

# Saturable Binding of Indisulam to Plasma Proteins and Distribution to Human Erythrocytes

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**DMD #8326**

**Running title**

Saturable blood distribution of indisulam

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Number of text pages (Abstract-Acknowledgments): 16

Number of tables: 2

Number of figures: 7 (1,2,3,4A,4B,5,6)

Number of references: 26

Number of words in Abstract: 250

Number of words in Introduction: 617

Number of words in Discussion: 884

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### Abstract

The anti-cancer agent indisulam has a non-linear pharmacokinetic profile, which may be partly related to saturable binding to blood constituents. To gain insight into the complex non-linear behavior of indisulam, we investigated binding to plasma proteins and erythrocytes. The purpose of the study was to develop a physiological model for the distribution of indisulam in blood. Concentrations of radiolabeled indisulam were measured in vitro 1) in total plasma and in ultrafiltrate to investigate plasma protein binding, 2) in erythrocytes and in plasma to investigate distribution to erythrocytes, 3) in erythrocyte membranes to investigate non-specific binding in erythrocytes. For in vivo assessment, 21 patients received 400-900mg/m<sup>2</sup> indisulam in a one- or two-hour infusion. Total and free concentrations in plasma and concentrations in erythrocytes were determined at multiple time points. In vitro plasma protein binding was described by a Langmuir model with a maximal binding capacity ( $B_{\max}=767\ \mu\text{M}$ ) and an equilibrium dissociation constant ( $K_D=1.02\ \mu\text{M}$ ). The maximal capacity of plasma protein binding in vivo corresponded to albumin levels. The bound concentration in erythrocytes was described by a two site model, comprising a saturable and a nonspecific binding component. The saturable component ( $B_{\max}=174\ \mu\text{M}$ ) may correspond to binding to carbonic anhydrase. The physiological model adequately described the non-linear disposition of indisulam in whole blood. Indisulam was bound to plasma proteins and distributed to erythrocytes in a saturable manner. These saturable processes may be attributed to binding to albumin (in plasma) and to carbonic anhydrase (in erythrocytes).

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### **Introduction**

Indisulam (*N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide, E7070) is a synthetic sulfonamide derivative, which is being developed as an anti-cancer agent. The compound has anti-tumor activity *in vitro* and *in vivo* against a wide variety of human tumor cell lines and xenografts.(Ozawa et al., 2001) In the A549 human non-small cell lung cancer cell line, treatment with indisulam leads to disruption of the cell cycle progression in the G1/S boundary accompanied by hypophosphorylation of the retinoblastoma protein and a reduction in the expression of cyclins A, B1 and cyclin-dependent kinase 2. Indisulam was also associated with upregulation of p53 and p21 and subsequent apoptosis.(Fukuoka et al., 2001) As shown by recent studies, indisulam is a potent inhibitor of cellular dehydrogenases. Hence, downstream events such as G1/S arrest and cell death are likely to occur due to interference with the malate-aspartate shuttle, glycolysis and gluconeogenesis.(Oda et al., 2003) The precise mechanism of action remains, however, under investigation.

In a phase I clinical program, indisulam was administered in four different treatment schedules (single one-hour infusion every 3 weeks (Raymond et al., 2002), daily times 5 one-hour infusion every 3 weeks (Punt et al., 2001), weekly times 4 one-hour infusion every 6 weeks (Dittrich et al., 2003), continuous infusion over 5 days every 3 weeks (Terret et al., 2003)). Phase II clinical studies are currently ongoing to evaluate the efficacy of indisulam as a single agent and in combination with standard therapies. Indisulam had moderate single agent anti-tumor activity in patients with colorectal cancer, metastatic breast cancer, head and neck cancer, non-small cell lung cancer, renal cell cancer or metastatic melanoma who did not respond to previous chemotherapy.(Fumoleau et al., 2003; Haddad et al., 2004; Mainwaring et al., 2002; Raftopoulos et al., 2004; Smyth et al., 2005; Talbot et al., 2002) The pharmacokinetic profile of indisulam is highly non-linear, characterized by increasing clearance with decreasing plasma concentration. Systemic exposure (expressed as the area under the plasma concentration versus time curve) has been shown to increase disproportionately with dose in four phase I studies.(Dittrich et al., 2003; Punt et al., 2001; Raymond et al., 2002; Terret et al., 2003) A population pharmacokinetic model has been developed previously.(van Kesteren et al., 2002) This empirical model comprised saturable distribution and two elimination pathways: a linear pathway and

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a saturable pathway. It adequately described the individual pharmacokinetic profiles for all four treatment schedules tested in phase I. It has been postulated that metabolic products of indisulam may be formed through saturable enzymatic processes, which may underlie the saturable elimination pathway.(van Kesteren et al., 2002) Furthermore, it has been suggested that the saturable distribution pathway may be related to distribution to erythrocytes.(van Kesteren et al., 2002) This latter hypothesis was supported by the results of an *in vitro* pharmacokinetic study, which demonstrated that the partition coefficient between erythrocytes and plasma of indisulam decreases with increasing incubation concentration.(van den Bongard et al., 2003) However, conclusive physiological explanations for the observed non-linear pharmacokinetic processes have not been given yet.

Reversible neutropenia and thrombocytopenia were the major and dose-limiting toxicities of indisulam.(Dittrich et al., 2003; Punt et al., 2001; Raymond et al., 2002; Terret et al., 2003) It has clearly been demonstrated that hematological toxicity is related to the exposure to indisulam.(van Kesteren et al., 2005) The non-linear pharmacokinetics of this drug may be an important factor in the observed variability in pharmacokinetics and hematological toxicity. Therefore, we considered it of crucial importance to characterize the non-linear properties of this drug. The objective of the current study was to develop a non-dynamic physiological model to describe the *in vitro* distribution of indisulam in whole blood. This was identified as an important step for the full elucidation of the non-linear pharmacokinetic profile of indisulam.

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### Materials and methods

Three *in vitro* experiments were conducted to investigate plasma protein binding (exp. 1), uptake in erythrocytes (exp. 2) and non-specific binding to erythrocyte membranes (exp. 3). The combined results were used to define a physiological model for the distribution of indisulam *in vitro* in whole blood. Plasma protein binding and uptake in erythrocytes were also assessed in a clinical study. *In vivo* applicability of the model was evaluated using data from 21 patients.

In all *in vitro* experiments, carbon-14 ( $^{14}\text{C}$ -) radiolabeled indisulam (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) was used and concentrations of  $^{14}\text{C}$ -indisulam were measured using liquid scintillation counting. The concentration ranges used for the *in vitro* experiments corresponded to clinically observed plasma concentrations of indisulam.

#### *In vitro* plasma protein binding (exp. 1)

Total and free plasma concentrations of  $^{14}\text{C}$ -indisulam in plasma were measured *in vitro*, at 6 concentration levels in triplicate. The difference between total and free concentrations corresponded to the concentration of  $^{14}\text{C}$ -indisulam bound to plasma proteins. The relationship between the free plasma concentration ( $C_{\text{free}}$ ) and the protein bound fraction ( $C_{\text{bound}}$ ) of indisulam was examined.

Fresh whole blood samples from 3 male healthy volunteers were collected from an antecubital vein into heparinized tubes. Blood samples were centrifuged at 3000 rpm for 10 minutes, at 4°C to obtain plasma. A 52 mM stock solution of  $^{14}\text{C}$ -indisulam in DMSO (radiochemical purity 97.5-98.1%) was prepared. This was diluted with DMSO to obtain  $^{14}\text{C}$ -indisulam working solutions of 0.26, 0.52, 5.2, 13, 26 and 39 mM. Five  $\mu\text{L}$  of  $^{14}\text{C}$ -indisulam DMSO working solutions of 0.26, 0.52, 5.2, 13, 26, 39 and 52 mM were added to 0.495 ml of human plasma (final drug concentrations: 0.26, 0.52, 5.2, 13, 26, 39 and 52  $\mu\text{M}$ ). For a control sample, 5  $\mu\text{L}$  of  $^{14}\text{C}$ -indisulam DMSO solution (0.26 mM) were added to 0.495 ml of phosphate buffered saline (pH=7.4, PBS) and mixed (final concentration: 2.6  $\mu\text{M}$ , which was expected to be in the range of free drug concentrations in the plasma samples).

Equilibrium dialysis was performed in 1 ml modules (Yazawa Kagaku Co. Ltd., Tokyo, Japan) fitted

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with Seamless Cellulose Tubing (UC20-32-100, Size: 20/32) with a molecular weight cut off of 12000~14000 dalton (Sanko Junyaku Co. Ltd., Tokyo, Japan). Each sample (0.5 ml) was equilibrated against 0.5 ml of PBS at room temperature for 24 hours. Plasma and PBS were sampled from each side of the cell to determine radioactivity of  $^{14}\text{C}$ -indisulam in each matrix using the Liquid Scintillation Analyzer LSA-2700TR (Packard Co., Ltd., Tokyo, Japan). Fifty  $\mu\text{l}$  plasma were dissolved in 1 ml of a solution containing tissue solubilizer Soluene-350 (Packard, Meriden, USA) and 2-propanol (1:1). After storage for a day at room temperature, 15 ml of hydrophilic scintillator (Instagel, Packard, Meriden, USA) containing 10% 0.5 M HCl (v/v) were added to the sample and radioactivity was measured. To 50  $\mu\text{l}$  of PBS, 15 ml of hydrophilic scintillator (ACS II, Amersham) were directly added and radioactivity was measured.

### *In vitro distribution to erythrocytes (exp. 2)*

The uptake of indisulam in erythrocytes was studied at various incubation times and at various concentrations as previously described in detail by Van den Bongard et al. (van den Bongard et al., 2003) Concentrations of indisulam were measured in plasma and in erythrocytes. The fraction of indisulam in erythrocytes versus the total incubation concentration was investigated. For implementation in a physiological distribution model, the relationship between the free concentration (calculated from the total plasma concentration, using the data from exp. 1) and the concentration of indisulam bound to erythrocytes was established in the current analysis.

In brief, whole blood from healthy volunteers was incubated at  $37^{\circ}\text{C}$  with  $^{14}\text{C}$ -indisulam at a final concentration of  $518\ \mu\text{M}$  ( $n=6$ ). Samples were taken immediately after the addition of  $^{14}\text{C}$ -indisulam, at 10 and 30 minutes and at 1, 2, 3, 4, 5, 6, 7, 8 and 25 hours after the addition of  $^{14}\text{C}$ -indisulam.

Another series of whole blood samples was incubated at  $37^{\circ}\text{C}$  with  $^{14}\text{C}$ -indisulam at concentrations of 10.4, 51.8, 130, 259, 389,  $518\ \mu\text{M}$  ( $n=3$ ) during 2 hours. After centrifugation, plasma (50  $\mu\text{l}$ ) and erythrocytes were isolated from all samples. Erythrocytes (200  $\mu\text{l}$ ) were washed with ice-cold isotonic PBS and were dissolved and decolorized using Solvable<sup>TM</sup> (Packard, Groningen, The Netherlands) (1 ml), 0.1M EDTA (100  $\mu\text{l}$ ) and hydrogen peroxide (500  $\mu\text{l}$ ). Both plasma and erythrocyte samples were

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mixed with 10 ml of Ultima Gold cocktail (Packard, Meriden, USA) and the detection of beta radiation was performed by a liquid scintillation counter (Tri-CARB 2100 CA) (Packard, Meriden, USA).(van den Bongard et al., 2003)

### *In vitro binding to erythrocyte membranes (exp.3)*

This experiment was conducted to investigate binding of indisulam to erythrocyte membranes and to quantify the attribution of this process to total uptake in erythrocytes. For this objective, both the total concentration in erythrocytes and the membrane bound concentration were measured. Whole blood samples from 3 male healthy volunteers were collected from an antecubital vein into heparinized tubes. Unlabeled indisulam (Eisai Co., Ltd., Kashima, Japan) and  $^{14}\text{C}$ -indisulam (9:1) were dissolved in DMSO to obtain a 259 mM stock solution (radiochemical purity: 97.57–99.23%). The stock solution was diluted with DMSO in order to obtain working solutions of 2.6 and 26 mM. Five  $\mu\text{l}$  of indisulam solution (concentration 2.6, 26 and 259 mM) were added to 4.995 ml of whole blood of each volunteer and mixed (final concentration 2.6, 26 and 259  $\mu\text{M}$ ). Blood was incubated at 37°C in a water bath and samples of 2 ml were taken after 5 minutes and 6 hours incubation. Blood samples were immediately centrifuged at 3,000 rpm for 10 minutes at 4°C. Erythrocytes were washed twice with 4 ml of ice-cold saline and centrifuged at 2,000 rpm for 3 minutes at 4°C.

Twenty  $\mu\text{l}$  of washed erythrocytes were collected for measurement of the total erythrocyte concentration of indisulam. To 100  $\mu\text{l}$  of erythrocytes, 0.8 ml distilled water was added for hemolysis. After vigorous shaking, the suspension was centrifuged at 20,000 rpm for 20 minutes at 4°C (TL-100,100.3, Beckmann). The pellet (erythrocyte membrane) was washed with 1 ml of distilled water and then centrifuged at 20,000 rpm for 20 minutes at 4°C (TL-100,100.3, Beckmann). The erythrocyte membranes were dissolved in 1 ml of a solution containing tissue solubilizer Soluene-350 (Packard, Meriden, USA) and 2-propanol (1:1). Radioactivity was determined in triplicate samples of erythrocytes and erythrocyte membranes of each concentration and sampling time. In addition, radioactivity was measured in supernatant fractions, which were separated from the membranes. All samples were decolorized by the addition of 200  $\mu\text{l}$  of 30% hydrogen peroxide. After storage for a day



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at room temperature, 15 ml of a hydrophilic scintillator (Instagel, Packard) containing 10% 0.5 mol/L HCl (v/v) were added, and the radioactivity was measured using the Liquid Scintillation Analyzer LSA-2700TR (Packard Co., Ltd., Tokyo, Japan).

### *In vivo blood distribution*

As the final model should essentially describe the *in vivo* blood distribution of indisulam, binding to plasma protein and uptake in erythrocytes was assessed in patients. Pharmacokinetic data were obtained from a phase I dose-escalation study in Japanese patients.(Yamada et al., 2005) This study was performed after a larger phase I program in Caucasian patients had been finalized. The study was reviewed by the Medical Ethics Committee of the National Cancer Center Hospital in Tokyo (Japan) and was performed in accordance with the Declaration of Helsinki.(World Medical Association Declaration of Helsinki, 2002) All 21 eligible patients gave written informed consent prior to inclusion in the study. Indisulam was administered at 5 different dose levels (400 (n=3), 600 (n=3), 700 (n=6), 800 (n=6) and 900 (n=3) mg/m<sup>2</sup>). The lower doses (400 and 600 mg/m<sup>2</sup>) were administered as one-hour infusions and the higher doses as two-hour infusions. Pharmacokinetic assessment was performed according to a full sampling schedule. Total plasma concentrations and concentrations in erythrocytes of indisulam were measured in whole blood samples taken at 30 minutes after the start of infusion, at 0, 10 and 30 minutes after the end of infusion and at 1, 2, 4, 6, 10, 24, 48, 72, 96, 120, 168 and 240 hours after the end of infusion. From each patient, two additional samples were taken at the end of infusion and at 1 hour after the end of infusion for measurement of total and free plasma concentrations of indisulam. Whole blood samples were centrifuged at 3000 rpm for 10 minutes at 5°C. Plasma samples were transferred to a glass tube (0.50 ml) and to ultrafiltration devices (Centrifree) (Amicon Corporation, Danvers, USA) (0.95 ml). In order to obtain 0.50 ml of ultrafiltrate, samples were ultracentrifuged at 1670 g for 20-45 minutes. Plasma ultrafiltrate samples were transferred to a plastic vial (0.50 ml). Phosphate buffer (1.0 ml, 0.1 M, pH 6.8) was added to the plasma and plasma ultrafiltrate samples. Erythrocytes (0.50 ml) were frozen in methanol for 10 minutes and thawed at room temperature for 10 minutes. This freeze-thaw cycle was performed three

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times. Boric acid buffer (2.0 ml, 0.6 M, pH 6.8) was added and the mixture was boiled for 1 minute. Indisulam was extracted using ethylacetate. After centrifugation, the organic layer was transferred into a glass tube and evaporated under nitrogen. Samples were reconstituted in 0.20 ml of CH<sub>3</sub>CN / phosphate buffer (6.7 mM, pH 6.6) (360:640 v:v). The concentration of indisulam was measured by high pressure liquid chromatography with UV detection.(Yamada et al., 2005) The method was linear between 0.052 μM and 130 μM and the assay accuracy and precision were <15.5%. N-(3-chloro-7-indolyl)-4-(N-methylsulfamoyl)benzenesulfonamide (ER-67771) was used as an internal standard.(Owa et al., 1999)

### *Data analysis*

Data were analyzed using the NONMEM program (version V, level 1.1) (Globomax LLC, Hanover, USA).(Beal et al., 1988) The first-order conditional estimation (FOCE) method with interaction between interindividual, intraindividual and residual variability was applied. PDx-Pop (version 1.1j release 4) (GloboMax LLC, Hanover, USA) was used as an interface for data and output processing and for modeling management. The adequacy of the models was evaluated by graphical plots of observed versus model predicted concentrations and bound concentrations versus free concentrations (overlay of observed and model predicted values). Statistical discrimination between hierarchical models was based on the log-likelihood ratio test.

Results from *in vitro* studies were analyzed to define a model for distribution of indisulam in whole blood, comprising plasma protein binding and binding of indisulam to erythrocytes. For both of these components, models for linear binding kinetics (equation 1) and models for saturable binding equilibriums (equation 2) were tested.

$$C_{\text{bound}}=N*C_{\text{free}} \quad (\text{Eq.1})$$

$$C_{\text{bound}}=B_{\text{max}} *C_{\text{free}}/(C_{\text{free}}+K_D) \quad (\text{Eq.2})$$

In these equations, N is the non-specific binding constant, B<sub>max</sub> corresponds to the maximal binding capacity and K<sub>D</sub> is equilibrium dissociation constant. If the free concentration is equal to K<sub>D</sub>, the bound concentration is half-maximal.

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The physiological model was developed to describe the *in vitro* distribution in whole blood under equilibrium conditions. It was assumed that binding between indisulam and protein binding sites occurred instantaneously. Thus, the fractions bound and free indisulam within plasma and within erythrocytes were assumed to be in equilibrium at any moment. Free concentrations in plasma and in erythrocytes were not in equilibrium at any time, as distribution to erythrocytes was shown to be time dependent previously. Van den Bongard et al. have demonstrated that equilibrium was reached after 2 hours of incubation *in vitro*.(van den Bongard et al., 2003) Therefore, only results obtained after incubation for 2 hours or longer were considered in the current analysis.

Model development was conducted in a step-wise approach. Initially, a relationship between free plasma concentrations and protein bound plasma concentrations was established. Data from experiment 1 were used for this analysis. Measurement of free plasma concentrations was not performed in experiments 2 and 3. Therefore, the protein binding model was subsequently applied to total plasma concentrations that were measured in experiments 2 and 3 to calculate free plasma concentrations. *In vitro*, free concentrations in the liquid interior of erythrocytes were assumed to be equal to calculated free plasma concentrations. This may be reasonable because equilibrium conditions were ascertained. Finally, a model for binding of indisulam to erythrocytes was developed to describe the relationship between free and bound concentrations of indisulam in washed erythrocytes. Clinical data were used to evaluate the *in vivo* applicability of the non-dynamic physiological model. *In vivo*, steady state conditions did not apply and thus equilibrium conditions could not be fully guaranteed. However, interference by dynamic processes was minimized by selection of data measured after completion of indisulam infusion. Parameters describing the plasma protein binding of indisulam and its binding to erythrocytes were re-estimated with *in vivo* data.

## Results

### Model development

#### *In vitro plasma protein binding*

*In vitro* protein binding results were included in the data analysis for concentration levels 52, 130, 259, 389 and 518  $\mu\text{M}$  only, as the free indisulam concentrations at incubation concentration 5.2  $\mu\text{M}$  were below the lower limit of quantitation. Plasma protein binding of indisulam was high, varying from 99.6% to 99.9% in the concentration range 52-518  $\mu\text{M}$ . The free fraction increased with increasing incubation concentration, which indicates that protein binding is saturable.

In order to further investigate this observation, concentrations of protein bound indisulam ( $C_{\text{plasma, bound}}$ ) were calculated by subtracting free concentrations ( $C_{\text{plasma, free}}$ ) from total plasma concentrations ( $C_{\text{plasma, total}}$ ). The relationship between  $C_{\text{plasma, bound}}$  and  $C_{\text{plasma, free}}$  was best described by a saturable binding model. (Eq.2) The maximal plasma protein binding capacity  $B_{\text{max, plasma}}$  was estimated to be 767  $\mu\text{M}$  and binding was half-maximal at a free concentration of 1.02  $\mu\text{M}$ . ( $K_{\text{D plasma}}$ , Table 1) Figure 1 shows that the concentrations observed *in vitro* were adequately described by the model. Figure 2 represents the physiological model for the distribution of indisulam in whole blood. *In vitro*, free concentrations in erythrocytes ( $C_{\text{erythrocytes, free}}$ ) were assumed to be equal to free plasma concentrations ( $C_{\text{plasma, free}}$ ) and could be calculated from total plasma concentrations using equation 3, which is a rearrangement of equation 2 (with  $B_{\text{max}} = 767 \mu\text{M}$  and  $K_{\text{D}} = 1.02 \mu\text{M}$ ).

$$C_{\text{free}} = \frac{1}{2} * (((K_{\text{D}} + B_{\text{max}} - C_{\text{plasma, total}})^2 + 4 * K_{\text{D}} * C_{\text{plasma, total}})^{\frac{1}{2}} - (K_{\text{D}} + B_{\text{max}} - C_{\text{plasma, total}})) \quad (\text{Eq.3})$$

#### *In vitro distribution to erythrocytes*

The combined results of *in vitro* experiments 2 (distribution to erythrocytes) and 3 (binding to erythrocyte membranes) were used to define the relationship between  $C_{\text{erythrocytes, free}}$  and  $C_{\text{erythrocytes, bound}}$ . Rosenthal analysis revealed that binding comprised a saturable component. (Fig.3) A single site saturable binding model was tested first. This model resulted in underestimation of  $C_{\text{erythrocytes, bound}}$  in

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the higher concentration ranges and could thus not adequately describe the observed data. Binding of indisulam to erythrocyte membranes, which was interpreted as non-specific binding to erythrocytes, was 0.6% - 2.9% of the total concentration in erythrocytes. Non-specific binding was incorporated in the model by extending it to a two site binding model, comprising one class of saturable binding sites and one class of non-specific binding sites. This approach resulted in a statistically significant improvement of the model ( $P=0.0001$ ). The relationship between  $C_{\text{erythrocytes, bound}}$  and  $C_{\text{free}}$  was well described by equation 4, which was used to estimate  $B_{\text{max erythrocytes}}$  ( $174 \mu\text{M}$ ),  $K_{\text{D erythrocytes}}$  ( $0.087 \mu\text{M}$ ) and the non-specific binding coefficient  $N_{\text{erythrocytes}}$  (7.4). (Table 1) The precision the estimation of the model parameters (expressed as the coefficient of variance, Table 1) was moderate for  $N_{\text{erythrocytes}}$  and good for all other parameters.

$$C_{\text{erythrocytes, bound}} = B_{\text{max}} * C_{\text{free}} / (C_{\text{free}} + K_{\text{D}}) + N * C_{\text{free}} \quad (\text{Eq.4})$$

As plasma was obtained from multiple volunteers, it was taken into account that the maximal binding capacity for binding of indisulam to erythrocytes could differ from one volunteer to another.

Interindividual variability (IIV) of  $B_{\text{max erythrocytes}}$  was estimated to be 14%. The model adequately described the observations. (Fig.3)

### Application to *in vivo* data

In total, 350 samples were taken from 21 patients for the *in vivo* assessment of the blood distribution of indisulam. Samples taken during infusion ( $n=21$ ) were excluded from data analysis (see Data Analysis in the Materials and Methods section).

### *In vivo plasma protein binding*

The model developed with *in vitro* data, could describe the corresponding results from the *in vivo* study reasonably well. (Fig. 4A) The fit between observed data from the *in vivo* study and the model significantly improved upon addition of the individual albumin level (ALB ( $\mu\text{M}$ )) as a covariate for  $B_{\text{max plasma}}$ . This important finding indicates that the albumin level of an individual patient may be predictive for the pharmacokinetic behavior of indisulam in that particular individual.

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In equation 5, the number of binding sites per albumin molecule is represented by  $n$ .

$$C_{\text{bound}} = \text{ALB} * n * C_{\text{free}} / (C_{\text{free}} + K_{\text{D plasma}}) \quad (\text{Eq.5})$$

The number of binding sites was not statistically significantly different from 1 and  $n$  was consequently fixed at 1. Using *in vivo* results only,  $K_{\text{D plasma}}$  was re-estimated to be 0.65  $\mu\text{M}$ . (Table 2) This adjusted model for plasma protein binding described the *in vivo* observations very well. (Fig. 4B)

### ***In vivo distribution to erythrocytes***

In 287 of 329 available samples, total plasma concentrations and concentrations in washed erythrocytes were measured. Free concentrations in plasma were calculated using equation 5. The model for *in vitro* distribution to erythrocytes did not correctly describe the results from the clinical study. However, when the parameters describing the uptake in erythrocytes were re-estimated using the *in vivo* data, the two site binding model proved to be adequate. (Figure 5) Table 2 lists the parameter values after re-estimation using *in vivo* data from 21 patients. All parameters were precisely estimated. The non-specific binding coefficient  $N_{\text{erythrocytes}}$  was estimated to be higher *in vivo* than *in vitro* (70.1 vs. 7.4) and the equilibrium dissociation constant  $K_{\text{D erythrocytes}}$  was lower *in vivo* than *in vitro* (0.00379  $\mu\text{M}$  vs. 0.087  $\mu\text{M}$ ). Other *in vivo* parameter estimates corresponded well to the *in vitro* model.

Figure 6 shows the non-linear time profile of the total plasma concentration of indisulam of a patient who received a one-hour infusion of 1000  $\text{mg}/\text{m}^2$ . Saturation of binding to plasma proteins and erythrocytes occurred within the therapeutic concentration range. The concentrations at which the binding of indisulam to plasma proteins and erythrocytes were half-maximal, are depicted in figure 6. At a total plasma concentration of 300  $\mu\text{M}$ , the free plasma concentration was 0.65  $\mu\text{M}$  and plasma protein binding was half-maximal. At a total plasma concentration of 3.5  $\mu\text{M}$ , the free plasma concentration was 0.004  $\mu\text{M}$  and erythrocyte binding was half-maximal.

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### Discussion and conclusion

A physiological model for the distribution of indisulam in whole blood was successfully developed. We have demonstrated that indisulam binds to plasma proteins and to erythrocytes in a saturable manner, which may be attributed to binding to albumin (in plasma) and to carbonic anhydrase (in erythrocytes).

In a previous study, protein binding of indisulam was reported to be high (98-99%) and no saturation in the concentration range 52-518  $\mu\text{M}$  could be demonstrated. However, in that study the precision was insufficient (standard deviation was 0.05-0.66% vs. 0.006-0.027% in the current experiment) to entirely preclude saturation of protein binding.(van den Bongard et al., 2003) The currently presented *in vitro* and *in vivo* studies have clearly shown that plasma protein binding of indisulam is a saturable process. The maximal binding capacity was highly dependent on individual plasma albumin levels. Therefore, albumin may be the major plasma binding protein of indisulam in the concentration range studied. The albumin level of an individual may be predictive for the pharmacokinetic profile of indisulam. Hypoalbumineamia, which is common in cancer patients (Mariani et al., 1976), may result in a reduction of albumin-bound indisulam concentrations and a consequent increase in free plasma concentrations. The clinical implications of this finding will need further investigation. In the current study, plasma protein binding in blood from cancer patients (*in vivo*) was lower than in blood from healthy volunteers (*in vitro*). This explains the bias in figure 4A: plasma protein binding in patients was underestimated by the *in vitro* model, which was based on data from healthy volunteers. The estimated 767  $\mu\text{M}$  for  $B_{\text{max}}$  (*in vitro*) corresponds to an albumin level of 50.6 g/L, which is within the normal range for healthy male volunteers (34-54 g/L) (U.S.National Library of Medicine and the National Institutes of Health, 2005).

Not only binding to plasma proteins, but also binding to erythrocytes was shown to be saturable. As carbonic anhydrase is abundant in erythrocytes (Demir et al., 1997) and as indisulam is a potent inhibitor of this enzyme (Abbate et al., 2004), it was hypothesized that binding to carbonic anhydrase I and II may explain the saturable character of distribution to erythrocytes. Binding parameters describing the distribution of indisulam to erythrocytes were in agreement with the total concentration

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of carbonic anhydrase I and II (CA I and CA II). For the structurally related carbonic anhydrase inhibitors acetazolamide, dorzolamide and para-iodo-benzenesulfonamide, maximal binding capacities for CA I and CA II in erythrocytes were 117-166  $\mu\text{M}$  and 16.1-19.9  $\mu\text{M}$  respectively (total 133-186  $\mu\text{M}$ ). (Bayne et al., 1979; Hasegawa et al., 1994; Singh and Wyeth, 1991) The estimated 174  $\mu\text{M}$  (*in vitro*) and 146  $\mu\text{M}$  (*in vivo*) values for indisulam correspond well to these previously reported concentrations.

Binding of indisulam to erythrocytes was higher *in vivo* than *in vitro*. This may be due to differences in the binding affinity of indisulam related to different protein conformations *in vivo* and *in vitro*.

Another explanation for the difference between the *in vivo* and *in vitro* erythrocyte binding parameters may be a potential pH gradient. Indisulam is a potent carbonic anhydrase inhibitor and can therefore cause an intracellular pH increase. As indisulam is an acid ( $\text{pK}_a$  7.01), a pH increase will result in a decrease of neutral indisulam molecules. As only neutral molecules can diffuse across the erythrocyte membrane, inhibition of carbonic anhydrase could cause the ratio between  $C_{\text{erythrocytes, free}}$  and  $C_{\text{plasma, free}}$  to increase. Consequently, the estimates of  $N_{\text{erythrocytes}}$  and  $K_{D \text{ erythrocytes}}$  for the *in vivo* distribution of indisulam may have been over- and underestimated, respectively. Finally, the differences in erythrocyte binding parameters between *in vitro* and *in vivo* conditions may be partially explained by the lack of steady state conditions. Due to relatively slow redistribution of indisulam from erythrocytes back to plasma, the ratio between  $C_{\text{erythrocytes, free}}$  and  $C_{\text{plasma, free}}$  may have been higher *in vivo* than in the closed *in vitro* system. Hence, the values of  $N_{\text{erythrocytes}}$  and  $K_{D \text{ erythrocytes}}$  may also have been over- and underestimated, respectively, to some extent due to the lack of steady state conditions. The observed ratios of  $N_{\text{erythrocytes}}$  and  $K_{D \text{ erythrocytes}}$  between *in vitro* and *in vivo* conditions were 0.11 and 23 respectively. As the observed differences were large, it is likely that changes in protein conformations and/or environmental differences between *in vivo* and *in vitro* conditions can not be ruled out by a potential lack of equilibrium.

In conclusion, the blood distribution of indisulam *in vivo* and *in vitro* is highly determined by saturable processes. It is likely that these processes comprise binding to albumin (in plasma) and to carbonic anhydrase I and II (in erythrocytes). Saturable plasma protein binding may have impact on the higher ranges of the concentration-time profile of indisulam, while saturable erythrocyte binding may affect



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the terminal elimination phase. The saturable character of plasma protein binding and distribution to erythrocytes may thus provide a partial explanation for the non-linearity in the pharmacokinetic profile. However, *in vivo*, indisulam is not only distributed within the blood compartment, but to other body fluids and tissues as well. Furthermore, indisulam is continuously metabolized and eliminated from the body. As distribution beyond the blood compartment and elimination are dynamic processes, it is essential to include the blood distribution in a physiological pharmacokinetic model, in order to fully understand the exceptional non-linear pharmacokinetic behavior of indisulam.

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## Acknowledgments

The authors would like to acknowledge Desirée van den Bongard (The Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital), Sanae Yasuda, Motoharu Kakiki, Tomio Takamatsu, Yuichi Inai, Tsutomu Yoshimura (Eisai Co., Ltd.), Yasuhide Yamada and Tomohide Tamura (The National Cancer Center Hospital, Tokyo) for their contributions to this project.

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## Footnotes

### Source of financial support

This research was supported by a grant from the Eisai network of companies.

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### Legends to the Figures

- Figure 1. Plot of protein bound versus free plasma concentrations of indisulam obtained from *in vitro* experiments (exp. 1). Both observed (○) and model predicted concentrations (—) are depicted.
- Figure 2. Graphical representation of the physiological model of the blood distribution of indisulam. The compound is bound to plasma protein and distributed to erythrocytes.  $B_{\max}$  is the maximal binding capacity,  $K_D$  is the equilibrium dissociation constant of the binding equilibrium and  $N$  is the non-specific binding constant.
- Figure 3. Concentrations of indisulam bound to erythrocytes versus free plasma concentrations *in vitro*. Observed values (○) corresponded well to individual predicted values (◆). Binding becomes partially saturated at higher concentrations, but remains to slightly increase due to a non-specific binding component.
- Figure 4. Observed protein bound versus free plasma concentrations of indisulam (○) obtained from *in vivo* experiments. A: The model derived from *in vitro* results (—) described the corresponding *in vivo* results reasonably well. B: Inclusion of the albumin plasma concentration as a covariate for the maximal binding capacity resulted in an improved model (◆).
- Figure 5. Concentrations of indisulam bound to erythrocytes versus free plasma concentrations *in vivo*. Individual predicted values from the two site binding model (◆) corresponded well to the observed values (○).
- Figure 6. Plasma concentration versus time profile of an individual Caucasian patient after administration of 1000 mg/m<sup>2</sup> indisulam in a one-hour infusion. The pharmacokinetic profile is profoundly non-linear. The concentrations at which the binding of indisulam to plasma proteins and erythrocytes are half-maximal, are within the therapeutic concentration range.



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Table 1. Parameter estimates for the model describing the *in vitro* blood distribution of indisulam.

	Estimate	(CV% <sup>1</sup> )	IIV
B <sub>max plasma</sub>	767 μM	(2.2%)	-
K <sub>D plasma</sub>	1.02 μM	(4.9%)	-
B <sub>max erythrocytes</sub>	174 μM	(6.2%)	14%
K <sub>D erythrocytes</sub>	0.0866 μM	(9.0%)	-
N <sub>erythrocytes (non-specific binding)</sub>	7.4	(44%)	-

CV= coefficient of variance, IIV= interindividual variability

<sup>1</sup> obtained with the COVARIANCE option of NONMEM

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Table 2. Parameter estimates for the model describing the *in vivo* blood distribution of indisulam.

	Estimate	(CV% <sup>1</sup> )	IIV
$B_{\max \text{ plasma}}$	[albumin] ( $\mu\text{M}$ )	-	-
$K_D \text{ plasma}$	0.65 $\mu\text{M}$	(4.6%)	-
$B_{\max \text{ erythrocytes}}$	146 $\mu\text{M}$	(3.9%)	17.5%
$K_D \text{ erythrocytes}$	0.00379 $\mu\text{M}$	(4.3%)	-
$N_{\text{erythrocytes (non-specific binding)}}$	70.1	(8.4%)	-

CV= coefficient of variance, IIV= interindividual variability

<sup>1</sup> obtained with the COVARIANCE option of NONMEM

Figure 1.

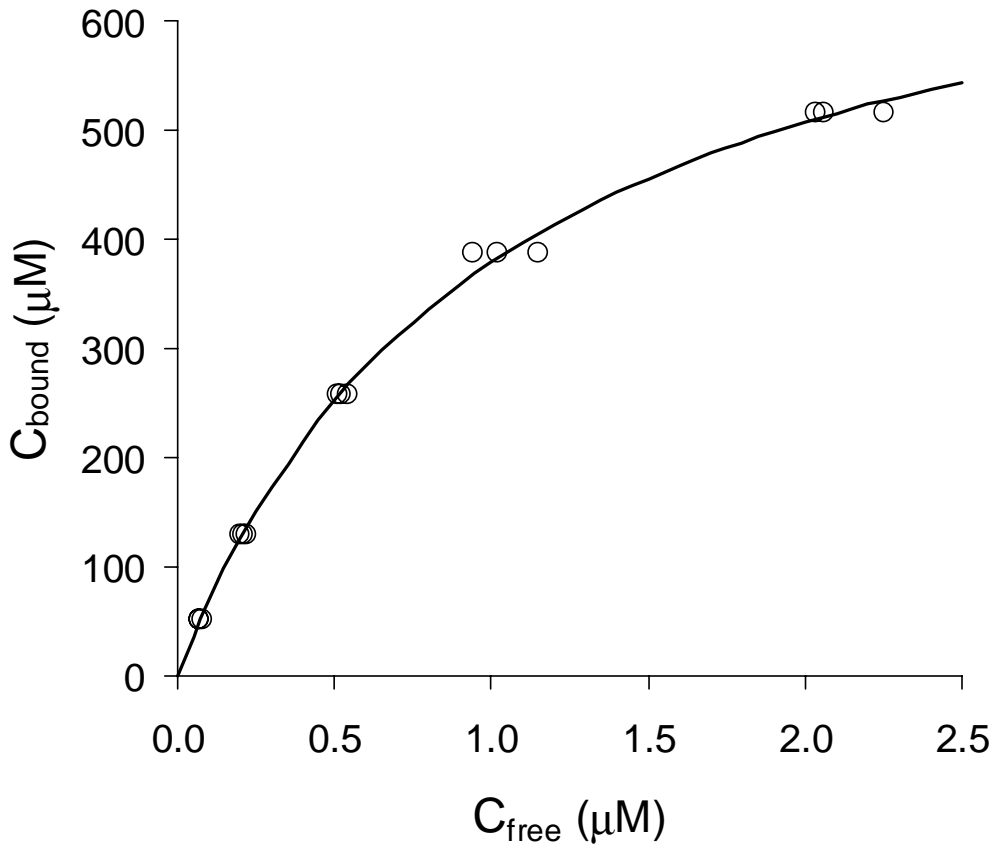


Figure 2.

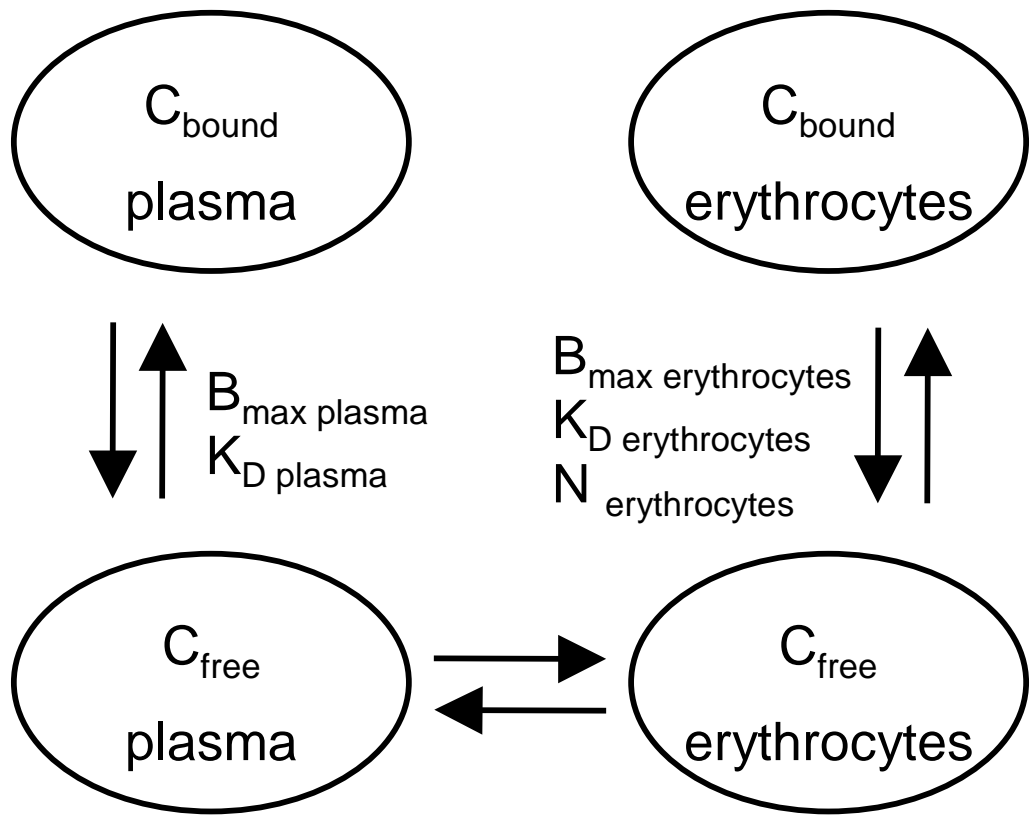


Figure 3.

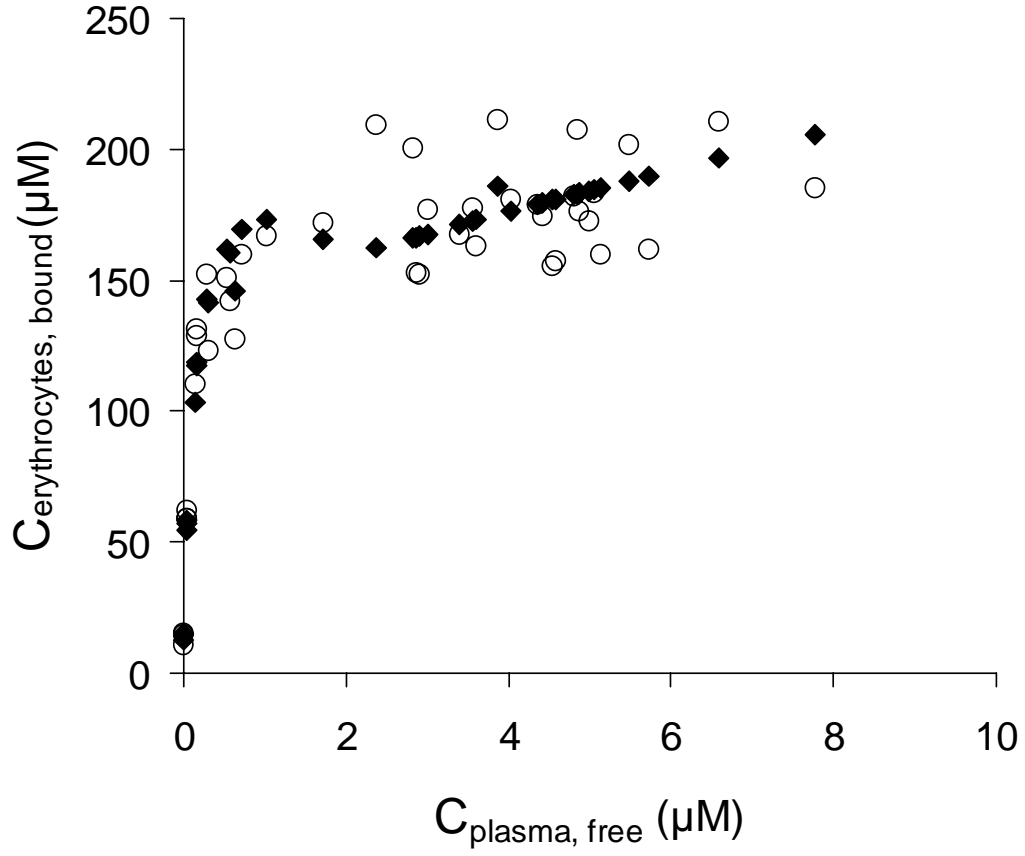


Figure 4A.

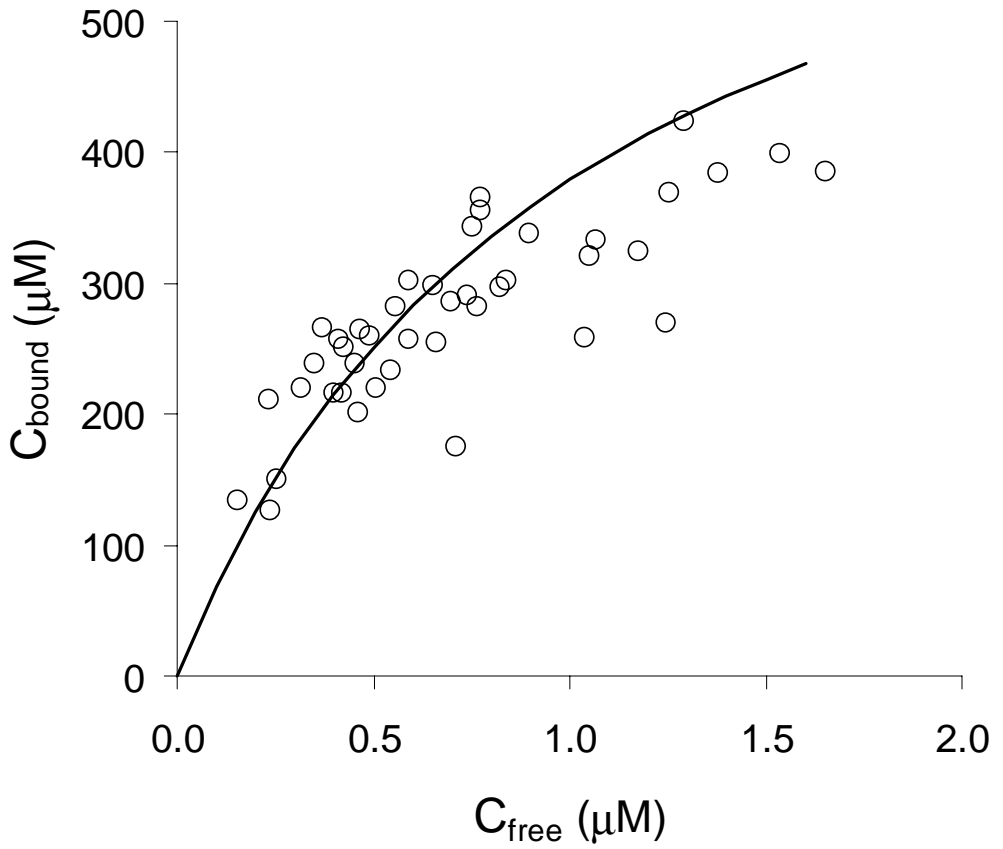


Figure 4B.

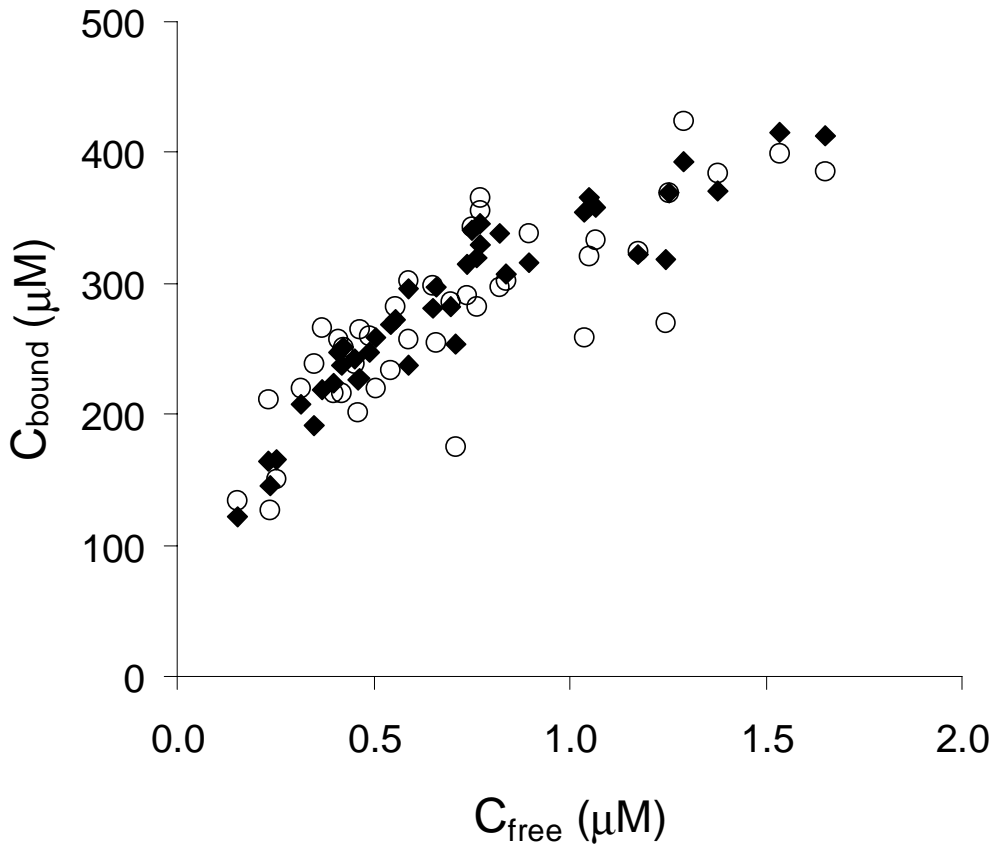


Figure 5.

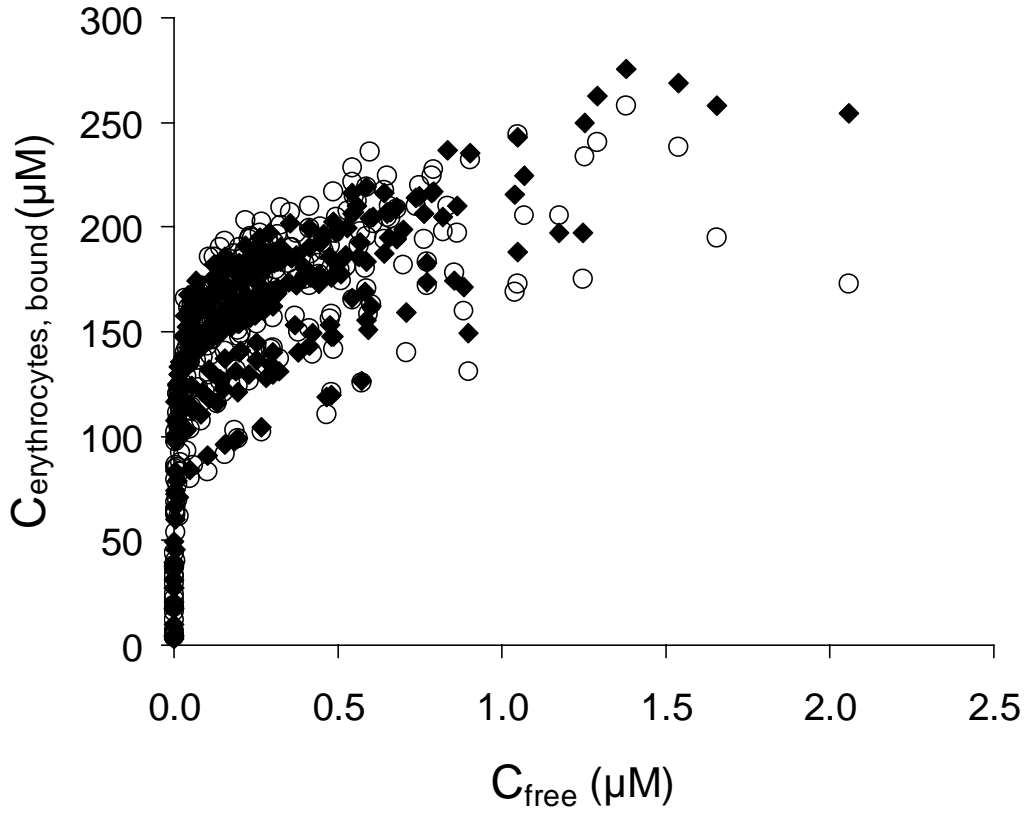




Figure 6.

