Falcon tubes. After one wash step of the RBC with ice-cold isotonic PBS wash-solvent (4500 μl), the RBC were centrifuged at approximately 1610 *g* for 15 min (at 0°C) and the supernatant was discarded. Then, 200 μl of the washed RBC samples was dissolved and decolorized using Solvable (1 ml), 0.1 M EDTA (100 μl) and hydrogen peroxide (500 μl) in a plastic vial.

Efflux of ^{14}C-radiolabeled E7070 from RBC to plasma

After one wash step of the RBC with ice-cold isotonic PBS, the RBC were incubated (in triplicate) with ^{14}C-radiolabeled E7070 to yield final concentrations of 0 (blank), 6, 50 and 100 μg/ml in 15-ml Falcon tubes in a water bath at 37°C with gentle shaking. After 2 h of incubation, 4 ml of blank plasma was added to each Falcon tube with the incubated RBC. At each incubation concentration, one sample (2 ml) was taken just before the addition of blank plasma, and samples (2 ml) were taken at 20 min, and 3 and 24 h after the addition of blank plasma. Samples were centrifuged at 1610 *g* for 5 min (at 4°C). After centrifugation, 100 μl of plasma was transferred from each Falcon tube to a plastic vial, and the rest of the plasma layers and buffy coats were carefully removed with sufficient margins. Then, 200 μl of the RBC samples was transferred to 15-ml Falcon tubes. After one wash step of the RBC with ice-cold isotonic PBS wash-solvent (1800 μl), the RBC were centrifuged at approximately 1610 *g* for 15 min (at 4°C), and the supernatant was discarded. Then, 200 μl of the washed

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RBC samples was dissolved and decolorized using Solvable (1 ml), 0.1 M EDTA (100 µl) and hydrogen peroxide (500 µl) in a plastic vial.

Plasma protein binding of ^{14}C-radiolabeled E7070

The binding of E7070 to plasma proteins was determined by the ultrafiltration method after incubation of plasma with ^{14}C-radiolabeled E7070 in a gently shaking water bath. The entire procedure was conducted at 37°C (in triplicate). Amicon micropartition systems (Amicon, Danvers, MA) with membrane disks with a cut-off level of 30 kDa (Millipore; Amicon, Beverly, MA) to separate bound from free E7070 in plasma. Preliminary experiments revealed negligible binding to the ultrafiltration device (3%). After pre-incubation of plasma for 10 min, ^{14}C-radiolabeled E7070 was added to plasma to yield a final concentration of 200 µg/ml and incubated for 24 h in 50 ml Falcon tubes to determine the ratio between the total and free E7070 concentrations in plasma, with time. Plasma samples were taken just before the addition of ^{14}C-radiolabeled E7070, at 5, 10, 20, 30, 45 and 60 min, and at 2, 3, 4, 5, 6, 7 and 24 h after the start of the incubation. After the plasma samples (50 and 100 µl) were taken and transferred to a plastic vial, 1 ml plasma samples were immediately transferred into ultrafiltration devices in duplicate. The plasma samples were ultracentrifuged at approximately 1118 *g* for 15 min at room temperature and approximately 200 µl of plasma ultrafiltrate was obtained; 100 µl of plasma ultrafiltrate was transferred to a plastic vial.

After establishing the time when equilibrium is achieved between the total and free drug concentration in plasma in the previous experiment, plasma was incubated (in triplicate) with ^{14}C-radiolabeled E7070 in a concentration range of 20–200 µg/ml and at 0 µg/ml. Plasma samples were taken after the time to reach equilibrium between the total and free drug concentration in plasma.

At each incubation concentration, the plasma samples (50 and 100 µl) were taken and transferred to a plastic vial. The rest of the plasma was immediately transferred to two ultrafiltration devices and centrifuged at approximately 1118 *g* for 15 min at room temperature. Approximately 200 µl of plasma ultrafiltrate was obtained; 100 µl of plasma ultrafiltrate was transferred to a plastic vial.

Bio-analysis

The detection of β radiation in plasma, RBC and PBS wash solvent was performed by a liquid scintillation counter (LSC) (Tri-CARB 2100 CA; Packard, Meriden, CT) with an energy range of 0–2000 keV on the same day as the experiments. Each sample was mixed with 10 ml of Ultima Gold cocktail (Packard) in a plastic vial. The counting time was 5 min per vial.

The samples were analyzed together with calibration standards and quality control (QC) standards in the LSC. Calibration curves were fitted using least-squares regression analysis. According to the disintegration per minute (d.p.m.) level in the *in vitro* samples and the QC control samples as determined by the LSC, the equation of the calibration curve was used to calculate the E7070 concentration in all samples. The calibration standards consisted of 1, 2, 4, 10, 20, 40, 100, 200, 400 and 1000 µg/ml E7070 dissolved in 0.9% NaCl. The QC standards consisted of 20, 100 and 500 µg/ml E7070 dissolved in 0.9% NaCl. Results of batches analyzed were accepted if at least two of the three QC standards were within ± 20% of their respective nominal values. Sample analysis was performed in duplicate and was repeated if the deviation in measured d.p.m. was more than 10%.

Data analysis

A partition coefficient value between RBC and plasma ($P_{RBC/plasma}$) was defined, and used as an indicator for the distribution of E7070 between the RBC and plasma under the various tested conditions. The $P_{RBC/plasma}$ was calculated by the ratio of the E7070 concentration in RBC (C_{RBC}) and plasma (C_p): $P_{RBC/plasma} = C_{RBC}$ (µg/ml)/C_p (µg/ml).

The uptake of E7070 to RBC was calculated as the E7070 fraction in whole blood bound to RBC (F_{RBC}) taking into account the differences in hematocrit level (Ht) of the blood samples. $F_{RBC} = [C_{RBC}$ (µg/ml) × Ht]/[C_{RBC} (µg/ml) × Ht + C_p (µg/ml) × (1 – Ht)].

The plasma protein binding of E7070 was calculated by dividing the free (C_f) and total (C_p) E7070 concentrations in plasma. Plasma protein binding (%) = [C_p – C_f (µg/ml)/C_p (µg/ml)] × 100.

Statistical analysis

Statistical analysis was performed with SPSS for Windows, version 10.0.7 (SPSS, Chicago, IL).

Differences between the RBC uptake under different conditions were determined using Student's *t*-test with a significance level of < 0.05.

Results

The E7070 concentrations in plasma and RBC were calculated from the radioactivity measurements. No degradation of radioactivity was observed in human RBC and plasma during a 24-h incubation at 37°C. Validation experiments of the coupled liquid chromatography–tandem mass spectrometry method to determine the E7070 concentrations in human plasma, urine and feces showed that E7070 was stable for 24 h at room temperature [16]. Consequently, we assumed that the radioactivity determinations are an accurate reflection of

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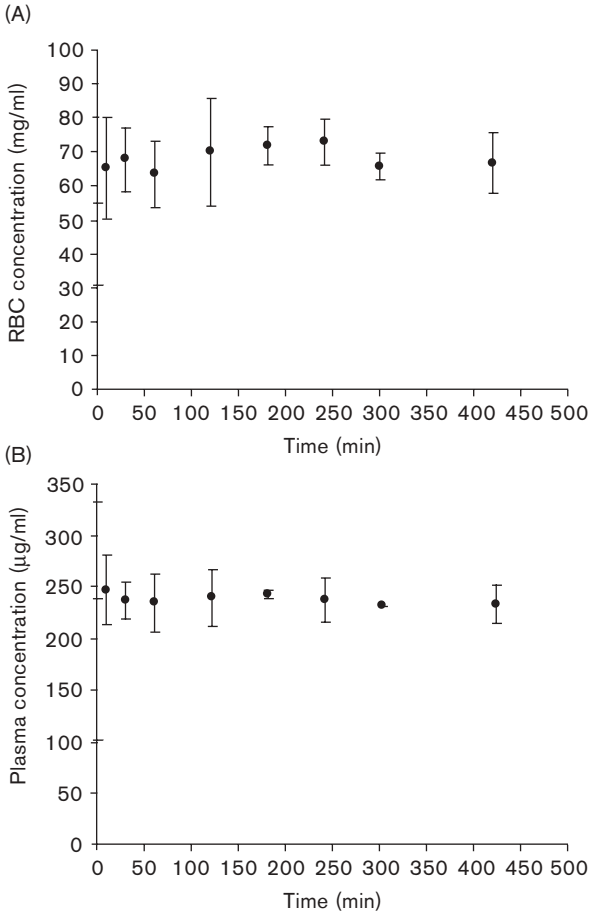
the E7070 concentrations in human RBC and plasma in this study.

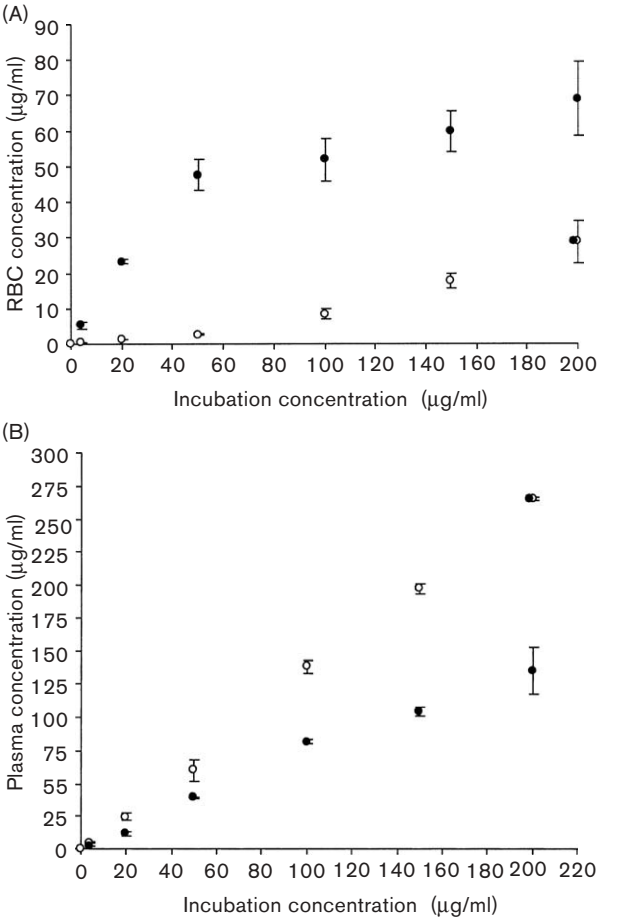
After the addition of E7070 to whole blood *in vitro* at 37°C the drug was taken up by RBC from plasma (Fig. 2). The addition of sodium azide to deplete ATP did not result in a significant inhibition of the uptake (data not shown). It was impossible, however, to quantify the ATP depletion (caused by the addition of sodium azide to whole blood) by a luciferase assay due to interference by hemoglobin. Previous reports indicate that a sodium azide concentration of 20 mM would be sufficient to deplete ATP in RBC [14]. Equilibrium between the E7070 concentration in RBC and plasma was achieved within 2 h at 37°C. After the incubation of whole blood with various E7070 concentrations for 2 h at 37°C, the E7070 concentration in RBC achieved a plateau at higher incubation concentrations (Fig. 3A). In contrast, the plasma concentration continued to increase at higher incubation concentrations (Fig. 3B). Simultaneously, a non-linear increase of the E7070 concentration in PBS (that was used in the washstep of RBC) was observed at higher incubation concentrations in whole blood (data not shown). This indicates that at higher incubation concentrations of E7070 in whole blood, more E7070 was lost in the wash step. This may be explained by non-linear binding of E7070 to other components of whole blood (excluding plasma) at higher incubation concentrations, e.g. leukocytes or platelets.

Fig. 2



E7070 concentration in RBC (A) and in plasma (B) during the incubation with 200 µg/ml E7070 in whole blood at 37°C *in vitro*. Each value is the mean of six experiments.

Fig. 3



E7070 uptake in RBC (A) and E7070 concentration in plasma (B) following incubation with E7070 in whole blood for 2 h at 37°C (solid symbols) and at approximately 0°C (open symbols). Each value is the mean ± SD of three experiments.

The drug uptake in RBC was significantly decreased at approximately 0°C compared to 37°C with a corresponding higher E7070 concentration in plasma at 0°C compared to 37°C (Fig. 3).

The *in vitro* partition coefficient ($P_{RBC/plasma}$) between RBC and plasma decreased at higher incubation concen-

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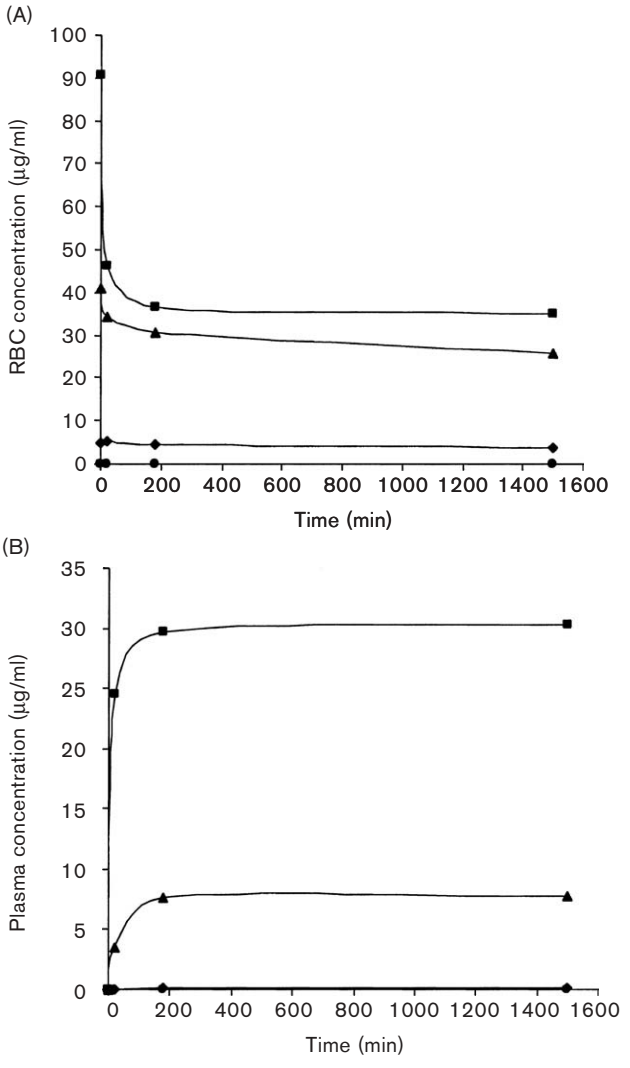
Table 1 The *in vitro* partition coefficient between RBC and plasma ($P_{RBC/plasma}$) and the fraction of E7070 in RBC following incubation in whole blood for 2 h at 37°C ($n = 3$)

Incubation concentration (μg/ml)	$P_{RBC/plasma}$ (mean ± SD)	Recovery in RBC (%) (mean ± SD)
4	2.37 ± 0.42	61.8 ± 6.3
20	2.03 ± 0.28	58.2 ± 5.4
50	1.22 ± 0.10	45.8 ± 4.0
100	0.48 ± 0.07	25.0 ± 3.3
150	0.34 ± 0.02	19.1 ± 0.5
200	0.31 ± 0.04	17.6 ± 0.5

Fig. 4



The *in vitro* E7070 efflux from RBC (A) to plasma (B) at various concentrations at 37°C: 0 (circles), 6 (diamonds), 50 (triangles) and 100 (squares) μg/ml. Each value is the mean of three experiments.

trations at 37°C (Table 1). Since the hematocrit of the blood samples ranged from 0.39 to 0.43, the E7070 recovery percentage in whole blood bound to RBC following incubation for 2 h at 37°C was determined.

Table 2 Plasma protein binding of E7070 after incubation of plasma with E7070 for 30 min at 37°C ($n = 3$)

Incubation concentration (μg/ml)	Mean plasma protein binding ± SD (%)
20	98.0 ± 0.66
50	98.8 ± 0.16
100	99.0 ± 0.05
150	99.0 ± 0.16
200	99.0 ± 0.07

This percentage decreased from 61.8 ± 6.3% at 4 μg/ml to 17.6 ± 0.5% at 200 μg/ml (Table 1). These data confirm that there is a non-linear increase of the drug uptake into RBC at higher drug concentrations. The results of the efflux experiments are summarized in Fig. 4. The E7070 efflux from RBC to plasma takes place in a concentration- and time-dependent manner.

E7070 showed a high plasma protein binding (98–99%) which was constant over the measured time period of 24 h after the addition of 200 μg/ml E7070 to plasma (data not shown). The *in vitro* binding was independent of the incubation concentration over a wide range of 20–200 μg/ml after 30 min of incubation with E7070 (Table 2).

Discussion

The results of these *in vitro* incubation experiments with E7070 in human blood revealed that the equilibrium between RBC and plasma is reached within approximately 2 h. The addition of sodium azide caused no significant decrease of the E7070 uptake in RBC. We may thus conclude that the influx of E7070 from plasma into RBC occurs by passive diffusion. The E7070 uptake in RBC occurred in a concentration-dependent manner and RBC became saturated in the concentration range studied (4–200 μg/ml). This non-linearity also appears from the lower mean partition coefficient values between RBC and plasma (2.37 at the 4 μg/ml to 0.31 at the 200 μg/ml concentration), and the lower E7070 fraction in RBC in whole blood (61.8 ± 6.3% at 4 μg/ml to 17.6 ± 0.5% at 200 μg/ml) at higher incubation concentrations. This indicates that E7070 is preferentially bound to RBC when present in whole blood, and that the distribution in the RBC compartment can become saturated. The protein binding of E7070 in plasma was high (98–99%) and showed no signs of non-linearity or saturation in the concentration range of 20–200 μg/ml.

From these experiments we can conclude that the non-linear distribution of E7070 in blood is probably, in part at least, caused by a saturable distribution to the RBC compartment. Sulfonamide agents bind to carbonic anhydrase, an intracellular enzyme that is present throughout the body, although RBC account for more than 90% of this enzyme [11–13]. Recently, several novel

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sulfonamides with high affinity for the enzyme carbonic anhydrase were synthesized that resemble E7070 and showed antitumor activity [12]. It is possible that E7070 binds to carbonic anhydrase and this binding becomes saturated when blood concentration of drug is higher than the available binding sites of the enzyme in the RBC. This can result in a non-linear distribution of E7070 in the RBC compartment, as has been described for another sulfonamide agent, the carbonic anhydrase inhibitor MK-417 [11].

Recently, a mass balance study of ^{14}C-radiolabeled E7070 in patients with solid tumors has been completed in our institute [17]. The maximal concentration of total radioactivity in plasma was higher compared to that in RBC during and shortly after the infusion of ^{14}C-radiolabeled E7070. At later time-points the radioactivity concentration in both plasma and RBC were in the same range. Consequently, the exposure to radioactivity in plasma was higher than in RBC. We assume that the binding sites of the enzyme carbonic anhydrase in RBC become saturated when relatively high E7070 levels are reached, during and shortly after infusion. Moreover, according to this observation in the mass balance study, and considering the results of the *in vitro* E7070 efflux experiments, we may conclude that E7070 will diffuse from the RBC into plasma during the elimination process in humans. The drug in RBC may not be easily available for extraction by the eliminating organs (i.e. the kidneys and the liver), and the expression of the pharmacologic activity may be influenced [18,19]. We observed no degradation of radioactivity in RBC during 24 h of incubation with ^{14}C-radiolabeled E7070. However, we do not know whether E7070 alone or E7070 and its metabolites contribute to this radioactivity. Consequently, we cannot exclude that there might be metabolism of E7070 in the RBC.

In conclusion, E7070 is preferentially bound to RBC in whole blood. This binding is a saturable process at higher concentrations in whole blood. There is no evidence of saturation of protein binding of E7070 in plasma at the concentrations tested. It is postulated that the binding site of E7070 within the RBC is the enzyme carbonic anhydrase.

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